REKOMBINANTE SPINNENSEIDENPROTEINE UND BIOMINERALISATION FÜR TECHNISCHE UND BIOMEDIZINISCHE ANWENDUNGEN

DISSERTATION

ZUR ERLANGUNG DES AKADEMISCHEN GRADES EINER

DOKTORIN DER NATURWISSENSCHAFTEN (DR. RER. NAT.)

IN DER BAYREUTHER GRADUIERTENSCHULE FÜR MATHEMATIK UND NATURWISSENSCHAFTEN (BAYNAT)

DER UNIVERSITÄT BAYREUTH

VORGELEGT VON

VANESSA JUTTA NEUBAUER

AUS LICHTENFELS

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It doesn’t matter how beautiful your theory is,

it doesn’t matter how smart you are.

If it doesn’t agree with experiment,

it’s wrong.

Richard Phillips Feynman

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Zusammenfassung

Die Übertragung von Prinzipien aus der Natur half dem Menschen in der Vergangenheit bei bahnbrechenden technischen und medizinischen Neuerungen, die nun unseren Alltag erleichtern. Eine genaue Studie der Vorgänge der Biomineralisation, wie sie in unser aller Knochen ablaufen, kann verschiedene Anwendungen enorm bereichern. Das Verständnis der zu Grunde liegenden Prozesse, wie die Interaktion von Zellen mit dem umliegenden mineralisierten Gewebe, die Steuerung der Kristallkeim- und Strukturbildung durch Proteintemplate oder der osmotischen Balance während der Mineralabscheidung aus übersättigter Lösung spielen dabei auch in ihrer Interaktion untereinander eine wichtige Rolle. Der Brückenschlag zwischen dem natürlichen Vorbild und der späteren Applikation findet dabei insbesondere bei biomedizinischen Anwendungen statt: Im biomedizinischen Anwendungsbereich gelten derzeit nach wie vor Implantation oder Transplantation als Goldstandardlösungen für den Ersatz von mineralisierten Geweben. Dabei stellen sich vermehrt Probleme wie die Knappheit an Spenderorganen, eine nachteilige Interaktion von Implantaten mit dem umliegenden Ersatzgewebe oder die Übertragung von Krankheiten aus tierischen Produkten dar. Um diese Hindernisse zu überwinden, können artifizielle Gewebe, die einer Biomineralisation des Materials unterliegen, geeignete Lösungen im Sinne des *Tissue Engineering* darstellen. In diesem Fabrikationsansatz werden personalisierte Ersatzgerüste erstellt und in den Patienten eingebracht. In technischen Anwendungen ist in Zeiten der Energiewende die möglichst effiziente Nutzung und klimafreundliche Erzeugung von Energie ein wichtiges Argument. Um die erneuerbare Energieerzeugung zu unterstützen und effizienter zu gestalten, kommen häufig keramische Katalysatoren zum Einsatz. So kann im Zuge der Wasserstoffwirtschaft die katalytische Spaltung von Wasser als Grundlage für die Gewinnung des Energieträgers Wasserstoff dienen. In ausgefeilten Mineralisationsprozessen können gerichtet Materialien entstehen, die diese Reaktion katalysieren und großtechnisch umsetzbar machen.

Um diese unterschiedlichen Ziele miteinander zu verbinden und basierend auf einer gemeinsamen Grundlage individuell zu lösen, wurden in der vorliegenden Dissertation nach dem Vorbild der natürlich ablaufenden Biomineralisation Lösungsansätze erstellt. Als gemeinsame Ausgangsmaterialien wurden dafür rekombinante Spinnenseidenproteine gewählt. Basierend auf funktionellen Peptidmotiven der repetitiven Kerndomäne des Abseilfadens der Gartenkreuzspinne *Araneus diadematus* lag bereits das rekombinante

Spinnenseidenprotein eADF4(C16) vor, das biotechnologisch großtechnisch hergestellt werden kann. Es ermöglicht neben einer molekularbiologischen Modifikation des Ausgangsmaterials ein Maßschneidern von Morphologien für unterschiedliche Anwendungen. Anhand diesen flexiblen Materials als Grundlage wurden verschiedene Fragestellungen zum Themenkomplex Biomineralisation beleuchtet, im Speziellen darunter Ersatzmaterialien für den Sehnen-Knochen-Übergang, der Enthese.

Der erste Teil dieser Arbeit fokussierte sich auf das Verständnis mechanistischer Vorgänge während der bioinspirierten Mineralisation von Mangancarbonat unter kontrollierten Bedingungen in Gegenwart von verschiedenen Additiven. Darüber hinaus wurde der Ladungseinfluss von zwei Spinnenseidenvarianten untersucht, die mit unterschiedlichen Peptid-Funktionalisierungen ausgestattet waren und als klassisches Proteintemplat fungierten. Weiterhin wurde die ebenfalls geladene, synthetische Polyacrylsäure in die Reaktion als Struktur-dirigierendes Polymer eingebracht. Dabei wurde herausgefunden, dass neben einem Ladungseinfluss der Komponenten auch die kolloidale Stabilität der eingebrachten Spinnenseidenpartikel das Mineralisationsergebnis beeinflusst. Eine Abweichung der gebildeten Spezies äußerte sich in unterschiedlich starker Interaktion der Spinnenseidenpartikel mit den unter diesen Bedingungen typischerweise gebildeten Mangancarbonat-Würfeln. Außerdem konnte eine Polymer-induzierte Flüssigphase ähnlich bereits beschriebener Strukturen in Gegenwart von Polyacrylsäure generiert werden. Somit konnten aus diesen Erkenntnissen Aussagen über das Mineralisationsgeschehen mit Spinnenseidenproteinen getroffen werden und eine Einschätzung der gebildeten Materialien für eine Anwendung als Katalysatormaterial abgeleitet werden.

Im zweiten Teil wurden Antworten zu Fragestellungen zu mineralisiertem Gewebeersatz erarbeitet. Da Spinnenseide ein biokompatibles Biomaterial darstellt, das nicht-toxisch und bioabbaubar ist und keine Immunreaktion auslöst, eignet es sich für derartige biomedizinische Anwendungen. Im Falle des teilmineralisierten Gewebes des Sehnen-Knochen-Übergangs sind neben einer graduellen Mineralisation hin zur Knochenseite weitere Designkriterien wichtig, die die Mechanik des Gewebes zur Kraftübertragung und Zellbesiedlung betreffen.

Es wurde eine biomimetische Biomineralisation von rekombinanten Spinnenseidenvarianten untersucht, die Mineralisierungs- und Kollagenbindemotive aus der Mineralisation im Knochen zu Grunde liegenden SIBLING Proteinen trugen. Eine

Materialstudie zeigte das Mineralisationsverhalten der Varianten und ihre Zellverträglichkeit. Durch eine Prozessierung in ein Gradientenmaterial wurde das unterschiedliche Zellverhalten von Osteoblasten mit einer Präferenz zu Gunsten des Kollagenbindemotivs sichtbar. Dies bestätigte eine Eignung für Materialanwendungen im graduellen Sehnenersatz an der Knochenseite.

Ein weiterer Ansatz realisierte Spinnenseiden-Kompositmaterialien mit anorganischen Füllstoffen. Vorteil dieser Herangehensweise war das gerichtete Einbringen von Keramiken in ein Matrixmaterial, das dann zusätzlich mit Zellen angereichert wurde. Die Entwicklung eines neuartigen Verfahrens im 3D Druck erlaubte das einfache Verarbeiten dieser Biotinten in Gradientenmaterialien. Somit wurde erfolgreich nach Vorbild der Enthese ein rekombinantes Spinnenseidenhydrogel mit einem Gradienten, beladen an Zellen und Fluorapatit-Partikeln, gedruckt und eine Zellviabilität nach dem Prozess bestätigt.

In einem dritten Teil wurde ein neuer Fabrikationsansatz für Spinnenseidengele entwickelt. Faltung und Selbstassemblierung von rekombinanten Spinnenseidenproteinen basierten in mischbaren, wässrig-organischen Zwischenphasen auf der Ausbildung von Wasserstoffbrücken und hydrophoben Effekten. Neben zu Grunde liegenden mechanistischen Betrachtungen wurde eine höhere Materialsteifigkeit der Spinnenseidengele in wässrig-organischen Mischphasen mit Dimethylsulfoxid erzielt. Sie eignen sich durch ihren organischen Lösungsmittelanteil als injizierbare oder druckbare Depots zur Formulierung von wasserunlöslichen Wirkstoffen.

Summary

The transfer of principles occurring in nature to technical and biomedical revolutions, which facilitate daily life, was a benefit for humankind throughout history. A detailed study of biomineralization processes undergoing in our bones can therefore beneficially contribute to future fields of application. The understanding of underlying processes such as cell interaction in mineralized tissues, nucleation and structure-directing effects of protein templates or osmotic balance during mineral growth propagation from saturated solution, and their interaction among each other play a crucial role therein. Regarding this natural blueprint, typical applications lie in the biomedical field: In biomedical applications, the reconstruction of lost mineralized tissues is currently based on transplantation or implantation solutions. However, issues can occur with respect to donor organ shortage, improper implant-host tissue interaction and disease transmission from animal sources. Tissue engineering approaches can overcome these obstacles upon providing artificial tissues from intrinsic mineralized materials. This fabrication method yields personalized scaffolds, which are then implanted into patients. The technical application field concerns a climate-friendly generation and efficient energy consumption during energy revolution. Strategies for efficient renewable energy generation often involve ceramic catalyst technologies. Moreover, they can deliver catalytic water splitting to produce hydrogen as potential new energy carrier. Controlled mineralization processes yield catalyst materials, which enhance the water splitting reaction and allow an industry-scale production.

This dissertation aims at solution approaches based on bioinspired biomineralization processes to combine the aforementioned aims and also to target them individually. Recombinant spider silk proteins served as common basis for these studies. The eADF4(C16) recombinant spider silk protein was previously designed based on functional peptide motives extracted from the repetitive core domain of the *Araneus diadematus* garden spider dragline silk and can be produced biotechnologically in large scale. Modifications of the material can be realized via molecular cloning and tailor-made processing into various morphologies. This flexible material allowed multidirectional approaches concerning biomineralization in general and among them specifically at the tendon-bone-insertion, the enthesis site.

The first part of this thesis focussed on bioinspired mineralization mechanisms during manganese carbonate mineralization under controlled conditions and in the presence of

various additives. Among them, two spider silk variants were studied concerning their peptide-tag charge influences as classical protein template. Moreover, the structure-directing, charged synthetic polymer poly(acrylic acid) was added to the reaction. As a result, it was found that not only the components' charge plays an important role but also the colloidal stability of the spider silk template particles influences the mineralization outcome. Cubic mineralized manganese carbonate species differed in their spider silk particle content depending on particle charge. Further, poly(acrylic acid) yielded the formation of a polymer induced liquid phase similar to reported structures. From these findings, the behaviour of spider silk proteins in mineralization setups could be translated. Their suitability as new catalyst materials could be estimated.

The second thematic complex was related to mineralized tissue regeneration topics. Spider silk renders a biocompatible biomaterial for such applications, as it is non-toxic, biodegradable and shows no immune reaction. Other design criteria for only partially mineralized tissues at the tendon-bone interface, which transfers high loads, are gradual mineralization and cell population towards the bone.

Biomimetic mineralization studies of recombinant spider silk hybrid variants with fusion peptides from mineralization and collagen binding sites of SIBLING proteins in bone were conducted. The material study enlightened their mineralization behaviour and their cell compatibility. Upon processing two variants into a gradient surface, a clear preference of osteoblast cells towards the collagen binding motive was visualized. This confirmed their suitability for gradual tendon to bone replacement applications.

Further, a recombinant spider silk protein composite material with inorganic fillers was realized. The advantage of this method is the controlled composition of matrix and mineral, which allowed even the incorporation of cells. The development of a new break-through 3D printing technique enabled the easy to handle processing of these bioinks into gradient materials. Therefore, recombinant spider silk hydrogels loaded with cells and fluorapatite particles could be gradient printed, mimicking the natural enthesis, and cell viability after the process was maintained.

In the third part, a new fabrication route for spider silk gels was developed. Folding and self-assembly of recombinant spider silk proteins in miscible aqueous-organic interphases was based on hydrogen bonds and hydrophobic interactions. Besides unraveling the underlying mechanism of structure formation, also higher material stiffness for spider

silk gels was yielded in aqueous-organic interphases with dimethylsulfoxide. Due to their organic solvent content, they can be applied as injectable or printable depots for the formulation of water-insoluble drugs.

1 Einleitung

1.1 Seide

Seidenmaterialien werden überwiegend von Spinnen und Insekten zu unterschiedlichen Zwecken, wie unter anderem Beutefang, Hausbau oder Fortpflanzung, hergestellt.[1] Trotz ihrer konvergenten evolutionären Entwicklung[1-3] haben Seidenmaterialien gemein, dass sie aus Proteinen mit einer repetitiven Kerndomäne, flankiert von nicht-repetitiven Termini, bestehen.[1] Betrachtet man ihre Größe und Struktur, finden sich diverse Unterschiede: Spinnenseidenproteine haben ein Molekulargewicht von bis zu 350 kDa[4, 5], wohingegen Seidenspinnerseidenproteine bis zu 400 kDa[2, 6, 7] aufweisen. Beide zeigen einen hohen Anteil an β-Faltblattstrukturen.[1] Abweichend davon stellen sich Wespen- oder Bienenseidenproteine dar, die mit 30 kDa und überwiegend α-helikaler *coiled coil* Struktur herausstechen.[1, 8] Die Florfliegenseide hingegen zeigt überwiegend *cross-*β-Struktur mit Proteinen von 60 und 100 kDa.[9, 10] Die mechanischen und biochemischen Eigenschaften von Seiden wurden vom Menschen schon vor langer Zeit für sich genutzt. Beispiele dafür sind edle Kleidungsstücke aus Seide des Seidenspinners bis hin zu Fischernetzen aus Spinnenseide.[1]

1.1.1 Natürliche Spinnenseide

Die weibliche Radnetzspinne *(Araneae)* kann bis zu sieben verschiedene Seidentypen ausbilden, die sich in ihrer Zugfestigkeit unterscheiden.[11] Sie werden nach der jeweiligen Drüse, aus der sie stammen, benannt (Abbildung 1).[11, 12] So ist die Seide der Großen und Kleinen Ampullendrüse am Aufbau des Netzes mit Ausbildung der Querfäden und der Hilfsspirale beteiligt.[11] Die innere Netzspirale wird durch hoch elastische flagelliforme Seide gebildet, die den Insektenflug bremst,[11] und ist mit klebriger Aggregat Seide ausgestattet.[11] Das Netz wird mit Pyriform Seide befestigt. Die beiden Seidenarten der Cylindriform Seide und Aciniform Seide stechen heraus, da sie nicht am Netz beteiligt sind. Erstere bildet die Kokonhülle, um die Spinneneier zu schützen. Letztere dient als weiche Innenschicht des Kokons und dem Einwickeln der Beute zum späteren Verzehr.[4, 5]

Die außergewöhnlichen mechanischen Eigenschaften der Spinnenseide rühren aus ihrer natürlichen Funktion her, Insekten im Flug zu stoppen und im Netz zu fangen.

Seidenfasern können beispielsweise eine hohe Elastizität mit Ausdehnung von bis zu 450 % (Flagelliform Seide) und gleichzeitiger Zugfestigkeit (bis 1,5 GPa der Seide der Großen Ampullendrüse) bei Durchmessern im geringen Mikrometer-Bereich aufweisen.[13]

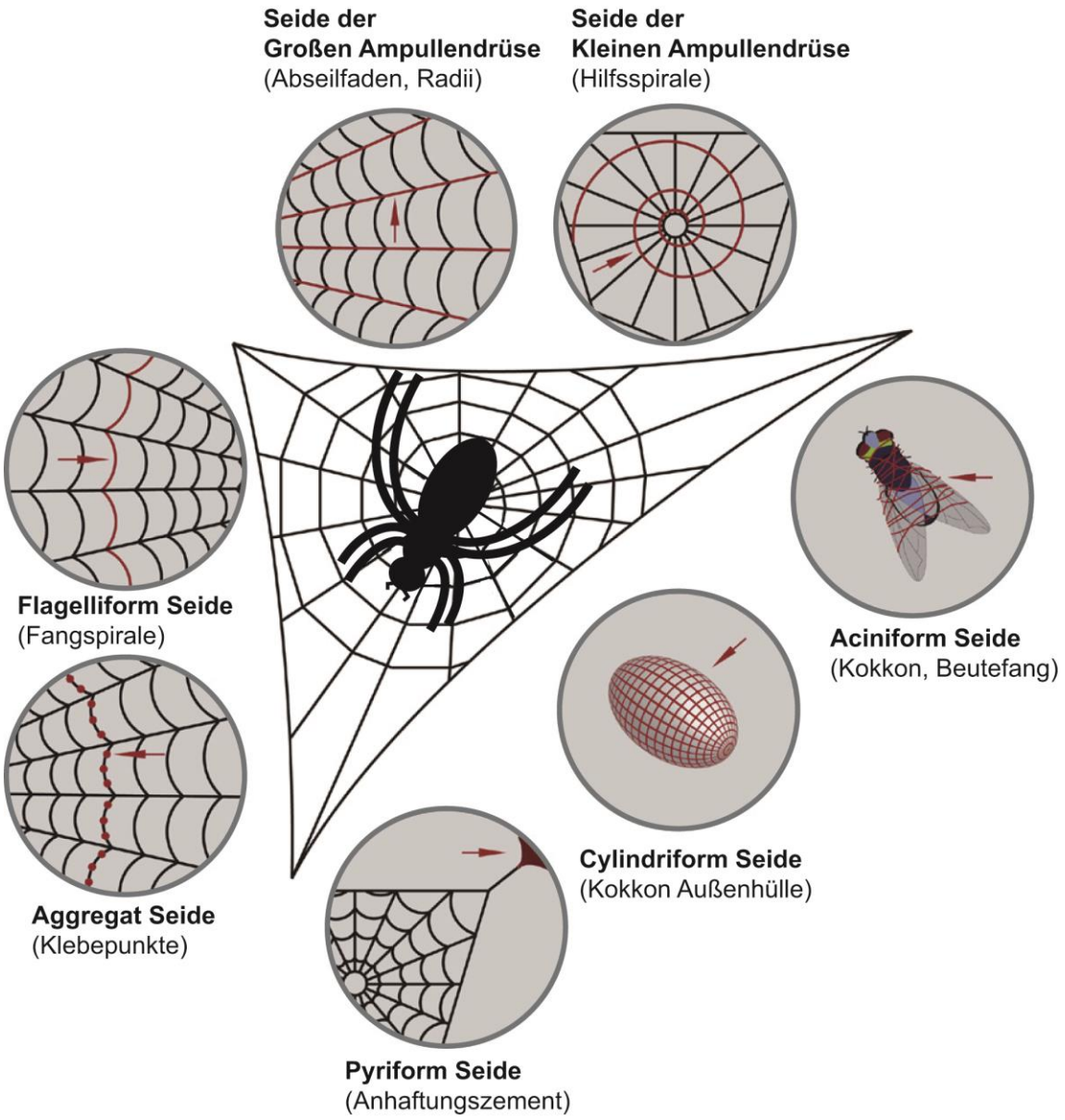

Abbildung 1: Darstellung der verschiedenen Seidenarten, die von weiblichen Radnetzspinnen (*Araneae*) hergestellt werden können. Die jeweilige Funktion ist in rot dargestellt. Nachdruck unter Genehmigung modifiziert nach Heidebrecht, A.; Scheibel, T., Recombinant Production of Spider Silk Proteins. In *Advances in Applied Microbiology*, Sariaslani, S.; Gadd, G. M., Hrsg. Elsevier Academic Press Inc: San Diego, **2013**, *82*, 115-153. Copyright (2013) Elsevier.

Den Fasern liegen Spinnenseidenproteine, auch Spidroine genannt, zu Grunde. Diese besitzen meist zwei globuläre, nicht-repetitive Domänen, die N- und C-terminal zu finden sind. Im Mittelteil der Proteine befindet sich meist eine repetitive Kerndomäne.[12] Bisher konnten Struktur-Eigenschaftsbeziehungen besonders für Proteine der Großen Ampullendrüse schlüssig hergeleitet werden: Im Abseilfaden wurden bereits zwei Proteine dieser Drüse, das Spidroin 1 und 2 (*Major Ampullate Spidroin 1* und *2,* MaSp1 und MaSp2) identifiziert, die sich in ihrem Prolin-Gehalt und ihrer Hydrophobizität unterscheiden. Ihre Kerndomäne weist bis zu 100 Wiederholeinheiten von Sequenzmotiven mit 40-200 Aminosäuren auf.[4] Darunter ist beispielsweise das Motiv eines Polyalanin-Blocks, das über hydrophobe β-Faltblattstrukturen als kristalline Anteile für hohe Zugfestigkeit verantwortlich ist.[4] Ein weiteres Motiv besteht aus Glycin-Glycin-X-Einheiten, wobei X Tyrosin, Leucin oder Glutamin darstellen kann. Sie bilden amorphe Strukturen zwischen den Teilkristallen.[12] Ihre Dehnbarkeit erhält diese Spinnenseide durch Glycin-Prolin-Glycin-XX-Motive, die meist elastische, helikale Strukturen bilden.[4]

Die Faserherstellung aus MaSp-Proteinen erfolgt durch Sekretion ihrer löslichen Form. Diese wird in der Spinndrüse hoch konzentriert gelagert und liegt bei Konzentrationen von über 50 Gewichtsprozent in einer Flüssigkristallphase vor.[12] Die nicht-repetitiven Domänen dienen der Stabilisierung der Proteine in Lösung.[14] Während des Spinnprozesses werden entlang des Spinntrichters Ionen ausgetauscht, Wasser entzogen und der pH gesenkt. Kosmotrope Salze wie Kalium und Phosphat nehmen den Platz von chaotropen Salzen wie Natrium und Chlorid ein.[12] Somit werden die gelösten Proteine durch chemische und mechanische Stimuli gefaltet und assembliert, indem sich ihre jeweiligen globulären Domänen aneinander anlagern und sich die repetitiven Kernsequenzen horizontal zur Spinnrichtung ausrichten. Die C-terminalen Domänen liegen bereits während der Lagerung parallel dimerisiert und über Disulfid-Brücken verbunden vor. Die N-terminalen Domänen hingegen lagern sich erst während des Spinnprozesses antiparallel an. Somit entsteht ein intrinsisch ausgerichtetes Konstrukt des Spinnenseidenfadens.[4, 15, 16] Final wird aus der Spinndrüse, nach zunehmender Entwässerung und intra- und intermolekularen Wechselwirkungen, ein wasserunlöslicher Faden gesponnen,[17] der erst durch die Scherkräfte eines enger werdenden Spinnkanals und durch Verstrecken mit Hilfe der hinteren Spinnenbeine seine mechanischen Eigenschaften durch Ausrichten der Proteine annimmt.[12]

Die mechanischen Eigenschaften des Abseilfadens, der die Rettungsleine der Spinne darstellt, basieren außerdem auf seinem hierarchischen Aufbau (Abbildung 2).[4] Der Kern

besteht aus Spinnenseidenfibrillen aus MaSp-Proteinen, die kristalline β-Faltblattstrukturen in einer amorphen Matrix bilden. Unter Luftfeuchtebedingungen über 60 % können solche Spinnenseidenfäden einer Superkontraktion unterliegen, bei der der Faden quillt und in seiner Länge entsprechend abnimmt. Das hat jedoch eine ungeordnete intrinsische Anordnung dieser kristallinen Regionen der Seidenproteine der Großen Ampullendrüse zur Folge und die mechanischen Eigenschaften verschlechtern sich.[12] Die Hülle besteht aus Glykoproteinen und Seidenproteinen der Kleinen Ampullendrüse, die jedoch nur eine lose Schutzschicht für die innen liegenden Fibrillen darstellt.[11] Eine Lipid-haltige, 10-20 nm dicke Schicht stellt die äußerste Hülle dar und kann ebenfalls Pheromone enthalten.[11]

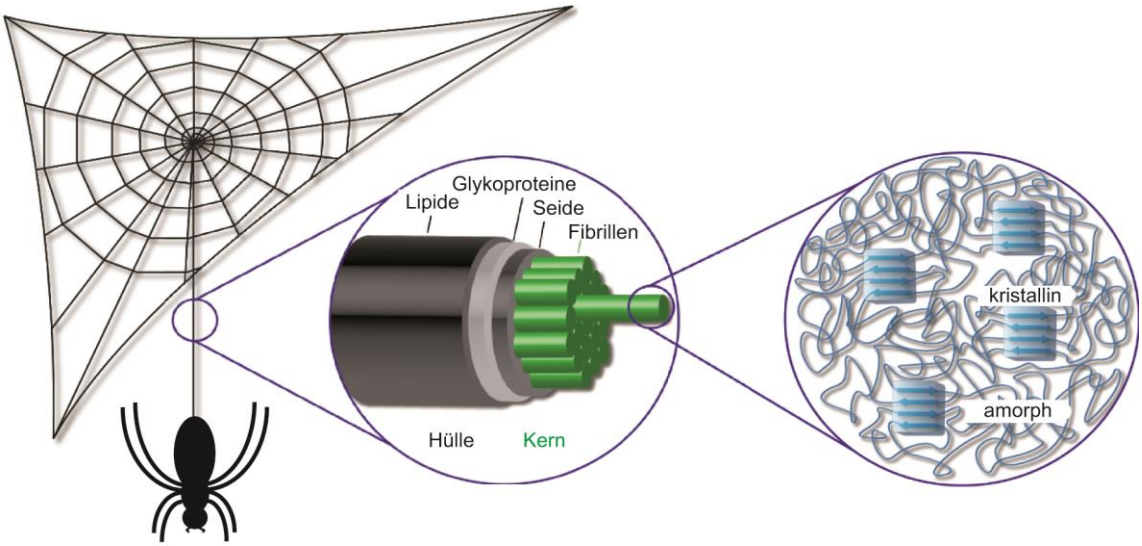

Abbildung 2: Modellhafte Darstellung des Kern-Hülle Aufbaus des Abseilfadens von Radnetzspinnen. Die innen liegenden Fibrillen werden von einer Hülle aus Seidenproteinen, Glykoproteinen und Lipiden umgeben. Die Fibrillen aus MaSp-Proteinen enthalten kristalline und amorphe Anteile. Nachdruck unter Genehmigung modifiziert nach Heidebrecht, A.; Scheibel, T., Recombinant Production of Spider Silk Proteins. In *Advances in Applied Microbiology*, Sariaslani, S.; Gadd, G. M., Hrsg. Elsevier Academic Press Inc: San Diego, **2013**, *82*, 115-153. Copyright (2013) Elsevier.

1.1.2 Rekombinante Spinnenseide

Auf Grund eines stark territorialen und kannibalistischen Verhaltens der meisten Spinnen ist eine Zucht ähnlich der des Seidenspinners zur Seidengewinnung in großem

Maßstab nicht möglich. Die Seidengewinnung wurde durch Melken von Spinnen mittels Abwickeln des Abseilfadens getestet,[12] stellt aber Stress für das Tier dar, was die Qualität der Seide beeinflussen kann.[4, 11]

Aus diesen Gründen wurde eine biotechnologische Herstellung mittels biomimetischer Spinnenseidengene etabliert. Dies ermöglicht eine Tier-unabhängige Produktion von rekombinanten Spinnenseidenproteinen in skalierbarem Maßstab und mit gleichbleibender Qualität.[12] Grundlage für diese Art der Herstellung war die Identifikation von Aminosäuresequenzmotiven natürlicher Spinnenseidenproteine[18, 19] und ihre Übersetzung in synthetische Gene unter Codon-Optimierung für den Wirtsorganismus.[11] Diese Gene können dann über molekularbiologische Methoden in einen Vektor eingebracht und vervielfältigt werden, um ähnlich repetitive Sequenzen wie im natürlichen Vorbild zu erzeugen (Abbildung 3).[20]

Als Expressionsorganismus können bei rekombinanter Herstellung verschiedene Wirte verwendet werden. Darunter befinden sich Bakterien, Pflanzenzellen, Hefen, Insektenzellen und transgene Tiere wie zum Beispiel Ziegen.[11] Voraussetzung für die Eignung als Wirt sind eine genetische Stabilität der eingebrachten Gensequenzen und ein schneller Nachschub an häufig verbrauchter tRNA beziehungsweise deren Aminosäuren in den repetitiven Einheiten.[5] In der Regel bietet sich aber *Escherichia coli* wegen seiner hohen Teilungsrate und schnellen Erzeugung von Biomasse an.[4, 11] Jedoch kann es während der Produktion zu unerwünschten Effekten wie Verkürzung insbesondere bei repetitiven Zielproteinen kommen. Weiterer Nachteil ist, dass *E. coli* keine typischen eukaryotischen posttranslationalen Modifikationen ausführen kann.[11]

Rekombinante Spinnenseidenproteine können nach der Fermentation gelöst im Zellplasma oder aggregiert in Einschlusskörperchen vorliegen. Nach etabliertem Protokoll schließen sich Reinigungsschritte wie Hitzefällung von restlichen *E. coli*-Proteinen bei gleichzeitig hitzestabilen Spinnenseidenproteinen und eine Ammoniumsulfat-Fällung des Zielproteins an.[11] Die so gewonnenen Proteine sind wasserunlöslich und werden nach Waschschritten und Gefriertrocknen als Rohmaterial erhalten.[20] Dieses kann dann in verschiedene Morphologien entsprechend der jeweiligen Anwendung prozessiert werden.[4]

Für diese Herstellungsweise wurde bereits ein MaSp-Protein der Gartenkreuzspinne *Araneus diadematus* herangezogen.[20] Die Konsensussequenz der Kerndomäne, das sogenannte C-Modul mit der Aminosäuresequenz im Einbuchstabencode von

GSSAAAAAAAASGPGGYGPENQGPSGPGGYGPGGP, wurde 16-mal wiederholt und als *engineered Araneus diadematus* Fibroin 4, kurz eADF4(C16), bezeichnet (Abbildung 3).[20] Weitere rekombinante Varianten basieren zum Beispiel auf dem Fibroin 3 der Großen Ampullendrüse (ADF3) der Gartenkreuzspinne.[21]

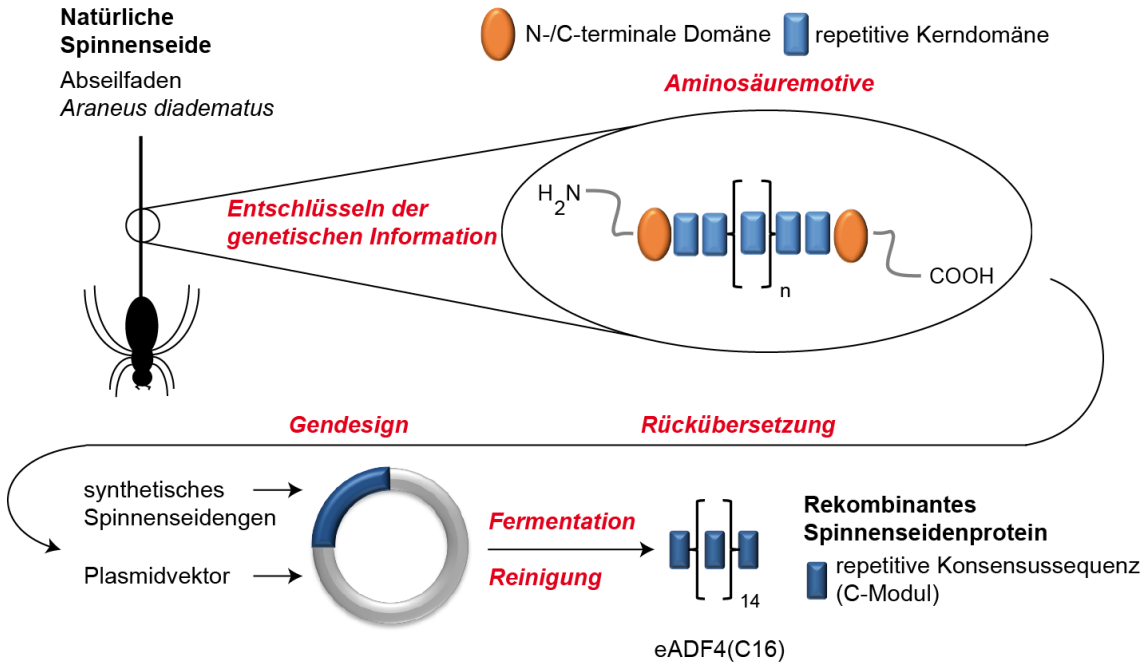

Abbildung 3: Darstellung der Vorgehensweise von der natürlichen Spinnenseide des Abseilfadens der Gartenkreuzspinne bis hin zur Herstellung des rekombinanten Spinnenseidenproteins eADF4(C16): Nach DNA-Extraktion und Entschlüsseln der genetischen Information des natürlichen Vorbilds konnten Aminosäuremotive identifiziert werden. Deren Rückübersetzung folgte ein Gendesign hin zum synthetischen Spinnenseidengen. Mittels Fermentation und Reinigung wurden dann rekombinante Spinnenseidenproteine erhalten. (Eigene Darstellung)

1.1.3 Funktionalisierung von rekombinanter Spinnenseide

1.1.3.1 Modifikation der Proteinsequenz

Molekularbiologische Techniken erlauben durch Klonieren das Maßschneidern von Proteinen. Eine gerichtete Erstellung von repetitiven Gensequenzen erlaubt so das rekombinante Darstellen von Proteinen ähnlich des natürlichen Vorbilds.[22] Durch

enzymatisches Schneiden an strategisch sinnvollen Stellen können DNA-Sequenzen vervielfältigt oder neu kombiniert werden.[20]

Schon kleine Veränderungen der repetitiven Einheiten gestalten Proteine mit unterschiedlichen Eigenschaften. Sie können sich hinsichtlich der Anzahl der Wiederholeinheiten unterscheiden, was Einfluss auf die mechanischen Eigenschaften der künstlichen Fasern aus diesen Materialien hat.[23] Da das C-Modul nur eine geladene Aminosäure (Glutaminsäure) enthält und so in der Repetition das polyanionische eADF4(C16) Protein gestaltet, hat der Austausch dieser Aminosäure eine Veränderung der Nettoladung und des Verhaltens zur Folge, beispielsweise in Bezug auf Wirkstofffreisetzung[24] oder mikrobieller Besiedelung[25].

Durch genetisch basierte oder chemisch konjugierte Anbindung von Peptiden oder Oligonukleotiden[26] an C- und/oder N-terminaler Position von rekombinanten Proteinen können verschiedenartige Funktionen erzeugt werden. Eine klassische Anwendung ist die Funktionalisierung mit dem Arginin-Glycin-Asparaginsäure-Tripeptid (kurz RGD), das für die Vermittlung von Zelladhäsion auf Oberflächen über Integrinbindung ursächlich ist.[27] Dies ist für den Einsatz als Material in der Geweberegeneration essentiell. Auch lokalisierte Ladungen in Peptid-*tags* können spezifische Effekte mit sich bringen, zum Beispiel eine Corona-Bildung von Blutproteinen auf Spinnenseidenpartikeln, was für die Materialwahl für biomedizinische Produkte eine Rolle spielt.[28] Auch in der technischen Anwendung, wie in der katalytischen Wasserstoffproduktion zur Energiegewinnung, können maßgeschneiderte Spinnenseidenproteine durch Bindung von Gold- und Titandioxid-Nanopartikeln zur Photokatalyse herangezogen werden.[29]

Die Erstellung von chimären Fusionsproteinen zeigt zusätzliche Funktionalität. Zwei individuelle Proteine können miteinander verbunden werden und ihre jeweiligen Eigenschaften kombinieren, wie beispielsweise Spinnenseide in Fusion mit dem *Green Fluorescent Protein* (GFP).[30] Das Fusionsprotein GFP-eADF4(C16) zeigte nach Elektrospinnen aus wässrigem Medium Fluoreszenzsignal, was eine Verarbeitung von Biomolekülen unter Erhalt ihrer Aktivität modellhaft zeigte.[31]

Eine zukünftige Herangehensweise könnte gezielt Kernfunktionen auf Peptidsequenzen zurückführen und diese Bausteine für ein maßgeschneidertes Materialdesign heranziehen.[22]

1.1.3.2 Variationen der Proteinprozessierung

Neben Modifikationen auf DNA-Ebene, die direkt bei der Herstellung unterschiedlich funktionalisierte Proteine liefert, können auch aus demselben Material verschiedene Morphologien entstehen („eines für alles"-Prinzip), die dann in unterschiedlichen Bereichen Anwendung finden (Abbildung 4).

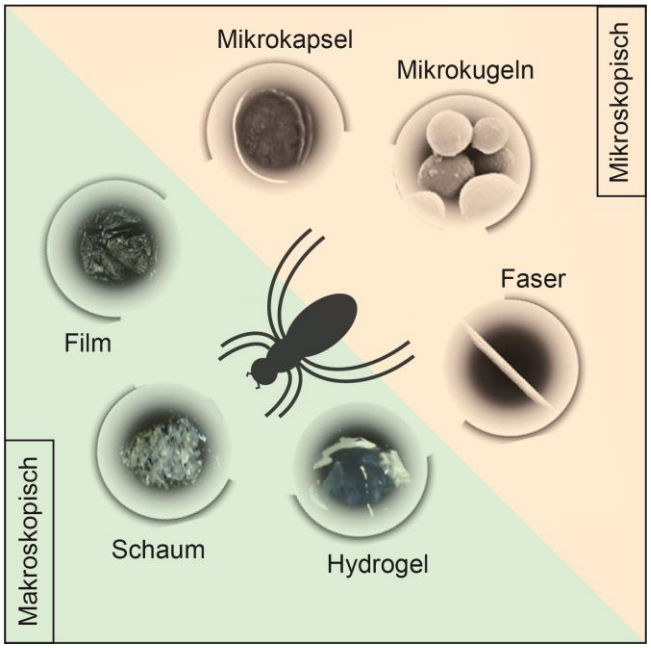

Abbildung 4: Darstellung verschiedenartiger Morphologien, die aus dem rekombinanten Spinnenseidenprotein eADF4(C16) hergestellt werden können. Nachdruck unter Genehmigung modifiziert nach Leal-Egana, A.; Scheibel, T., Silk-based materials for biomedical applications. *Biotechnol. Appl. Biochem*. **2010**, *55*, 155-167. Copyright (2010) John Wiley & Sons.

Ähnlich ihrem natürlichen Vorbild sind auch die Strukturen rekombinant hergestellter Spinnenseidenproteine sensitiv gegenüber Salzen. Hohe Konzentrationen an Natriumchlorid unterdrücken die Aggregation und eignen sich zur Lagerung der Proteine („*salting in*" Effekt), wo hingegen Kaliumphosphat Aussalzungseffekte hervorruft („*salting out*" Effekt).[14] Die aussalzende Wirkung von Kaliumphosphat auf gelöste Spinnenseidenproteine ist abhängig von der Salzkonzentration: Es wurde ein Übergangsbereich zwischen der Ausbildung von Fibrillen bei bis zu 300 mM und Mikrokugeln ab 400 mM beobachtet.[32] Diese Art der Mikrokugeln kann als Wirkstoffdepot für eine gezielte Verabreichung aktiver Moleküle an einem Wirkort herangezogen werden.[33-35]

Neben dem Einfluss von Salzen auf rekombinante Spinnenseidenproteine kann auch an der wässrig-organischen Grenzfläche eine Strukturausbildung durch Phasenseparations-induzierte Selbstassemblierung beobachtet werden. Die dabei gebildeten Mikrokapseln besitzen eine dünne Außenhülle, die reich an β-Faltblattstrukturen ist.[36] Sie kann als semipermeable Membran mit einer Molekulargewichtsgrenze von 27 kDa funktionieren.[37]

Bei der Prozessierung von Spinnenseidenmaterialien liegt eine Faserherstellung nahe. Verschiedene Ansätze können mittlerweile sogar den natürlichen Spinnprozess nachempfinden und daraus mechanistische Aussagen ableiten.[23] Im Gegensatz dazu stehen Prozesse wie das Nassspinnen oder Elektrospinnen industriell umsetzbaren Lösungen näher und können Fasern für technische Anwendungen liefern.[38, 39]

Die einfachste Verarbeitung von Spinnenseidenproteinen ist das Gießen von Filmen als einfache 2D Morphologie. Dies ist aus wässriger Lösung, aber auch aus unverdünnter Ameisensäure oder 1,1,1,3,3,3-Hexafluoro-2-isopropanol realisierbar. Je nach Lösungsmittel nehmen die hergestellten Filme unterschiedliche Anteile an kristallinen Strukturen an.[40] Eine Nachbehandlung mit Kaliumphosphat-Puffer oder primären Alkoholen wie Methanol oder Ethanol erhöht den β-Faltblattgehalt und liefert wasserunlösliche Filme.[41, 42]

Biomedizinische Anwendungen, zum Beispiel für die Geweberekonstruktion, erfordern 3D Strukturen, die den Platz des ersetzten Gewebes einnehmen. Aus rekombinanten Spinnenseidenproteinen können Hydrogele hergestellt werden, die mit Zellen besiedelt die Grundlage für einen Gewebeersatz darstellen.[43] Durch additive Verfahren wie den 3D Dispensdruck kann das Material in die gewünschte Form gebracht werden.[44] Über Einstellung der Porengröße wird die Versorgung mit Nährstoffen und Metaboliten und eine Zellmigration ermöglicht.[43] Dies ist ebenfalls mit mesoporösen Schäumen aus rekombinanten Spinnenseidenproteinen der Fall, bei denen Natriumchlorid-Kristalle als Porogen die Porengröße vorgeben.[45]

1.2 Biomineralisation

Der Prozess der Biomineralisation unterliegt in höherem Maße der Kontrolle einer gerichteten Mineralisierung als reine Oxidations- oder Präzipitationsprozesse, die ebenfalls in der Natur zu finden sind. Dies führt zu einer höheren Ordnung der gebildeten Mineralphase in Hinblick auf Polymorphismus, da hierbei der biogene Kristall als Kompositmaterial aus organischen und anorganischen Anteilen entsteht. Somit können sich diese Minerale von ihren aus geologischen Prozessen gebildeten Gegenstücken stark unterscheiden.[46-48]

1.2.1 Biomineralisationsprozesse in der Natur

Biomineralisation ist ein Prozess, der in der Natur zum Beispiel zur Ausbildung von Stützstrukturen, Exoskeletten oder Zähnen dient (Tabelle 1).

Tabelle 1: Übersicht ausgewählter biomineralisierter Strukturen in Lebewesen.

Mineralart	**Taxon**	**Gewebe/ Vorkommen**	**Funktion**
Calciumphosphat (Apatit)[49, 50]	Vertebraten (Tetrapoden und Knochenfische)	Knochen/Zähne	Stütz- und Schutzfunktion, Lokomotion, Nahrungszerkleinerung
Calciumcarbonat[51, 52]	Vertebraten	Innenohr: Otokonien (Tetrapoden)/ Otolithen (Fische)	Gleichgewichtsorgan
Calciumcarbonat[53]	Vertebraten (Vögel, Reptilien)	Eierschale	Schutz des Embryos
Calciumcarbonat[54]	Mollusken	Mantel	Schutzfunktion
Calciumcarbonat[55]	Mollusken (Tintenfisch)	Schulp	Auftriebsorgan
Calciumcarbonat[56]	Echinodermaten (Seeigel)	Exoskelett	Schutz und Verteidigung
Calciumcarbonat[57]	Kalkflagellaten (Coccolithophorida)	Exoskelett	Schutz
Siliziumdioxid[58]	Kieselalgen (Diatomeen)	Exoskelett	Schutz
Eisenoxid (Magnetit)[59]	Bakterien (Magnetospirillum)	Zellorganell (Magnetosom)	Orientierung (Magnetotaxis)

Die zu Grunde liegenden Minerale sind hauptsächlich Calciumphosphat, Calciumcarbonat und Siliziumdioxid. Ferner können spezialisierte Organismen Magnetitpartikel für eine magnetotaktische Orientierung herstellen.[59, 60] Ein interessanter Aspekt der Biomineralisationsprozesse ist deren Ablauf unter milden Bedingungen, das bedeutet in wässrigem Medium, unter Atmosphärendruck und Umgebungs- oder Körpertemperatur aus lokal übersättigten Lösungen.[61] In der Regel bilden die beteiligten Zellen Membran-gebundene Matrixvesikel als Kompartimente aus, in denen die lokale Übersättigung stattfinden kann, ohne den Zellmetabolismus zu beeinträchtigen.[62]

Calciumcarbonat findet sich als Materialbestandteil überwiegend bei aquatischen Organismen, eindrucksvoll demonstriert in Korallenriffen, ist aber auch mineralischer Bestandteil der Eierschalen von Vögeln und Reptilien.[48, 53] In Mollusken sind gleich zwei Polymorphe an Calciumcarbonat nebeneinander vertreten, Calcit und Aragonit:[63] In der inneren Schicht der Muschelschale befindet sich Perlmutt. Dieses besteht aus regelmäßigen Aragonitlagen, die durch interlamellare Matrixanteile aus β-Chitin und assoziierten aziden Glykoproteinen verbunden sind.[64] Es trifft dann an der Grenzfläche zur weiter außen liegenden Prismenschicht auf Calcit.[65] Im Tintenfischschulp zeigen sich mikrostrukturierte Lagen an diesen beiden Calciumcarbonat-Polymorphen mit einer Protein- und Polysaccharid-haltigen organischen Matrix.[66] Die Mineralisationsprozesse beider Strukturen sind als Vesikel-gesteuerte extrazelluläre Mineralisation anzusehen.[67] Der Stachel eines Seeigels hingegen gleicht einem Einkristall mit eingeschlossenen Proteinanteilen in einer amorphen Ungleichgewichtsphase.[48, 56] Er wird typischerweise in einem vielzelligen Vesikel, einem Synzytium, gebildet und stellt den Übergang zwischen intra- und extrazellulärer Mineralisation dar.[67] Es wird angenommen, dass sich kristalline Phasen eines biogenen Minerals aus amorphen Vorläuferphasen bilden.[48] Diese unterliegen dann einer kinetischen Kaskade. Gerade amorphes Calciumcarbonat ist besser löslich als seine metastabilen Polymorphe Vaterit oder Aragonit, die sich dann wiederum in das thermodynamisch stabile Calcit umwandeln können.[48] Die weniger stabilen Vorläuferphasen konnten in synthetischen Ansätzen durch Polymere oder Aminosäuren stabilisiert werden, was diese Hypothese stützt.[68, 69]

Diatomeen sind einzellige Mikroalgen, die ihr komplex gestaltetes Außenskelett aus Siliziumdioxid, ähnlich der Schwämme, aufbauen.[70] Die Diatomeenzellwand hat den prinzipiellen Aufbau einer Petrischale mit zwei unterschiedlich großen Teilen, die ineinander greifen.[58] Neben einer sexuellen Vermehrung können sich Diatomeen auch

vegetativ durch Zellteilung fortpflanzen. Dabei muss auch der Neuaufbau der mineralisierten Zellwand garantiert werden. Dies geschieht durch eine Teilung der beiden Zellen, die anders als andere Zellen noch temporär zusammenbleiben, bis ihre neuen Zellwände mineralisiert sind.[71] Auch diese Biomineralisation ist über Membran-gebundene Vesikel gesteuert, die Kieselsäure als lösliche Form von Silizium enthalten, in der Zelle transportiert werden und nach Exozytose mit der Zellwand, der Silicalemma, verschmelzen.[58, 71] Eine Nanostrukturierung der Zellwand wird durch Silaffin-Proteine und langkettige Polyamine gesteuert, wobei nur die polykationischen Silaffine Silikatbildung fördern.[58, 70, 71]

Um im Magnetfeld der Erde navigieren zu können, bilden magnetotaktische Bakterien sogenannte Magnetosomen aus.[72] Es handelt sich in diesem Falle um rein intrazelluläre Mineralisation. Diese Organelle enthalten Magnetit-Nanopartikel, die ebenfalls von einer Membran kompartimentiert sind.[59] Sie sind typischerweise aneinandergereiht im jeweiligen Individuum zu finden.[72] Die Größe und Form der Nanopartikel ist genetisch programmiert und liegt bei dem Modellorganismus *Magnetospirillum gryphiswaldense* bei kuboktaedrischen Partikeln mit 35 nm Kantenlänge.[73] Eine Beteiligung von Proteinen am Biomineralisationsprozess ist unbestritten. Jedoch konnte den 18 identifizierten Proteinen in der Magnetosomenmembran derzeit noch keine spezifische Funktion zugewiesen werden.[73, 74] Im Vergleich der Sequenzen zu Proteinen, die bei anderen Mineralisationsprozessen eine Rolle spielen, konnten diejenigen Mam-Proteine (Magnetosom-abundante Membranproteine) erkannt werden, die den Transport von Metallkationen steuern.[73, 75] Durch genetische Manipulation der Membran[76] und der damit verbundenen Biomineralisation können neue Anwendungen der Magnetosomen wie zum Beispiel als Kontrastmittel[77] in der Kernspintomographie entstehen.[72]

1.2.2 Biomineralisationsprozesse in Vertebraten

Die Ausbildung eines mit Calciumphosphat mineralisierten Gewebes von Vertebraten erfolgt durch die gewebespezifischen Zellen im umliegenden Gewebe.[47, 78] Zu Beginn sind Vorläuferzellen beteiligt, die die Aufgabe der Initialisierung des Mineralisationsprozesses übernehmen:[62] Dieser wird zunächst über Matrixvesikel vermittelt, die von der Prä-Osteoblasten-Zellmembran abgelegt werden und akkumulierte Ionen enthalten.[67] Anschließend läuft der Prozess als extrazelluläre Mineralisation ab.[62, 79] Erste Apatitphasen, die sich in den Matrixvesikeln bilden, werden an die Umgebung abgegeben und binden an

spezifische Proteintemplate. Diese zunächst amorphen Vorläuferphasen werden durch diese Proteine stabilisiert und ein gerichtetes Kristallwachstum von Hydroxylapatit ($Ca_5[(PO_4)_3OH]$) findet an Kollagenfibrillen statt.[79]

Durch matrixgesteuerte Differenzierung werden diese Templat-Proteine in konzertierten Mustern exprimiert, die für eine anabolistische, aber nicht final pathogene Mineralisierung notwendig sind.[62] Es sind stark geladene Proteine der SIBLING (*Small Integrin Binding Ligand N-Linked Glycoprotein*) Familie,[80] die auch für eine Stabilisierung der zunächst gebildeten amorphen Calciumphosphat-Phase verantwortlich sind.[46] Darunter sind Osteopontin,[81] Osteocalcin,[82] Osteonectin[83] und Sialoprotein,[84] die die Biomineralisation im Knochen und Zahn steuern. Im speziellen Fall der Zahnmineralisierung sind ebenfalls Dentinmatrixprotein-1 und Dentinphosphophoryin involviert.[85] Es ist zu erwähnen, dass diese Proteine durch post-translationale Modifikation oft stark phosphoryliert sind, was die Attraktion von Calciumionen unterstützt.[80, 85] Die SIBLING-Proteine stellen Nukleierungszentren dar und exponieren in den meisten Fällen lokalisierte, negative Ladungen durch Peptidsequenzen reich an Glutaminsäure und Asparaginsäure.[80, 86-89] Im Speziellen werden Osteopontin und Sialoprotein als Promotoren der Mineralisierung angesehen,[90] wobei Arginin-haltige Proteine als Inhibitoren fungieren.[91] Generell findet die Kontrolle des Kristallwachstums oder Inhibierung über die Anteile an löslichen Proteinen statt, die Ionen komplexieren und der Mineralisation entziehen, und immobilisierten Proteinen, die die Mineralisierung voran treiben.[80, 91] Peptidsequenzen der nicht-kollagenen Proteine können als Motive zur Mineralisierung oder Kollagenbindung für biomimetische Mineralisationsansätze genutzt werden oder entsprechend ihrer Ladungseigenschaften als Vorbild für geladene synthetische Polymeradditive in Mineralisationsprozessen dienen.[92]

Diese nicht-kollagenen Proteine stellen jedoch nur bis zu 10 % der organischen Matrix des Knochenkomposits dar.[80] Kollagen Typ I, das den Hauptbestandteil der organischen Matrix und entsprechend 20-30 % der Gesamtmasse ausmacht, mineralisiert selbst nicht.[80] Die Zahnmineralisierung gestaltet sich sogar noch komplexer, da dabei zwei mineralisierte Phasen vorliegen: Zum einen der Schmelz, der keinerlei Kollagen enthält, zum anderen das Zahnbein, das dem Knochenmaterial ähnelt.[61] Außerdem ist im Zahnschmelz als weiteres Mineral Fluorapatit ($Ca_5[(PO_4)_3F]$) vorhanden.[93] Kollagen Typ I bildet eine tripel-helikale Struktur, die sich zu Fibrillen assembliert.[80] Durch gleichzeitige Bindung der nicht-kollagenen Proteine an die Kollagenfibrillen entsteht das organisch-anorganische Knochen-

Verbundmaterial. Es ist dabei eine geordnete Struktur zu erkennen, da Mineralisation in den Stoßregionen der Kollagenfibrillen zueinander und untereinander stattfindet (Abbildung 5). Somit wachsen Hydroxylapatit-Minerale von dort aus parallel entlang der Kollagenfibrillen. Diese Organisation liefert ein hochstrukturiertes Nano-Verbundmaterial mit verbesserten mechanischen Eigenschaften.[80]

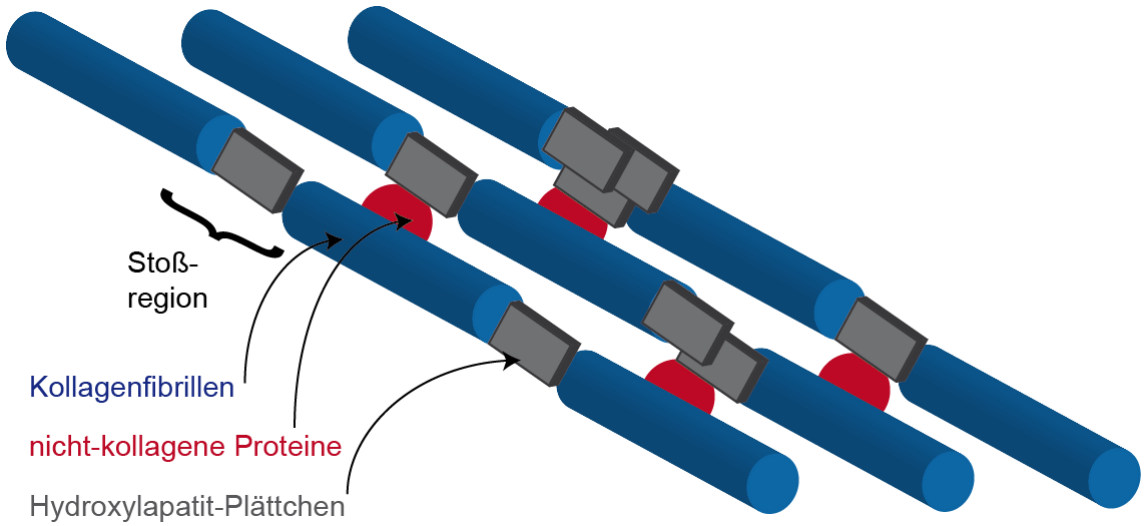

Abbildung 5: Modell der gerichteten Mineralisation in den Stoßregionen der Kollagenfibrillen. Dort findet die Bindung der nicht-kollagenen Proteine statt, von denen aus die Hydroxylapatit-Plättchen mineralisieren. (Eigene Darstellung)

Durch die starke Kontrolle des Mineralisierungsprozesses über Proteintemplate[94] bilden sich orientierte Mineralplättchen mit den Dimensionen 60-100 nm in der Länge, 30-50 nm in der Breite und 2-6 nm in der Höhe aus. Dies macht sie zu den kleinsten biogen vorkommenden Mineralen.[80] Die Bindung zwischen Kollagen und der mineralischen Phase ist noch nicht vollständig aufgeklärt, wird aber auf die Interaktion zwischen Aminosäureresten und Calciumionen zurückgeführt.[80] Mit zunehmender Mineralisierung verdichtet sich die Matrix des Gewebes und eingeschlossene Osteoblasten können nur noch passiv durch Diffusion versorgt werden. Sie sind dann im Stadium von voll differenzierten Osteozyten angekommen.[80] Da Diffusion von Sauerstoff und Metaboliten nur über eine begrenzte Wegstrecke (Mikro- bis Millimeter)[95] in Geweben erfolgreich ist, muss auch im Knochen eine Vaskularisierung vorhanden sein, die sich im neuen Knochenmaterial aus Endothelzellen bilden kann.[96] Da durch Wachstum eine flexible Anpassung des Skeletts an die Größe des jeweiligen Individuums von Nöten ist, übernehmen Osteoklasten die Aufgabe, Knochenmaterial stetig kontrolliert abzubauen.[97] Die Folge daraus ist, dass sich das gesamte Skelett eines Menschen während seiner Lebensdauer dynamisch anpasst.[61] Eine

altersbedingte Abnahme der Knochendichte ist im Normalfall auf ein vermehrtes Absterben von Osteozyten und Knochenumbau zurückzuführen.[98] Osteoporose hingegen ist eine pathogene Ausprägung dieser Indikation in Form einer metabolischen Knochenstörung. Sie führt neben verringerter Knochendichte ebenfalls zu architektonischen Veränderungen der Knochenstruktur. Das Auftreten kann in fortschreitendem Alter oder hormonellen Veränderungen, besonders bei Frauen, begründet sein.[98] Tritt solch eine Indikation auf, brechen Knochen bei Bewegung oder starker Last zunehmend schneller.[99] Auch die gegenteilige Ausprägung einer Fremdmineralisation von weichen Geweben stellt ein Krankheitsbild dar.[100] Eine Calcifizierung als Einlagerung von Calciumsalzen bis hin zur Ossifikation (Verknöcherung) wird in der Regel von regulatorischen Hormonen gesteuert.[101] In einer systemischen Störung stellt dies jedoch ein pathogenes Verhalten dar, das sich unkritisch äußern oder massive Beeinträchtigungen mit sich bringen kann.[102]

1.2.3 Mineralisierte Gewebe

Neben den vollständig mineralisierten Geweben der Knochen und der Zähne sind auch manche angrenzende Gewebe teilweise mineralisiert. Zu den teilmineralisierten Geweben zählen Knorpel, Bänder und Sehnen. Dies ist einem fließenden Übergang der zum Knochen hin angrenzenden Gewebe geschuldet, die weniger stark mechanisch belastet werden.[103]

Zwischen Knorpel und subchrondralem Knochen zeigt sich eine calcifizierte Knorpelschicht, die als mechanische und biochemische Barriere funktioniert.[104, 105] Der Knorpel weist so anstelle eines graduellen Übergangs zwei distinkte Schichten auf, die nicht-mineralisierte und die Mineral-aufweisende Knorpelschicht.[106] Dabei ist eine klare Abgrenzung der mineralisierten Anteile zum Knochen kaum zu treffen: Im Falle dieser Schicht zeigt sich die Ausbildung von dem Knochen ähnlich mineralisiertem Kollagen Typ II und Proteoglykanen als Hauptbestandteile des Knorpels.[104] Studien indizieren eine stärkere Mineralisierung dieser Schicht als im Knochen selbst, was auf ihre Funktion als eine abrasive Opferschicht hindeutet.[107, 108] Um diese Bedingungen in Ersatzgeweben wiederherzustellen, können Materialien mit distinkten Lagen[109, 110] oder Gradientenmaterialien zum Einsatz kommen.[106, 111]

Sehnen stellen das Bindeglied zwischen zwei stark unterschiedlichen Geweben, dem weichen Muskel und dem steifen Knochen, dar. Um eine Übertragung der Kräfte während

der Muskelkontraktion zu gewährleisten, muss die jeweilige Anbindung an den Gewebsübergängen graduell erfolgen, da sonst ein Ausriss der Sehne wahrscheinlich ist.[103] Der Mittelteil der Sehne besteht aus parallelen Kollagenfasern, die sich unter uniaxialem Zug strecken.[103] In Zugrichtung des Muskels werden 0,4 GPa übertragen, zur Knochenseite hin 20 GPa.[103] Der Übergangsbereich zwischen Sehnen und Knochen wird als Enthese bezeichnet.[112] Dabei können drei Zonen des Übergangs charakterisiert werden: Neben dem ersten, klar der Sehne zugehörigen Bereich, schließt sich ein Bereich mit faserigen Knorpelanteilen an. Der letzte Bereich vor dem Knochen selbst ist ein mineralisierter Faserknorpel.[103, 113] Generell lässt sich die Zunahme an Kollagenfibrillen-Desorientierung und Hydroxylapatitbildung zur Knochenseite hin beobachten (Abbildung 6).[114]

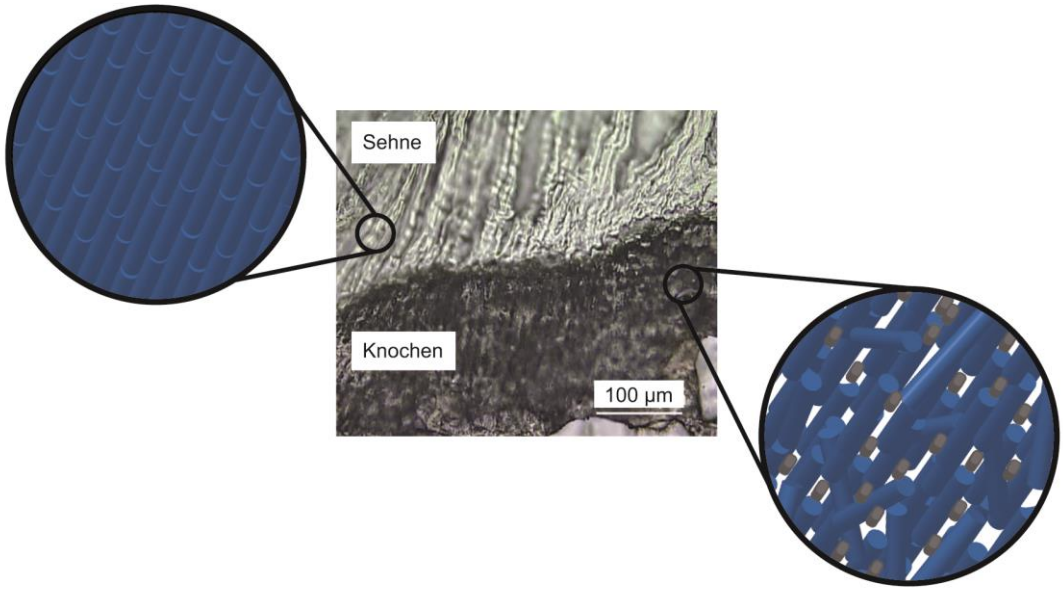

Abbildung 6: Lichtmikroskopische Aufnahmen von longitudinalen murinen Sehnenschnitten mittels Lasermikrodissektion am Knochenübergang. Schematische Darstellung: Der Bereich der Sehne zeigt regelmäßige Anordnung der Kollagenfibrillen (blau), der Knochenbereich ist zunehmend ungeordnet und mineralisiert (grau). Nachdruck unter Genehmigung modifiziert nach Deymier, A. C. *et al*., Micro-mechanical properties of the tendon-to-bone attachment. *Acta Biomater.* **2017**, *56*, 25-35. Copyright (2017) Elsevier.

Sehr ähnlich den Mineralisierungsprozessen im Knochen werden auch in der Sehne Matrixvesikel abgelegt, die für eine Mineralisierung notwendig sind. Da Sehnen aus longitudinalen Kollagenfibrillen bestehen, konnte die Verteilung dieser Vesikel mikroskopisch in Sehnenschnitten verfolgt werden.[79] Entsprechend ihrer graduellen Mineralisierung sind auch die Vesikel entlang der Sehne verteilt. In stärker mineralisierten

Regionen erscheinen die Abstände zwischen den Kollagenfibrillen größer, da hier Mineralisation ebenfalls extravesikular stattfindet und Apatitkristalle eingelagert werden.[79] Auch hier sind SIBLING Proteine an der Mineralisation beteiligt.[115]

Sollte durch zu hohe oder ruckartig auftretende Kräfte die Sehne, eventuell sogar samt Enthese, ausreißen, gestaltet sich eine natürliche Sehnenregeneration schwierig. Neben den mechanischen Eigenschaften, die auch in einer Rekonstruktion wiederherzustellen sind, weist das Gewebe nur wenige Blutgefäße und geringe Zellbesiedelung auf, welche für eine Gewebeheilung notwendig sind.[112] Somit wird das Sehnengewebe durch Narbengewebe ersetzt, das den mechanischen Ansprüchen nicht genügt.[103] Zwar reißen Sehnen seltener an der Enthese aus, jedoch ist eine möglichst narbenfreie Anbindung in das Muskel-Sehnen-Knochen-Konstrukt essentiell für den Erfolg der Sehnenrekonstruktion.[103, 113] In der Regel werden Sehnen aus anderen Körperregionen als Selbstspende verpflanzt.[103] Weiterhin sind künstliche Sehnenersatzkonstrukte im Einsatz, die maßgeschneiderte Lösungen mit gesponnenen Faserkonstrukten bieten sollen.[116-118]

Bänder verknüpfen weiche Gewebe oder Knochen je untereinander, indem sie diese lokal fixieren. Die Knochen-verbindenden Bänder sind ähnlich zu Sehnen aus parallelen Kollagenfaserbündeln aufgebaut und weisen dieselben Übergangsmechanismen hinsichtlich einer graduellen Mineralisation auf.[119] Ohne die Positionierung der Knochen durch Bänder kommt es zunehmend zu Verschleiß der betroffenen Gelenke, der sich vor allem im Knie äußert.[120, 121]

1.3 Übertragung natürlicher Prinzipien auf bioinspirierte Fabrikationsansätze

Seit jeher versucht der Mensch, Prinzipien aus der Natur zu verstehen, für sich zu nutzen und anwenden zu können. Eindrucksvoll zeigte sich dies beispielsweise im Erfindergeist Leonardo Da Vincis, was dessen Konstruktionszeichnungen zu Erfindungen wie der Flugmaschine, inspiriert durch den Vogelflug, belegen.[122] Neben einem bloßen Nachbau natürlicher Materialien steht auch das Verständnis der zu Grunde liegenden Anforderungen an beispielsweise die Mechanik, Statik oder Materialverarbeitung im Fokus.[122, 123] Diese Herangehensweise wird als *design by analogy* bezeichnet.[124] Im Falle, dass die Vorbilder der Biologie entspringen, spricht man von Biomimikry oder Bioinspiration.[124] Solche bioinspirierten Ansätze übertragen oft nur relevante Teilaspekte eines natürlichen Vorbilds auf einen neuen Fabrikationsansatz. Bekannte Anwendungen basierend auf Oberflächenstrukturierung sind unter anderem das Lotus-Prinzip[125] für selbstreinigende Oberflächen oder das Gecko-Prinzip[126] für kleberfreie Adhäsion.[122] Auch hier sei das Beispiel des rekombinanten Spinnenseidenproteins genannt, das auf Basis seines natürlichen Vorgängerproteins designt wurde und nun für verschiedenste Anwendungen zur Verfügung steht.[12]

1.3.1 Gradientenmaterialien

1.3.1.1 Funktionsprinzip natürlicher Gradientenmaterialien

Funktionelle Gradientenmaterialien sind in der Natur überall dort zu finden, wo zwei Materialien mit unterschiedlichen, meist mechanischen Eigenschaften miteinander verbunden werden. Eine graduelle Ausführung erlaubt dabei einen fließenden Übergang, der die lineare Ab- und Weiterleitung äußerer Kräfte von einer Materialseite zur anderen ohne Materialversagen ermöglicht. Treffen zwei ungleiche Materialien an einer Grenzfläche direkt aufeinander, wie es typischerweise in Kompositmaterialien der Fall ist, tritt Radialspannung auf und es kann zu einer „Sollbruchstelle“ kommen, an der das Material versagt.[127] Weiterhin ist neben einem Gradienten ein rein gradueller Übergang, auch als Stufengradient bezeichnet, möglich. Dies führt zu einer kleinstufigen Änderung der Eigenschaften. Im Optimalfall ist keinerlei distinkte Abstufung der Materialien entlang des

Gradienten zu erkennen.[127] Mit dem graduellen Verlauf von Eigenschaften geht auch ein Übergang von Strukturen (Morphologien, Porengrößen), unterschiedlichen Materialien oder Konzentrationen eines Materials in eine bestimmte Richtung einher.[128] Oft sind mehrere dieser Ansätze kombiniert und ergeben einen multidimensionalen Gradienten.[128] Als natürliches Vorbild sind hier der Byssusfaden der Miesmuschel, der Tintenfischschnabel und der Knochen-Sehnen-Übergang, die sogenannte Enthese, zu erwähnen (Abbildung 7). Die zu Grunde liegenden Gradientenverläufe werden hierzu näher diskutiert.

Neben seiner erstaunlichen Eigenschaft, mit einem Plaque Unterwasser-Adhäsion zu ermöglichen, ist der daran anknüpfende Byssusfaden der Miesmuschel (Gattung der *Mytilus*) ein Gradientenmaterial aus elastischen und fibrösen Proteinen.[129] Das Fadeninnere besteht aus zwei verschiedenen kollagenartigen Proteinen (sogenannte preCOLs), wobei die äußere Faserhülle *Mytilus foot protein-1* (Mfp-1) als Hauptbestandteil hat. Die Ausbildung eines chemischen Gradienten an 3,4-Dihydroxyphenylalanin (Dopa) von 10-15 mol-% in Mfp-1 mit höchster Konzentration in der Muschelfuß abgewandten, distalen Seite erfolgt post-translational.[130] Dopa wird in marinen Invertebraten als Adhäsionsvermittler verwendet und sorgt für die Anbindung an den Untergrund. Im Byssusfaden ist ein Gradient an distalen preCOLD- und proximalen preCOLP-Proteinen mit jeweils gegenläufig hohem Anteil zu finden, die einen mechanischen Gradienten erzeugen. Dies hat eine Versteifung des Materials hin zur distalen Seite, die am Untergrund ankert, zur Folge.[131]

Der Schnabel des Humboldt-Kalmars (*Dosidicus gigas*) ist wohl eines der härtesten, natürlich vorkommenden voll organischen Materialien. Selbst in seiner hydrierten Form können Kräfte von über zwei Größenskalen Unterschied (von 50 MPa bis 5 GPa) übertragen werden.[132, 133] Dies wird durch einen groß angelegten Steifigkeitsgradienten realisiert, da der Schnabel im weichen Gewebe verankert ist. Das Gradientenmaterial wird hier aus einem rein chemischen Gradienten bestehend aus Wasser, Chitinfasern und Histidin-reichen Proteinen, die Dopa-Seitenketten aufweisen, realisiert.[132, 133] Es findet eine graduelle Quervernetzung zwischen den Dopa- und Histidin-Resten hin zur härteren Schnabelseite statt.[132] Zusätzlich sinkt der Wasser- und Chitinanteil hin zur steiferen Seite. Eine optische Verfolgung des Gradienten ist anhand der Pigmentierung des Tintenfischschnabels möglich, da der Färbung Catechol zu Grunde liegt, das mit Dopa-reichen Proteinen in sklerotisierten Geweben von Insekten oder Wirbellosen in Verbindung steht.[132, 133]

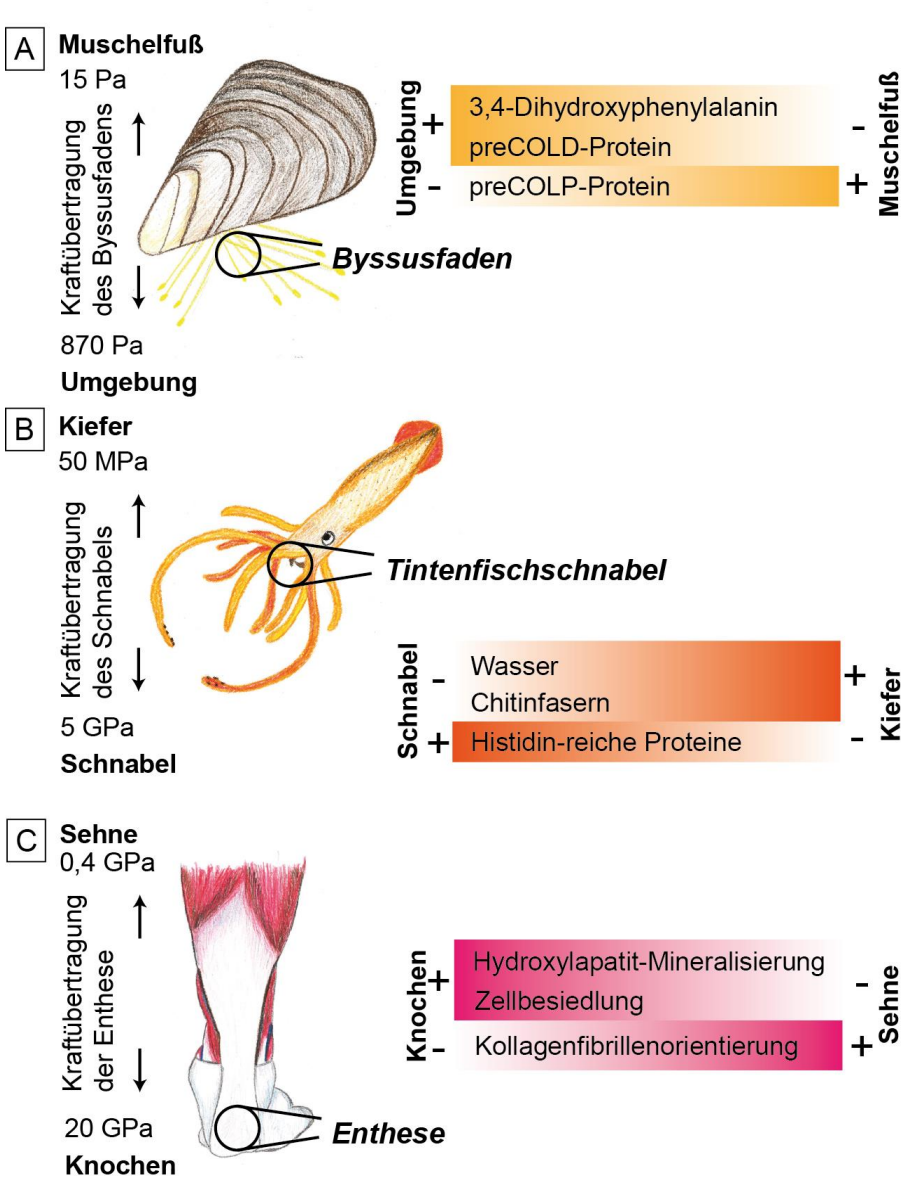

Abbildung 7: Beispiele für natürliche Gradientenmaterialien: A) Die Byssusfäden der Miesmuschel, B) der Papageienschnabel des Tintenfischs und C) die menschliche Enthese mit Angaben zu ihren jeweiligen mechanischen Eigenschaften und spezifischen Gradienten. (Eigene Darstellung, Werte nach Referenzen [103, 132, 134])

Die Enthese[112] ist durch den steilen graduellen Anstieg an Zellbesiedlung, Hydroxylapatit (Konzentrationsgradient) und einer Desorientierung der Kollagenfasern (struktureller Gradient) zur Knochenseite hin gekennzeichnet, um den Übergang von der weichen Sehnenseite hin zur steifen Knochenseite zu ermöglichen.[103, 135] Unter normaler Belastung wird so die bei Muskelbewegung auftretende Kraft von einem Material mit Steifigkeit im MPa-Bereich zu einem Material im GPa-Bereich übertragen. Das Gradientenmaterial, das nur einen Mikrometerbereich umspannt, wird somit aus einem

Kollagen-Hydroxylapatit-Komposit gebildet, der zum Knochen hin zunimmt.[114] Die graduelle Mineralisierung des Gewebes wird auch hier, ähnlich wie im Knochen selbst, von Proteinen der sogenannten SIBLING Familie gesteuert.[80, 103]

1.3.1.2 Erzeugung und Anwendung von Gradientenmaterialien

Das Funktionsprinzip von Gradientenmaterialien rückte ab den 1980er Jahren in den Fokus der Materialforschung. Es wurden Lösungen für technische Anwendungen gesucht und erstmals in der Raumfahrt durch einen Gradientenverlauf realisiert, um thermische Stressspitzen in Hitzeschildern zu minimieren.[136] Immer weitreichender wurden funktionelle Gradientenmaterialien (FGM) geschaffen und angewandt.[137] Dies kann über verschiedene Herstellungsmethoden geschehen, wie beispielsweise Pulvermetallurgie,[136, 138] Polymerisationsansätze,[139] Mikro-[140] und Makrofluidik,[116, 141, 142] Photolithographie[143] oder Elektrospinnen[118, 137]. Damit werden Gradientenmaterialien mit unterschiedlicher Materialzusammensetzung,[116, 138] Mechanik[118, 137, 144] oder Binnenstruktur[117, 145-147] generiert.

Gradientenmaterialien für die biomedizinische Anwendung werden vor allem im Zusammenhang mit porösen Knochenmaterialien oder mechanisch anspruchsvollen Sehnenersatzmaterialien benötigt. Graduell poröse Strukturen, die eine biologisch relevante Porengröße für die Knochenrekonstruktion abdecken, können verschieden ausgeführt werden.[145] Um eine personalisierte Lösung für Patienten herstellen zu können, rückt der 3D Druck als Methode der Wahl in den Vordergrund. Graduelle Konstrukte aus Biomaterialien werden so durch unterschiedlich angesteuerte Extrusion zweier Materialien generiert, von denen beispielsweise ein Material mit einem anorganischen Füllmaterial beladen ist.[148] Durch interne Steuerung des 3D Druckers[149] oder durch zwei gegenläufige Spritzenpumpen[141] werden beide Materialien mit unterschiedlicher Geschwindigkeit extrudiert (Abbildung 8). So kann beispielsweise eine graduelle Biomineralisation durch Füllstoffe in einer Komponente erzeugt werden, um die Materialsteifigkeit gezielt zu erhöhen. Die so entstandenen Konstrukte eignen sich für die Anwendung in der Entheserekonstruktion. Das graduelle Einbringen von zwei Füllstoffen unterschiedlicher Größe kann dem Zweck der Knochenrekonstruktion dienen. Die Vermischung findet dann in einer Mischkammer statt und das in seiner Konzentration vordefinierte, gemischte Material wird extrudiert.[111, 149, 150] Diese Art der graduellen Verarbeitung setzt aber in der

Regel die Modifikation des Gerätes mit Anbau eines Doppel-Kartuschen-Mischkopfes voraus, sodass oft ein kostspieliger Umbau notwendig wird und die weitergehende Nutzung eingeschränkt ist.[151]

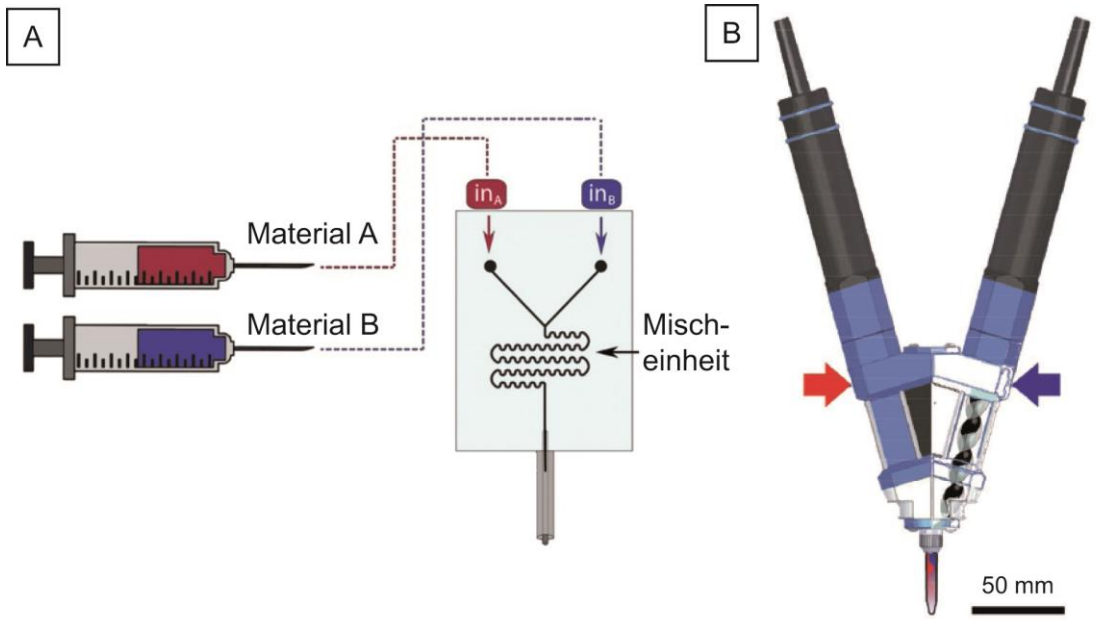

Abbildung 8: Schematische Darstellung der Realisierung von 3D Gradientenmaterialien. A) Spritzenpumpe befüllt mit unterschiedlichen Materialien A und B, die in einer Mischeinheit aufeinandertreffen. B) Extrusionsdüse mit zwei Einlässen für unterschiedliche Materialien. Nachdruck unter Genehmigung modifiziert nach A) Idaszek, J. *et al.*, 3D bioprinting of hydrogel constructs with cell and material gradients for the regeneration of full-thickness chondral defect using a microfluidic printing head. *Biofabrication* **2019**, *11* (4), 044101. Copyright (2019) IOP Publishing. B) Kokkinis, D.; Bouville, F.; Studart, A. R., 3D Printing of Materials with Tunable Failure via Bioinspired Mechanical Gradients. *Adv. Mater.* **2018**, *30* (19), 1705808. Copyright (2018) John Wiley & Sons.

1.3.2 Biofabrikation

Das Feld der Biofabrikation hat in den letzten Jahren rasant an Popularität gewonnen. Mit der Erstausgabe der Fachzeitschrift *Biofabrication* (Verlag IOP Publishing, Bristol, Großbritannien) im Jahr 2009, die sich diesem Themenkomplex widmet, wurde nach Mironov *et al.*[152] erstmals der Begriff definiert: Es handelt sich dabei um die Überschneidung der Fachdisziplinen Biologie, Materialwissenschaften und Maschinenbau.

Dabei entstehen „*lebende und nicht lebende biologische Produkte, die aus Materialien wie lebenden Zellen, Molekülen, extrazellulärer Matrix und Biomaterialien bestehen*“ [152].

Der Begriff Biomaterial definiert dabei ein Material, das nicht zwingend biologischen Ursprungs ist, aber vorteilbringend in den Körper eingebracht werden kann.[153, 154] Einerseits liefert die Orientierung an der Biologie bioinspirierte und biomimetische Ansätze.[152] Resultat dieser Fabrikation sind artifizielle Produkte, die von „Burgerfleisch aus der Petrischale“[155] bis hin zu künstlichen Geweben für die Biomedizin reichen. Darunter fallen auch Biosensoren zur medizinischen Überwachung, die ähnlich einem Pflaster auf die Haut aufgebracht werden können.[156] Eine Simulation von organartigen Funktionen für die Vorhersage von Verhaltensweisen in Bezug auf Wirkstoffverträglichkeit oder von Krankheitsmodellen kann mittels *organ on a chip* realisiert werden.[157] Weiterhin kann der Anspruch auch in der guten Verträglichkeit von pharmazeutischen Produkten, wie neuartigen Wirkstofftransportsystemen[33] oder einer bioinerten Beschichtung von medizinischen Schläuchen[158] oder Implantatoberflächen gegen Ausbildung von Kapselfibrose[159], liegen. In anderen Anwendungen ist die Bioaktivität von Oberflächen für die Integration von Implantaten explizit erwünscht, beispielsweise bei in den Knochensockel eingebrachten Titanimplantaten.[160] Andererseits zählen nach dieser Definition ebenfalls neue bioinspirierte Wege der Treibstofferzeugung[161] oder der Katalysatorforschung[162] dazu.

Folglich umfasst diese Forschung ein zunehmend größer werdendes Feld, wobei der Begriff Biofabrikation oft dynamisch über die Fortschritte in Technologien und Anwendungsbereichen definiert wird, was jedoch zu einer Inkonsistenz der Terminologie führt.[163] Eine Abgrenzung zu den Disziplinen der regenerativen Medizin und *Tissue Engineering* sowie der additiven Fertigung ist schwieriger geworden. Da diese Begriffe oft synonym verwendet wurden, wurde nach Groll *et al*.[163] eine erneuerte Begriffsdefinition angestrebt. Diese beinhaltet explizit die Schnittmenge der Disziplinen als „Biofabrikation für *Tissue Engineering* und die regenerative Medizin”. Diese Neudefinition dient einer Erweiterung der Begrifflichkeit und schließt dabei auch die Herstellungsverfahren für Zell-beladene Konstrukte mit ein.[163, 164] Es handelt sich um „*die automatisierte Herstellung von biologisch funktionellen Produkten mit einer strukturellen Organisation bestehend aus lebendigen Zellen, bioaktiven Molekülen, Biomaterialien, Zellaggregaten wie beispielsweise Mikro-Geweben oder hybriden Material-Zell-Konstrukten, die durch Biodrucken oder Bioassemblierung und anschließender Gewebereifung gewonnen werden*“ [163]. Im Folgenden

sollen nun die zu Grunde liegenden Prinzipien des Ansatzes *Tissue Engineering* näher erläutert werden.

1.3.2.1 Tissue Engineering

Unter dem Begriff *Tissue Engineering* versteht man Fabrikationsansätze mit dem Ziel, Gewebe teilweise oder vollständig zu ersetzen. Der Verlust eines Gewebes oder gar Organs führt zu Einschränkungen oder vollständigem Einstellen der zu Grunde liegenden Funktion.[165] Die daraus entstehende medizinische und ökonomische Problematik wurde Anfang der 1990er Jahre von Langer und Vacanti erkannt und *Tissue Engineering* als Lösung vorgeschlagen.[166] Der momentane Stand zum Gewebeersatz beruht nach wie vor auf Auto-, Allo- oder Xenotransplantation. Bei Autotransplantation können Teilgewebe des Spenders an einen anderen Ort im Körper verpflanzt werden, beispielsweise Hautlappen nach Verbrennungen. Dies kann jedoch an der Spenderstelle zu sekundärer Morbidität führen.[167] Allotransplantation bezieht sich auf Spenderorgane fremder, toter oder lebender Personen. Es besteht nach wie vor eine größere Nachfrage an Spenderorganen als diese zur Verfügung stehen.[168] In diesem Fall muss die Abstoßung des Gewebes oft für lange Zeit durch Immunsuppressiva unterdrückt werden.[169] Dies spitzt sich noch zu, wenn das Transplantat nicht vom Menschen stammt, sondern tierischen Ursprungs ist (Xenograft).[170] Dazu kommt das Risiko der Übertragung von tierischen Krankheiten auf den Menschen.[171, 172]

Aus den komplexen Anforderungen an den Gewebeersatz stellen sich einige Kernfragen, die bei der Gerüstherstellung berücksichtigt werden müssen. Größere Defekte oder gar Organverlust führen im Körper zum Kollabieren der umliegenden Strukturen. Das bedeutet, dass temporäre „Platzhalter“ geschaffen werden müssen, um eine Kavität für das neu entstehende Gewebe freizuhalten. Durch den voranschreitenden Gewebeaufbau kann dann das entsprechende Gerüst resorbiert werden.[165] Daher muss im idealen Fall der Bioabbau des Biomaterials synchron mit der entsprechenden Geweberekonstruktion ablaufen, was für den Fall von zum Beispiel Haut innerhalb von wenigen Tagen oder im Knochen von mehreren Wochen der Fall ist.[173]

Für diese Herausforderung steht eine Vielzahl an Biomaterialien zur Verfügung, die in der Regel biologischen Ursprungs oder bioinspiriert sind, beziehungsweise synthetisch

hergestellt werden. Synthetische Polymere, die häufig Anwendung in der Geweberekonstruktion finden, sind zum Beispiel Polyethylenglycol, Polymilchsäure oder Poly-ε-Caprolacton.[168, 174] Ihr entscheidender Nachteil ist jedoch, dass sie bioinert sind und eine Funktionalisierung nachträglich eingefügt werden muss.[165] Hinzu kommt, dass zwar nicht das Material selbst, aber seine Abbauprodukte, insbesondere von synthetischen Polymeren, toxisch sein können.[168] Im Falle von Biomaterialien wie Kollagen, Gelatine oder Fibronektin aus tierischen Quellen besteht, wie bei Xenotransplantaten, die Gefahr der Krankheitsübertragung.[172] Das Polysaccharid Alginat[175] kann ebenfalls verarbeitet werden und wird in der Regel bei Prozessierung zu Hydrogelen mit Calciumionen vernetzt.[176, 177] Chitosan wird aus Chitin der Krabbenschalen nach Deproteinierung und Decalcifizierung unter Verkürzung der Kettenlänge und Reduzierung des Acteylierungsgrads gewonnen.[178, 179] Wie bei anderen Materialien kommt es auch bei Chitosan zu Schwankungen zwischen den Materialchargen, was vor allem für die medizinische Anwendung streng kontrolliert wird.[180] Rekombinant hergestellte Proteine, unter denen sich Kollagene[181] und Seidenproteine[20, 182] befinden, lösen einige dieser Probleme und ermöglichen eine großtechnische Herstellung.

Um eine Abstoßung oder Unverträglichkeit zu vermeiden, müssen Gerüstmaterialien biokompatibel sein und nach Möglichkeit dem zu ersetzenden Gewebe weitestgehend ähneln. Ein Weg zur personalisierten Medizin ist es, Spenderzellen des Zielgewebes zu entnehmen, zu kultivieren und auf das in seiner Form, Größe und Gewebespezifität personalisierte Gerüst zu geben.[168] In der Regel sind bei diesem Vorgehen nur wenige Spenderzellen notwendig.[154] Bei ausreichender Zellbesiedlung und eventuell beginnender Differenzierung wird das Konstrukt an der Defektstelle eingebracht. Es können für diese Herangehensweise zwei Ansätze verfolgt werden: Zum einen können aus einzelnen Bestandteilen, wie zum Beispiel zellbeladenen Matrizen, Gewebe modular zu einem (3D) Konstrukt aufgebaut werden.[183] Zum anderen können Trägermaterialien 3D Strukturen vorgeben, die nachträglich mit aktiven Komponenten wie Zellen, Wachstumsfaktoren oder Wirkstoffen besiedelt werden. Das Verständnis von Struktur-Eigenschafts-Beziehungen ist dabei nützlich, um eine gerichtete Zelladhäsion zu erzielen. Zelladhäsion findet beim Aussäen auf eine Oberfläche in einem zweistufigen Prozess statt.[184] Zu Beginn ist die topographische Beschaffenheit der Oberfläche, wie seine Rauheit oder Strukturierung, von Bedeutung. Erst danach werden die chemischen Eigenschaften, wie die Bereitstellung von Bindungsmotiven, erkannt.[185] Solche fokalen Adhäsionsprozesse dienen zudem der

komplexen Signalübertragung von der extrazellulären Matrix zur Zelle: Sie finden mit der Zell-umgebenden Matrix über Integrine in der Zellmembran und dem Aktin-Zytoskelett statt.[186]

Die Größe und Form einer Defektstelle im Patienten kann oft mit Hilfe von Computertomographie genau bestimmt werden. Anhand der bildgebenden Verfahren wird mittels Computer-gestütztem Design (*computer aided design*, CAD) und Herstellung (*computer aided manufacturing*, CAM) ein personalisiertes Gewebekonstrukt erstellt (Abbildung 9).[187] In diesem Zuge ist der 3D Druck eine geeignete Wahl, um ein solches Vorgehen durch Ablegen der einzelnen Konstruktlagen in der additiven Fertigung zu realisieren.[183]

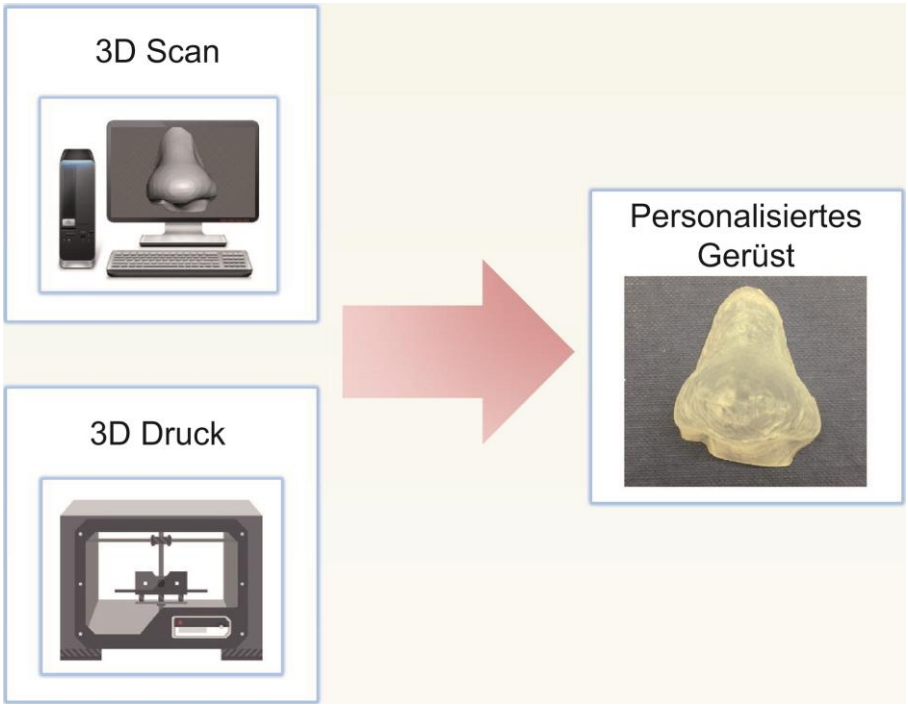

Abbildung 9: Prozess der Herstellung von personalisierten Gewebekonstrukten basierend auf einem 3D Scan mit Hilfe des 3D Drucks. Nachdruck unter Genehmigung modifiziert nach Goyanes, A. *et al.*, 3D scanning and 3D printing as innovative technologies for fabricating personalized topical drug delivery systems. *J. Control. Release* **2016**, 234, 41-48. Copyright (2016) Elsevier.

Einige Biomaterialien können zu Hydrogelen prozessiert werden, die sich verdrucken lassen.[165] Darunter befinden sich Seidenmaterialien, in die direkt Zellen eingekapselt werden können. So entstandene Biotinten können im 3D Druck direkt funktionale Konstrukte liefern.[44] Jedoch gibt es das ein oder andere Hindernis auf diesem Weg der Verarbeitung:

Die Druckgenauigkeit liegt bei Biopolymeren oft unter der von synthetischen Polymeren.[165] Außerdem ist die Viskosität eines zellbeladenen Materials entscheidend, da zwar stabile Konstrukte nötig sind, aber die Zellen während des Drucks bei steigender Viskosität immer stärkeren Scherkräften ausgesetzt sind.[188] Auch der Durchmesser der Drucker-Extrusionsdüse hat einen Einfluss auf das Zellüberleben und ihr späteres Verhalten.[189, 190] Die aktuelle Forschung beschäftigt sich mit der Detektion von Scherkräften während des Drucks in Modellen, um das Überleben von Zellen abschätzen zu können.[191]

1.3.2.2 Geweberekonstruktion mit rekombinanter Spinnenseide

Die Abdeckung von Wunden mit Spinnennetzen und ihre anti-inflammatorische Wirkung, ohne eine Immunantwort auszulösen, war schon bei den alten Griechen bekannte Praxis.[192] Somit sind die vorteilhaften Eigenschaften von Seidenmaterialien für biomedizinische Anwendungen schon lange bekannt. Neben Spinnenseide ist dabei auch die Seide des Seidenspinners *Bombyx mori* von Interesse. Im Gegensatz zu Spinnen kann dieser in großen Zuchtbetrieben auf Maulbeerblättern gehalten und seine Seide geerntet werden. Diese Rohseide muss dann einem Prozess unterzogen werden, der die äußere Beschichtung der Seidenfasern ablöst. Der Prozess wird als *degumming* bezeichnet und findet unter Kochen bei 60 °C in Lithiumbromid statt. Die dabei entfernten Sericine sind bekannt, Immun- und inflammatorische Antworten im Körper auszulösen. Das dann gewonnene Seidenmaterial kann gelöst und prozessiert werden.[7, 193]

Um eine einfache Herstellung in großen, homogenen Mengen und eine geeignete Prozessierung von Spinnenseidenproteinen zu ermöglichen, wird oft der Weg einer rekombinanten Herstellung gewählt. Die so gewonnenen Proteine eignen sich für biomedizinische Anwendungen. Eine Auswahl solcher Anwendungsbeispiele ist in Tabelle 2 zusammengefasst. Die Zelladhäsion ist für den Erfolg eines Biomaterials in der Geweberekonstruktion entscheidend. Durch molekularbiologische Methoden oder nachträgliche chemische Kopplung können rekombinante Spinnenseidenproteine mit Zellbindungsmotiven wie dem bekannten RGD-Tripeptid funktionalisiert werden.[27] Die somit entstandene Zytokompatibilität ermöglichte Zell-Matrix-Interaktion mit Spinnenseidenproteinen in unterschiedlichen Morphologien wie 2D Filmstrukturen[27] oder 3D Hydrogelen.[44]

Tabelle 2: Übersicht ausgewählter Anwendungsbeispiele von rekombinanter Spinnenseide in der Geweberekonstruktion.

Zielgewebe	Spinnenseiden-morphologie	Verwendete Zellen	Hergestellte Funktion
Herz-muskel[194, 195]	Film als Beschichtung von Glasträgern	Kardiomyozyten, Fibroblasten, Endothelzellen	Synchrone Kardiomyozyten-Kontraktion durch Zell-Zell-Kommunikation/ elektrische Signalweiterleitung, Proliferation
Gefäßsystem[196]	Fasern mittels Nassspinnen und Elektrospinnen	AV-loop als *in vivo* Rattenmodell	Neuausbildung von Vaskularisierung
Knochen[197]	Spinnenseiden-Polymer-*blend*-Filme	Humane mesenchymale Stammzellen	Osteo-Zelldifferenzierung
Knochen[198, 199]	Filme	Humane mesenchymale Stammzellen	Osteo-Zelldifferenzierung
Sehnen-Kochen-Übergang[200]	(Gradienten)Filme	Knochenvorläuferzellen der Ratte	Graduelle Zelladhäsion
Periphäres Nervensystem[201]	Tubulär gerollte, elektrogesponnene Vliese mit eingelegten Kollagen-Fasern	Neuroblastoma-Glia-Hybridzellen	Ausbildung von Synapsen und Membranpotentialen
Periphäres Nervensystem[202]	Selbstrollende Chitosan-Spinnenseiden-Filme mit eingelegten gesponnenen Vliesen oder Kollagen-Cryogelen	Neuronen	Zelldifferenzierung und Ausrichtung
Haut[203]	Poröse Filme/Membranen	*in vivo* Rattenmodell	Gesteigerte Wundheilung

Es ist bekannt, dass nicht nur chemische, sondern auch topographische Eigenschaften einer Substratoberfläche die Zelladhäsion und -differenzierung beeinflussen.[184, 204, 205] Strukturgebung kann durch Aufbringen von Schikanen wie Kavitäten, Erhebungen oder Rillen auf 2D Filmen erfolgen.[184, 206, 207] Es ist aber auch eine 2.5D nanofibrilläre Strukturierung, wie sie typischerweise durch Elektrospinnen entsteht, von Vorteil, um die extrazelluläre Matrix abzubilden.[184, 208] Solche 2.5D Strukturen bilden gesponnene Vliese aus Spinnenseidenproteinen, die durch Nachbehandlung mit primären Alkoholen wasserunlöslich gemacht werden.[209] In der Geweberekonstruktion können sie für die Regeneration von Hautschichten[210] oder als Wundauflage[209, 211] nach dem historischen

Vorbild dienen. Spinnenseidenproteine können zu Hydrogelen verarbeitet werden, was eine personalisierte Formgebung im 3D Druck ermöglicht. Bei der Herstellung von Biotinten aus eADF4(C16)-RGD konnten zudem Fibroblasten eingebracht und während der Gelierung verkapselt werden. Das Verdrucken des Materials lieferte homogen verteilte Zellen, die über einen längeren Zeitraum in Kultur gehalten werden konnten.[44] Dabei spielte vermutlich auch die intrinsische Porosität der Hydrogele[43] eine Rolle, die eine Versorgung mit Nährstoffen und Sauerstoff gewährleistet, sowie für den Abtransport von Metaboliten essentiell ist.

Für die Geweberekonstruktion spielen aber auch weitere Faktoren eine Rolle. So hat die Elastizität eines Gerüsts Einfluss auf Zelldifferenzierung, was als Mechanotransduktion bezeichnet wird. Im Falle von Seidenmaterialien wird die Steifigkeit durch den Gehalt an *β*-Faltblattstrukturen eingestellt, das heißt, sie steigt mit größerem Anteil an dieser Sekundärstruktur.[192] Ein Beispiel, bei dem die zu Grunde liegende Substratsteifigkeit Einfluss auf die (neu angesiedelten) Zellen hat, ist die Narbenbildung nach einem Herzinfarkt.[212] Das geschädigte Herzgewebe weist dadurch eine höhere Steifigkeit auf, sodass rekrutierte oder implantierte Stammzellen sich nicht zu Kardiozyten sondern Fibroblasten differenzieren.[213, 214] Die Nutzbarkeit von Materialien aus rekombinanten Spinnenseidenproteinen für die Regeneration von Herzgeweben wurde bereits materialwissenschaftlich untersucht.[194, 195] Damit könnte das Material beispielsweise für Herzmuskelauflagen zur Regeneration und Behandlung von Herzinsuffizienz eingesetzt werden.

1.3.2.3 Biomimetische und bioinspirierte Mineralisation

Biomedizinische Anwendungen

In manchen Fällen ist eine körpereigene Regeneration von mineralisierten Geweben nicht möglich. Dies ist der Fall bei Verlust größerer Mengen an Knochenmaterial, zum Beispiel als Folge von Krebserkrankungen oder Osteoporose. Entsprechend müssen Ersatzmaterialien an der Defektstelle eingebracht werden, um eine Stabilisierung und Regeneration des Gewebes zu erreichen.[215] Titanimplantate sind für viele Knochenanwendungen derzeit das einzige Mittel der Wahl, haben jedoch den entscheidenden Nachteil, dass sie sich oft schlecht mit dem umgebenden Gewebe verbinden und dies chirurgische Zweiteingriffe nach sich zieht.[160] Obwohl mittlerweile eine Vielzahl

an Biomaterialien zur Verfügung stehen,[154, 216] müssen verschiedene Kriterien für die Rekonstruktion mineralisierter Gewebe gegeben sein. Neben der geeigneten Mechanik sollte die Besiedlung durch gewebsspezifische Zellen und damit einhergehend Mineralisation möglich sein.

Betrachtet man ein Konstrukt zum Ersatz mineralisierter Gewebe, können drei Eigenschaften voneinander abgegrenzt werden: Die Osteoinduktion eines Materials bezeichnet den Stimulus auf pluripotente, nicht differenzierte Zellen, sich zu Osteoblasten zu differenzieren. Unter Osteokonduktion versteht man die Eigenschaft eines Materials, Knochen an seiner Oberfläche zu bilden. Zuletzt wird die vollständige Verankerung eines Materials in den Knochen als Osseointegration bezeichnet.[217]

Mineralisation kann in Konstrukten für die Geweberekonstruktion über drei Ansätze erfolgen: Zum einen können Materialien designt werden, die auf Grund ihrer Ladung Wechselwirkung mit Ionen des umgebenden Mediums eingehen und eine Mineralisation zur Folge haben (Abbildung 10A). Um diese Wechselwirkung zu verstärken und zu kontrollieren, können Bindungsmotive verwendet werden, die in natürlichen, mineralisierenden Proteintemplaten identifiziert wurden. Darunter befinden sich vor allem stark geladene Peptide, die aus SIBLING Proteinen wie Osteopontin[218, 219] oder Sialoprotein[220] stammen. Weiterhin wurden mittels Phagendisplay Peptidsequenzen identifiziert, die spezifisch an Hydroxylapatit binden und das Potential zur Biomineralisation bergen.[198] Diese wurden dann an die Gensequenz eines Proteinrückgrats mit beispielsweise repetitiven Motiven aus der Spinne *Nephila clavipes*[198] oder des Seidenspinners *Bombyx mori*[221] gekoppelt, rekombinant hergestellt und ihr Mineralisationsverhalten untersucht. Dazu wurden Calcium-, Phosphat- oder Carbonathaltige Mineralisierungslösungen auf den Materialien inkubiert.[197, 198, 222, 223] Weiterhin ist es möglich, eine biomimetische Mineralisation durch Inkubation in *Simulated Body Fluid* (SBF) zu erzeugen.[224, 225] SBF enthält eine Ionenkonzentration und -zusammensetzung, die der des menschlichen Blutplasmas stark ähnelt. Somit können Materialien *in vitro* auf ihre Tauglichkeit für den Knochenersatz getestet werden. In der Regel beginnt eine Mineralisation in diesem Aufbau schon nach wenigen Tagen und schreitet bis zu vier Wochen fort.[226] Das gebildete Mineral enthält häufig Calciumphosphat-Spezies, zum Beispiel Hydroxylapatit.[226] Neben einer induzierten (Bio-)Mineralisation kann direkt ein anorganischer Füllstoff in eine (Bio-)Materialmatrix als Kompositmaterial eingebracht werden (Abbildung 10B).[227-232]

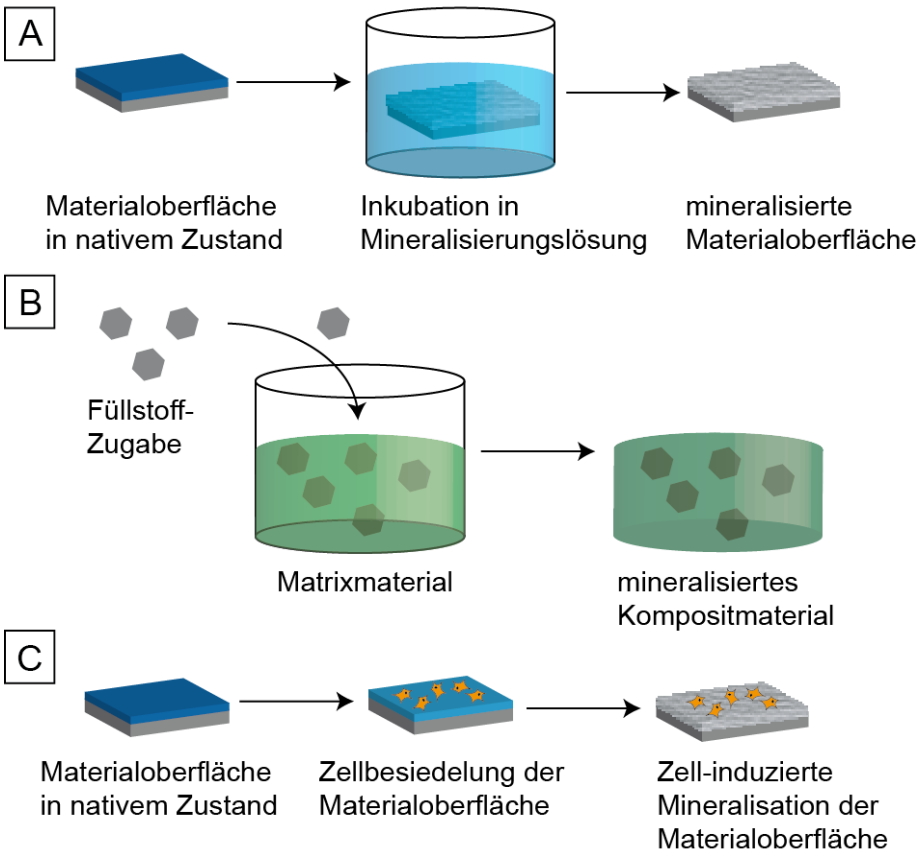

Abbildung 10: Herangehensweisen zur Mineralisation von Materialien. A) Induzierte Mineralisierung aus Lösung. B) Einbringen von Mineralphasen in ein vorgelegtes Matrixmaterial. C) Zellinduzierte Mineralisation. (Eigene Darstellung)

Dies hat den Vorteil, dass sofort eine mineralische Phase im Material vorhanden ist und das Gerüst nicht erst über längere Zeit nachbehandelt werden muss. Gerade bei simultanem Einbringen von Zellen ist eine solche Nachbehandlung nicht denkbar. Nichtsdestotrotz können auch solche Materialien mit Mineralisierungslösungen nachbehandelt werden.[227, 231] Dadurch kann Hydroxylapatit abgeschieden werden und die Zelladhäsion, -proliferation und -differenzierung, vor allem von Osteoblasten, verbessern.[198, 222, 233] Diese Art der Herstellung stellt eine einfache Variante dar, Füllmaterialien nach gewünschter Konzentration und Zusammensetzung in ein Biomaterial einzubringen. Das Material selbst kann zum Beispiel via 3D Druck oder Lyophilisation[228, 231] in die gewünschte Form gebracht werden.

Vergleicht man diese Art der biomimetischen Mineralisation mit den Biomineralisationsprozessen, die im Knochen stattfinden, ergeben sich Parallelen und Unterschiede. Man stellt fest, dass zuerst eine vergleichsweise weiche Matrix vorhanden ist,

in der sich Prä-Osteoblasten ansiedeln.[80] Die voranschreitende Mineralisation wird dann durch diese Zellen ausgelöst, indem nach konzertierten Genexpressionsmustern Proteine sekretiert werden.[85] Das Zusammenspiel und die zeitliche Abfolge der Expression führen dann zu einer gerichteten Mineralisation.[62] Auch hier können biomimetische Ansätze zur mineralisierten Geweberegeneration angreifen (Abbildung 10C). Über die Wahl der geeigneten Materialsteifigkeit[234], Vormineralisation[78, 234] oder Differenzierungsmedien[235-238] konnte Osteoblastendifferenzierung eingeleitet werden.

Betrachtet man die Rekonstruktion teilmineralisierter Gewebe, wie dem Knochen-Sehnen-Übergang oder den Knochen-Knorpel-Übergang, können fibröse Gerüste mit einer gewebten Innenstruktur anstelle von Mineralisation die benötigte Mechanik erreichen.[239]

Technische Anwendungen

Für Materialien in technischen Anwendungen sind vor allem Oxide und Carbonate von Interesse (Tabelle 3). Ihre Darstellung mittels Biomineralisation hat verschiedene Vorteile: So kann während des Prozesses auf sonst üblicherweise hohe Temperaturen und organische Lösungsmittel verzichtet werden.[240, 241] Zusätzlich spielen neben den ungiftigen Edukten in der Synthese auch reduzierte Kosten eine Rolle.[242, 243]

Tabelle 3: Übersicht ausgewählter Beispiele für biomineralisierte Produkte in technischen Anwendungen.

	Mineralspezies	Anwendung
Carbonate	$MnCO_3$	Kondensatoren,[244] Anodenmaterial in Lithiumionen-batterien[245]
	$CaCO_3$	Füllstoff in der Herstellung von Kunststoffen und Kautschuk,[246] CO_2-Bindung aus der Atmosphäre,[247] Verlängerung der Lebenszeit von Bausubstanzen[248]
Oxide	MnO	Abwasserreinigung[249]
	Fe_3O_4	Katalyse,[250] Wirkstofftransportsystem,[251] Diagnostik[252]
	ZnO	Solarzellen/Laserdioden,[253] antimikrobielle Materialien[254-257]
	Co_3O_4	Energieumwandlung,[258] Wirkstofftransport/Diagnostik/ Radiotherapie[259]
	CuO	Antibakterielle Materialien,[242] Abwasserreinigung[243]

Besonders vielseitig erwies sich Calciumcarbonat, das das Einbringen von bioinspirierten Templaten wie löslichen Polymeren zum Generieren von amorphen,

flüssigen Vorläuferphasen[260, 261] und hierarchischen Mesostrukturen[262] erlaubte. Dabei war auch eine Kontrolle über die gewünschte Kristallmorphologie möglich.[263, 264]

Durch Kenntnis des Kristallisationsprozesses von Calcit, der mit einer wenig kristallinen Vorläuferphase beginnt und durch Proteintemplate in seiner Nukleierung und Wachstumsphase weiter voranschreitet, konnten ähnliche Mechanismen für andere Minerale abgeleitet werden.[244, 253] In weiteren Mineralisationsansätzen konnten sogar Pflanzenextrakte[242, 243, 254-257] oder das Tabakmosaikvirus[265] als Template dienen.

2 Zielsetzung

Das Verständnis der Mechanismen der Biomineralisation eröffnet Möglichkeiten zur Lösung von Fragestellungen verschiedenster Art: Biomineralisierte Materialien finden Anwendung in Bereichen wie der biomedizinischen Regeneration von mineralisierten oder teilmineralisierten Geweben oder technisch nutzbaren Materialien, vor allem für die Katalyse in energiegewinnenden Prozessen. Grundlage für diese Dissertation stellten rekombinante Spinnenseidenproteine dar. Materialien daraus eignen sich auf Grund ihrer Biokompatibilität und Bioabbaubarkeit als Gerüstmaterialien in der Geweberekonstruktion. Ihre Funktionalisierbarkeit und Prozessierung ermöglichen außerdem weitere Anwendungen und Forschungsansätze.

Mangancarbonat-basierte Materialien eignen sich für technischen Anwendungen, beispielsweise in der Katalyse. Eine Studie beschäftigte sich mit mechanistischen Fragestellungen gerichteter Mangancarbonat-Mineralisation in einer definierten Atmosphäre, die nach bioinspiriertem Vorbild in Gegenwart von zwei rekombinanten Spinnenseidenprotein-Templaten unterschiedlicher Ladung in Form von Mikropartikeln stattfinden sollte. Der Einfluss der C-terminalen Peptidsequenzen der Spinnenseidenproteine, die eine negative oder eine positive Nettoladung trugen, sowie die Interaktion unter Zugabe einer weiteren Komponente in Form von Polyacrylsäure, sollte untersucht werden.

Das Biomineralisationsverhalten von funktionalisierten Spinnenseidenproteinen sollte auch auf biomedizinische Anwendungen übertragen werden. Im Bereich der Geweberekonstruktion stellte sich die Aufgabe, Materialien für den Sehnenersatz und dabei den Knochen-Sehnen-Übergang im Speziellen, zu entwickeln. Dieser Bereich ist durch eine zunehmende Zellbesiedlung, eine Mineralisation mit Hydroxylapatit und einem Steifigkeitsgradienten hin zur Knochenseite gekennzeichnet. Eine Teilstudie zielte auf die Induzierbarkeit von Hydroxylapatit-Biomineralisation auf Spinnenseidenoberflächen ab. Ähnlich dem natürlichen Vorbild im Knochen sollten Mineralisierungsmotive aus nicht-kollagenen Proteinen mit rekombinanten Spinnenseidenprotein als prozessierbares Trägermaterial kombiniert werden. Charakterisierung der Mineralphasen, Zellkulturstudien und die Herstellung von 2D Gradientenmaterialien sollten Auskunft über Materialeignung für Gewebegerüste zur Entheserekonstruktion liefern. Da artifizielle Biomineralisationsprozesse zeitlich langsam voranschreiten und die dabei herrschenden

osmotischen Bedingungen für Zellen ungünstig sind, sollte in einer weiteren Teilstudie ein Kompositmaterial mit Spinnenseidenmatrix und anorganischen Füllstoffen hergestellt werden. Dabei sollten mit Fluorapatit beladene Spinnenseidenhydrogele hergestellt und Mausfibroblasten in das Material eingebracht werden. Die Frage der Prozessierbarkeit in ein 3D gedrucktes Gradientenmaterial sollte mit Kooperationspartnern durch Strömungssimulation beantwortet werden.

Um Spinnenseidengele für den 3D Druck weiter zu optimieren, sollte in einer dritten Studie eine Alternativroute zur Gelherstellung über ein physikalisches Netzwerk in wässrig-organischen Mischphasen etabliert werden. Untersuchungen hinsichtlich der Sekundärstrukturausbildung, morphologischen Erscheinung und mechanischen Charakterisierung nach Gelbildung sollten der Aufklärung des zu Grunde liegenden Mechanismus der Gelierung dienen. Weiterhin sollte die Möglichkeit der Formulierung von wasserunlöslichen Wirkstoffen untersucht werden.

3 Synopsis

Diese Arbeit besteht aus fünf Teilarbeiten (siehe Publikationsliste Kapitel 5), die verschiedene Anwendungen von rekombinanten Spinnenseidenmaterialien für komplexe Ansätze, insbesondere rund um die Biomineralisation, zeigen (Abbildung 11).

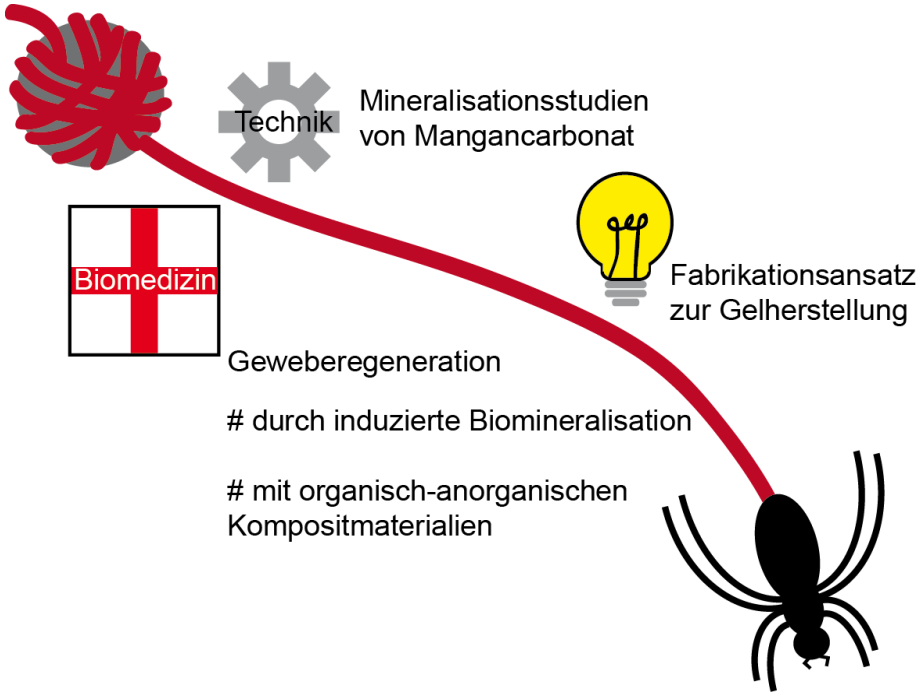

Abbildung 11: Rekombinante Spinnenseide zieht sich wie ein „roter Faden“ durch diese Arbeit, die sich von technischen Anwendungen über die Biomedizin bis hin zu neuen Fabrikationsätzen erstreckt. (Eigene Darstellung)

Zentraler Schwerpunkt dieser Dissertation war die Regeneration von Sehnen und ihr gradueller Übergang in den mineralisierten Knochen. Dem zentralen Fokus zu Grunde liegend, auch zum umfassenden Verständnis von Mineralisationsprozessen als Ausgangspunkt der nachfolgenden Studien, wurde im ersten Teil dieser Arbeit die Mineralisation von Mangancarbonat in Gegenwart von Additiven wie synthetischen Polymeren und Spinnenseidenpartikeln unter kontrollierten Bedingungen untersucht.

Ein Übersichtsartikel ermöglichte den Überblick zum Stand der Forschung für den Ersatz harter Gewebe mit Seidenmaterialien. Darauf aufbauend wurden im zweiten Teil dieser Arbeit zwei Ansätze mit gängigen Methoden der Biomineralisation von Materialien gewählt. In der ersten Teilarbeit wurde die induzierte Biomineralisation von funktionalisierten rekombinanten Spinnenseidenproteinen in unterschiedlichen

Mineralisierungsmedien untersucht, welche dem natürlichen Vorbild in einem biomimetischen Ansatz nahekommen. Die mineralisierten Oberflächen wurden charakterisiert und mit Hilfe von Zellkulturstudien auf Tauglichkeit als Gradientenmaterial für den Sehnen-Knochen-Übergang untersucht. Neben einer reinen Materialstudie konnte weiterhin ein Übergang von 2D auf 3D Materialien für Gewebegerüste ermöglicht werden. So wurde in der zweiten Teilarbeit ein Kompositmaterial hergestellt, das eine simultane Verarbeitung der relevanten Komponenten an Spinnenseidenhydrogelmatrix, Fluorapatit-Nanopartikeln und Zellen zu einem Gradientenmaterial im 3D Druck erlaubte.

Im dritten Teil dieser Arbeit wurde eine Materialstudie betrieben, die eine alternative Herstellungsmethode von Spinnenseidengelen mit neuen Eigenschaften für den 3D Druck etablieren kann. Dazu wurde die Selbstassemblierung von Spinnenseidenproteinen in mischbaren wässrig-organischen Zwischenphasen genutzt. Neben mechanistischen Betrachtungen der Strukturausbildung während der Gelierung wurden die gewonnenen Materialien morphologisch und mechanisch charakterisiert. Daraus ergab sich weiterhin ein System zur Formulierung von wasserunlöslichen Wirkstoffen.

Die folgenden Kapitel stellen eine Zusammenfassung der zentralen Aspekte dieser drei Arbeitsabschnitte dar. Aus den entsprechenden Publikationen können tiefergehende Zusammenhänge, Einordnungen und experimentelle Details entnommen werden.

3.1 Biomineralisation von rekombinanten Spinnenseiden-proteinen für technische Anwendungen

Biomineralisation in natürlichen Prozessen wird durch Proteine mit lokalisierten Ladungen als Template gesteuert. Sie akkumulieren gelöste Ionen aus dem umgebenden Medium und nukleieren so die Mineralisation. Durch fortschreitende Mineralisation entsteht dann ein organisch-anorganisches Kompositmaterial. In dieser Teilarbeit (siehe auch Kapitel 5, Teilarbeit I) wurde das bioinspirierte Mineralisationsverhalten von zwei Varianten des eADF4(C16) Proteins auf die Bildung von Mangancarbonat in einem Kooperationsprojekt mit der AG Jun.-Prof. Dr. Anna Schenk (Universität Bayreuth) untersucht.

Hierfür wurden zwei Spinnenseidenvarianten mit Peptid-*tags* aus je acht Wiederholungen der negativ geladenen Glutaminsäure für eADF4(C16)-E_8G, ähnlich den SIBLING Proteinen im Knochen, und des positiv geladenen Lysins für eADF4(C16)-K_8G als C-terminale Peptidsequenzen biotechnologisch hergestellt. Die Proteine zeigten so eine lokalisierte Ladung und wurden in Mikropartikel prozessiert. In ihrer Gegenwart wurde in einem geschlossenen System, das von Schenk *et al*.[265] bereits etabliert wurde, eine über die Gasphase induzierte Mineralisation von Mangancarbonat herbeigeführt (Abbildung 12A). In einer Abwandlung wurde der zusätzliche Effekt der negativ geladenen Polyacrylsäure als polymeres Additiv untersucht.

Im Vergleich zu reinem abgeschiedenen Mangancarbonat, das kubische Strukturen mit einer Größe von durchschnittlich 1,5 µm lieferte, wurden die Spinnenseidenpartikel, die selbst eine durchschnittliche Größe von 500 nm aufwiesen, auf unterschiedliche Weise in das Kompositmaterial inkorporiert. Die positiv geladene Variante eADF4(C16)-K_8G zeigte deutlich stärke Assoziation mit dem Mineral in engen Kavitäten. Einen eher losen Verbund bildete eADF4(C16)-E_8G mit Mangancarbonat-Würfeln (Abbildung 12B I-III). Materialcharakterisierung mittels *Attenuated Total Internal Reflection* Infrarotspektroskopie, Röntgenpulverdiffraktometrie und Thermogravimetrischer Analyse bestätigten eine stärkere Einbindung des positiv geladenen Proteintemplats in das Mineral. Das Vorhandensein von Spinnenseidenpartikeln beeinflusste die Mineralphase nicht. Eine Quantifizierung der Proteinanteile im Mangancarbonat erfolgte mittels Thermogravimetrischer Analyse. Dabei wurde für die negative Variante eADF4(C16)-E_8G ein Verlust an intrakristallinem und an der Oberfläche adsorbierten organischem Material von -2,7 Gewichtsprozent und für die positive Variante eADF4(C16)-K_8G

von -6,2 Gewichtsprozent für den Bereich zwischen 250-500 °C berechnet. Bei diesen Temperaturen kann eine Umsetzung von $MnCO_3$ zu Mn_2O_3 als hauptsächliches Calzinierungsprodukt angenommen werden.[266]

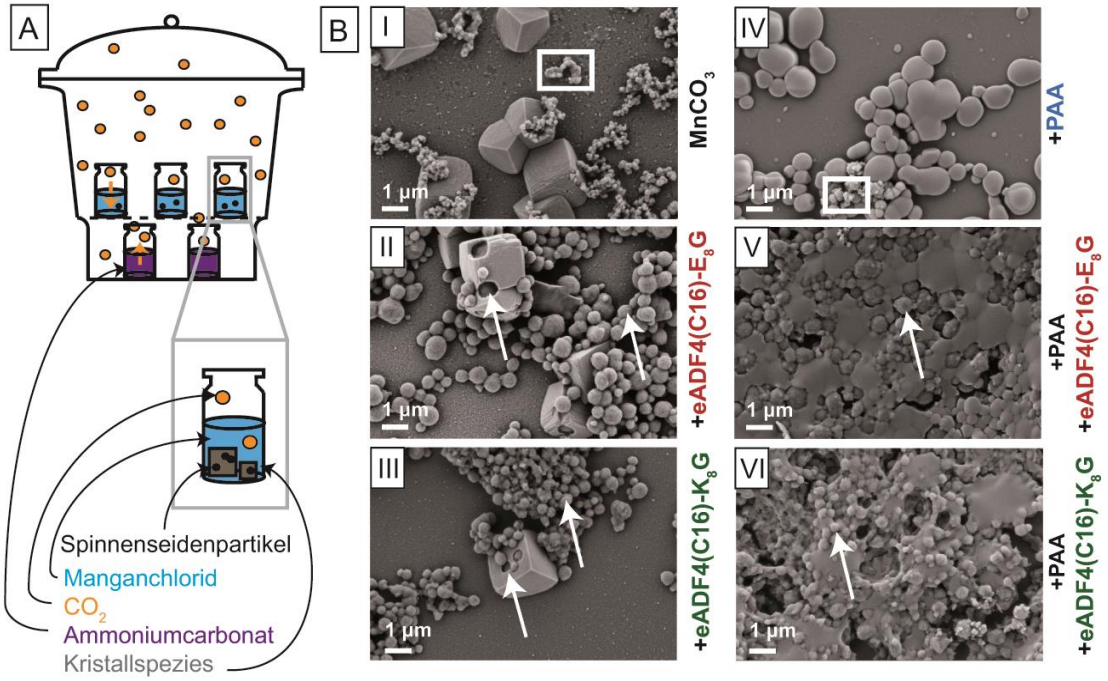

Abbildung 12: Herstellung von Mangancarbonat-Phasen unter Zugabe von verschiedenen Templaten. A) Schematische Darstellung der Mangancarbonat-Mineralisation über Gasdiffusion. B) Rasterelektronenmikroskopische Aufnahmen der $MnCO_3$ Mineralphasen I) ohne Additive, II) in Gegenwart von eADF4(C16)-E_8G Partikeln, III) in Gegenwart von eADF4(C16)-K_8G Partikeln, IV) in Gegenwart von Polyacrylsäure (PAA), V) in Gegenwart von eADF4(C16)-E_8G Partikeln und PAA, VI) in Gegenwart von eADF4(C16)-K_8G Partikeln und PAA. Maßstäbe wie angegeben. Pfeile zeigen eingebundene Spinnenseidenpartikel, Kästen Mineralisationsnebenprodukte an. Nachdruck unter Genehmigung modifiziert nach Neubauer, V. J.; Kellner, C.; Gruen, V.; Schenk, A. S.; Scheibel, T. Recombinant major ampullate spidroin-particles as biotemplates for manganese carbonate mineralization. *Multifunct. Mater.* **2021**, *4*, 014002. Copyright (2021) IOP Publishing.

Durch Zugabe eines weiteren, Struktur-dirigierenden Additivs, der negativ geladenen Polyacrylsäure, zum Mineralisationssystems wurde die Bildung von Polymer-induzierten Flüssigphasen[260] beobachtet (Abbildung 12B IV-VI). Es wurde bereits in Calciumcarbonat-Mineralisationsansätzen[267, 268] gezeigt, dass Polyacrylsäure stark Prozess-dominierende

Eigenschaften aufweist und amorphe Vorläuferphasen stabilisiert. Interessanterweise wurde der gegenläufige ladungsabhängige Trend hinsichtlich der Spinnenseidenpartikel-Inkorporation verglichen zu Ansätzen ohne Polyacrylsäure beobachtet und Partikel der negativ geladenen Spinnenseidenvariante stärker in das Mineral inkorporiert. Eine analytische Betrachtung der entstandenen Spezies bestätigte diese Vermutung, wobei wieder die Mineralphase unbeeinflusst blieb. Thermogravimetrische Analysen der Proben des mit Polyacrylsäure versetztem Mangancarbonats wurden nach Abzug des Carbonat-Beitrags mit einem organischen Anteil von zusätzlichen -3,0 Gewichtsprozent verortet. Weitere Gewichtsabnahmen in Gegenwart von Spinnenseidenpartikeln wurde mit -1,3 % für eADF4(C16)-K_8G und -5,4 % für eADF4(C16)-E_8G festgestellt.

Zeitlich aufgelöste Experimente gaben Auskunft über die zu Grunde liegenden Interaktionen der Einzelkomponenten. Mittels Dynamischer Lichtstreuung wurden die hydrodynamischen Radii der Komponenten in verschiedenen Kombinationsmöglichkeiten der Substanzen unter Ausschluss von Kohlenstoffdioxid zur Unterdrückung von Mineralisationsprozessen untersucht. Positiv geladene eADF4(C16)-K_8G-Partikel schienen erst mit Carbonatanionen während der Mineralisation zu interagieren, da keine Fluktuationen der hydrodynamischen Radii festgestellt wurden. Unter Zugabe von negativ geladener Polyacrylsäure bildeten sich Koazervate mit eADF4(C16)-K_8G, die den Mineralisationsprozess beziehungsweise die Proteineinbindung in das Mineral behinderten. Anders stellte sich der Mechanismus mit negativen Lokalladungen auf Spinnenseidenpartikeln dar. Für eADF4(C16)-E_8G-Partikel wurde eine zunehmende Akkumulation von Mangankationen angenommen, die nach Absättigung in einen Mineralisationsprozess übertraten. In Gegenwart von gelöst vorliegender Polyacrylsäure wurde dieser Effekt verstärkt und eine kolloidal stabile Phase wurde schneller erreicht. Daraus abgeleitet wurde die Hypothese, dass für ein erfolgreiches Einbringen von Additiven während der Mineralisation nicht nur die Ladung der Komponenten entscheidend war, sondern auch ihr Zustand in Lösung oder Suspension.

Die gebildeten mesoporösen Strukturen lieferten die Grundlage für zukünftige Forschung hinsichtlich Materialdesign für technische Anwendungen in der katalytischen Wasserspaltung. Durch Vergrößerung der Materialoberfläche durch Kavitäten kann die Katalysatoreffizienz gesteigert werden. Nach einer Biotemplat-geführten Mineralisation durch Spinnenseidenmorphologien kann durch Sintern ein neuartiges Katalysatormaterial entstehen. Zusätzlich wurden Einblicke in das Mineralisationsverhalten von

Spinnenseidenproteinen mit lokalen C-terminalen Ladungen gewährt, die als Grundlage für weitere Mineralisationsstudien dienten.

3.2 Biomineralisation von und mit rekombinanten Spinnenseidenproteinen für Anwendungen am Sehnen-Knochen-Übergang

Es wurde ein Übersichtsartikel erstellt, der den Stand der Technik hinsichtlich Seidenmaterialien für den Ersatz harter Gewebe wiedergibt (siehe auch Kapitel 5, Teilarbeit II). Im Folgenden wurden dann Lösungsansätze für Ersatzmaterialien für den Sehnen-Knochen-Übergang auf Basis von Spinnenseidenproteinen konzipiert. Dazu wurden zwei Ansätze genutzt, die induzierte Biomineralisation von Materialien aus Lösung und die Herstellung organisch-anorganischer Kompositmaterialien.

3.2.1 Induzierte Biomineralisation von rekombinanten Spinnenseidenvarianten

Die induzierte Biomineralisation von Materialien stellt einen Ansatz dar, der den natürlichen Prozessen während der Gewebemineralisation ähnlich ist. Dabei ist die Wahl des geeigneten Mineralisationsmediums entscheidend. Derartige Materialstudien erlauben vorläufige Aussagen über das Verhalten von neuen Materialien im Körper.[224] In dieser Teilarbeit (siehe auch Kapitel 5, Teilarbeit III) wurden biomimetische Peptidmotive zur Mineralisation und Kollagenbindung mit rekombinanter Spinnenseide fusioniert.

Durch molekularbiologische Methoden wurden Spinnenseidenhybride Gen-optimiert und biotechnologisch hergestellt. Darunter waren Fusionsproteine mit N- und C-terminalen Mineralisationssequenzen des Sialoproteins (eADF4(SN-C16) für Sialoprotein/N-terminal und eADF4(C16-SC) für Sialoprotein/C-terminal), der hoch konservierten Kollagenbindedomäne aus Osteopontin eADF4(C16-osteo) und deren Kombination eADF4(SN-C16-osteo) (Abbildung 13A).

Aus den Proteinen wurden Filme aus 1,1,1,3,3,3-Hexafluoro-2-isopropanol auf Polystyrol-Oberflächen gegossen. Mineralisationsstudien in *Simulated Body Fluid* mit physiologischer Salzzusammensetzung und -konzentrationen sowie Zellkulturmedium, das neben dieser Salzkomposition ebenfalls essentielle Proteine enthält, lieferten eine Einschätzung der Materialoberflächen. Nachbehandelte Filme mit Methanol zur Erhöhung

des kristallinen *β*-Faltblattanteils zeigten keine Tendenz zur Mineralisation auf der passivierten Oberfläche. Getrocknete Filme ohne Nachbehandlung zeigten hingegen Mineralisationsprozesse, die zu einer optischen Trübung der Filmoberfläche führten (Abbildung 13A). Rasterelektronenmikroskopische Aufnahmen (Abbildung 13A) und Wasserkontaktwinkelmessungen bestätigen die Bildung von Mineralen auf den Filmen. *Attenuated Total Internal Reflection* Infrarotspektroskopie, Energiedispersive Röntgenspektroskopie und Röntgenpulverdiffraktometrie dienten der Identifikation der Mineralphase.

Die Fusionsproteine wurden auf ihre Fähigkeit zur Zelladhäsion mit MC3T3 E1 murinen Prä-Osteoblasten untersucht. Dazu wurden getrocknete Filme und mineralisierte Filme als Substrat getestet. Im Vergleich zur internen Kontrolle zeigte keine der Varianten gesteigerte Zelladhäsion und nur wenige Zellen eine gespreitete Morphologie. Gerade dieses Ergebnis der mineralisierten Filme deutete auf nur geringe Mineralmengen hin, die in der Zellkultur noch keine signifikanten Unterschiede hervorriefen.

Durch gesteuerte Extrusion mittels Spritzenpumpen wurden Gradientenmaterialien aus Spinnenseidenfusionsproteinen hergestellt. Eine Durchmischung fand in einer konstruierten Mischkammer statt, die von zwei Seiten mit den Lösungen gespeist wurde. Die augenscheinlich wenig mineralisierende Variante eADF4(SN-C16) wurde gegen eADF4(C16-osteo) als Mineralisations- und Kollagenbindevariante in einen Gradientenfilm prozessiert. Murine MC3T3 E1 Prä-Osteoblasten zeigten während einer Beobachtung der Filmoberfläche über 21 Tage einen konservierten Zelladhäsionsgradienten entlang der steigenden Konzentration an eADF4(C16-osteo) (Abbildung 13B).

Daraus wurde geschlussfolgert, dass durch Zellen abgeschiedenes Kollagen mit dem Kollagenbindemotiv der eADF4(C16-osteo) Spinnenseidenvariante interagierte und eine Mikroumgebung[269] für die Zellen geschaffen hat. Erst durch das Gegenüberstellen der Spinnenseidenfusionsproteine konnte so ein favorisiertes Verhalten der Zellen beobachtet werden. Insgesamt lieferte diese Materialstudie Aussagen zu einer Eignung der Varianten für den Einsatz am Sehnen-Knochen-Übergang.

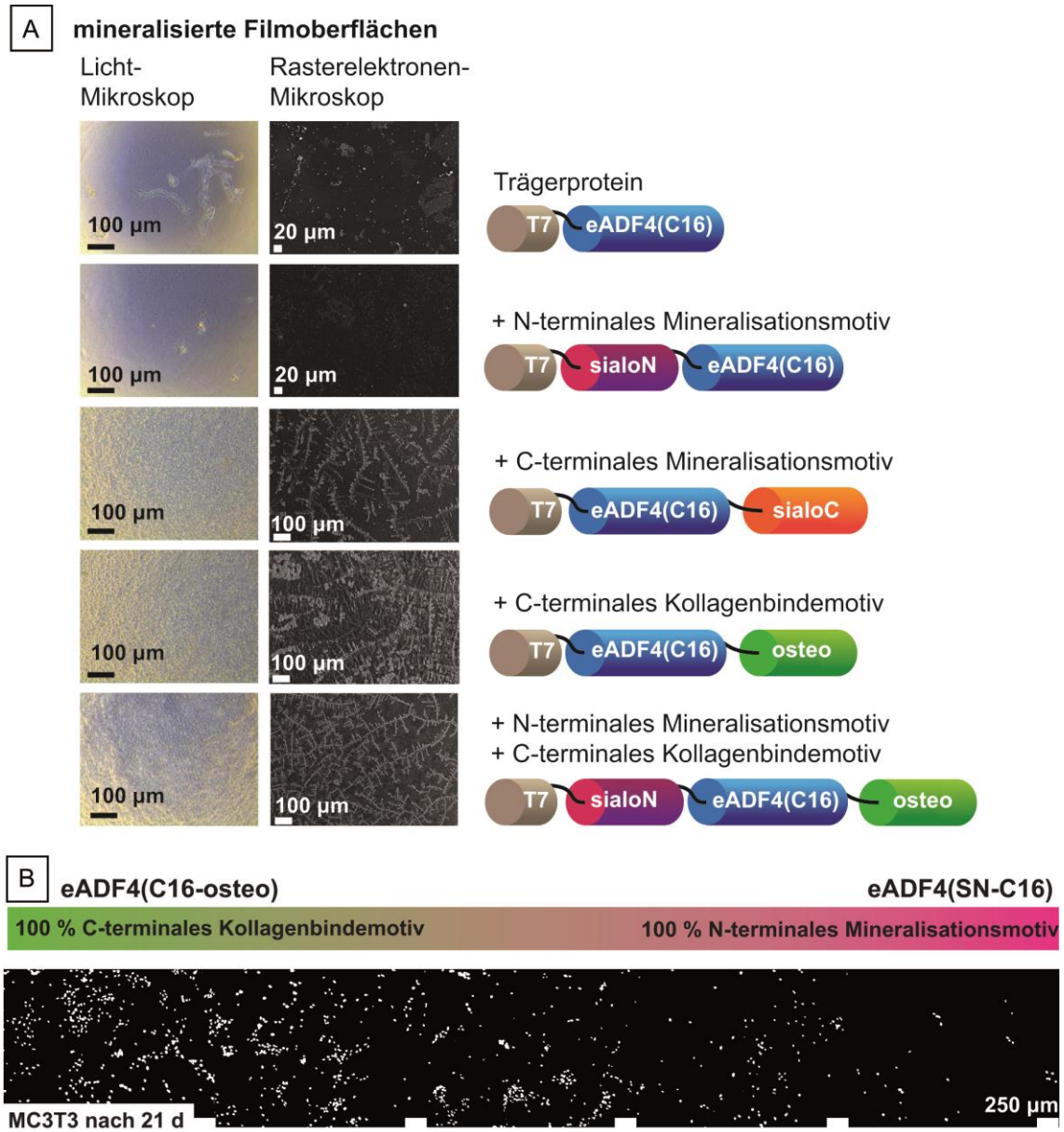

Abbildung 13: A) Darstellung der rekombinanten Spinnenseidenfusionsproteine (rechts) und lichtmikroskopische (links) sowie rasterelektronenmikroskopische Aufnahmen (Mitte) der Filmoberflächen nach biomimetischer Mineralisation in *Simulated Body Fluid*. Maßstab wie angegeben. B) Fluoreszenzmikroskopische Aufnahmen fünf separater Positionen auf einem eADF4(C16-osteo) zu eADF4(SN-C16) Gradientenfilm nach 21 Tagen Zellbesiedelung durch murine MC3T3 E1 Osteoblasten. Zellkerne der Zellen sind in weiß auf schwarzer Filmoberfläche dargestellt. Maßstäbe wie angegeben. Nachdruck unter Genehmigung modifiziert nach Neubauer, V. J.; Scheibel, T., Spider Silk Fusion Proteins for Controlled Collagen Binding and Biomineralization. *ACS Biomater. Sci. Eng.*, **2020**, *6* (10), 5599-5608. Copyright (2020) American Chemical Society.

3.2.2 Gradientendruck von rekombinanten Spinnenseidenproteinen mit anorganischen Füllmaterialien

Neben *in situ* Biomineralisation von Materialien können die Matrixbestandteile auch mit Mineralen während der Verarbeitung zu einem organisch-anorganischen Kompositmaterial zusammengefügt werden. Dies hat den Vorteil, dass definierte Anteile an Füllstoffen eingebracht werden können. Ziel dieser Teilstudie (siehe auch Kapitel 5, Teilarbeit IV) als Kooperationsprojekt mit der AG Prof. Dr.-Ing. Frank Rieg (Universität Bayreuth) war die Simulation und Umsetzung von graduell mineralisierten 3D Konstrukten via Dispensdruck von Kompositmaterialien aus rekombinanten Spinnenseidenhydrogelen für Anwendungen am Sehnen-Knochen-Übergang.

Basierend auf einem *feedback loop* mit Vorhersagen zu Materialverhalten durch numerische Annahmen einer Strömungssimulation (AG Rieg) wurde ein Konzept erarbeitet, das die *in situ* Generation eines 3D gedruckten Gradientenmaterials aus einer Kartusche ermöglicht. Dabei wurden die zu vermischenden Materialien als separate Blöcke nacheinander in eine Kartusche gefüllt und durch das U-Profil unter laminarer Strömung während des Drucks als Gradient extrudiert (Abbildung 14A). Experimentell wurde der Gradientendruck aus einer Druckerkartusche zunächst mit Gesichtscreme als Modellmaterial realisiert. Die Übertragung auf Spinnenseidenhydrogele stellte sich als realisierbar heraus. Dazu wurde ein mit einem Fluoreszenzfarbstoff versetztes Spinnenseidenhydrogel gegen ein unbeladenes Hydrogel gedruckt. Mittels Fluoreszenzdetektion wurde der graduelle Materialübergang qualitativ visualisiert und durch Fluoreszenzspektroskopie quantitativ validiert. Um einen Mineralisierungsgradienten zu generieren, wurden stäbchenförmige Fluorapatit-Partikel durch eine *Onepot*-Sonosynthese[270] hergestellt. Da sie ähnlich Hydroxylapatit ein Material darstellen, das im Körper in mineralisierten Geweben vorhanden ist,[271, 272] wurden sie für Materialstudien des Sehnen-Knochen-Übergangs ausgewählt. Die Partikel mit durchschnittlich 100 nm Länge und 20 nm Breite wurden durch transmissionselektronenmikroskopische Aufnahmen, Energiedispersive Röntgenspektroskopie und *Attenuated Total Internal Reflection* Infrarotspektroskopie charakterisiert. Sie wurden im Verbund mit Spinnenseidenhydrogelen aus eADF4(C16) rheologisch und rasterelektronenmikroskopisch betrachtet und als Gradientenmaterial wie oben beschrieben verdruckt (Abbildung 14B).

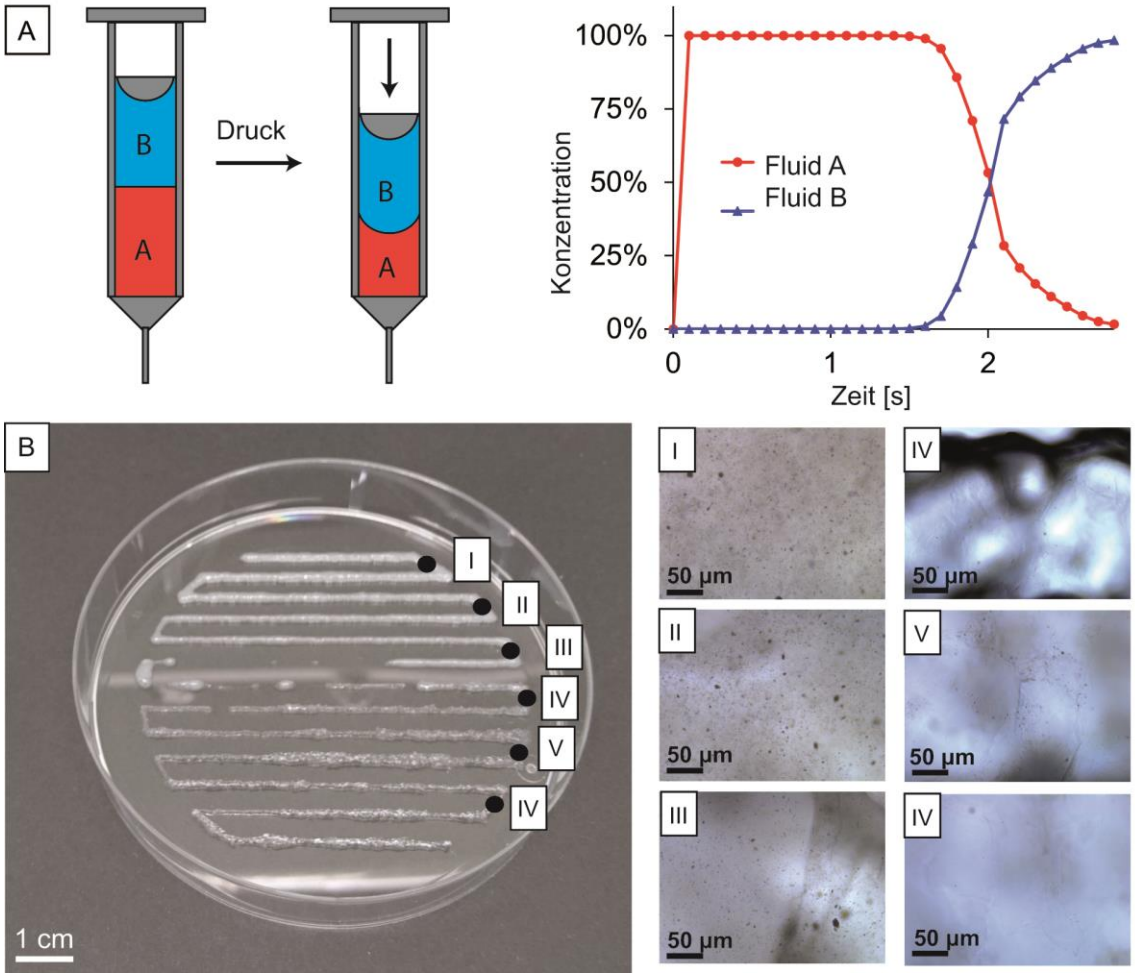

Abbildung 14: Gradientendruck mit Spinnenseidenhydrogelen in einem neuartigen Ein-Kartuschen-System. A) Darstellung der Gradientenerzeugung mittels U-Profil im laminaren Fluss bei Anlegen eines Drucks (links), sowie Extrusionsverlauf der beiden Materialien im Graphen rechts dargestellt. B) Photographische Abbildung eines 3D gedruckten Gradientenmaterials aus Spinnenseidenhydrogel mit Fluorapatitpartikeln (oben) gegen ein unbeladenes Hydrogel (unten) und lichtmikroskopische Detailaufnahmen sechs verschiedener Stellen auf dem Konstrukt, die eine graduelle Abnahme der Partikel zeigen. Maßstäbe wie angegeben. (Eigene Darstellung)

Um die Interaktion der negativ geladenen Fluorapatit-Partikel mit ebenfalls negativ geladenen eADF4(C16) Spinnenseidenhydrogelen zu steigern, wurden die Keramikpartikel mit polykationischer Spinnenseide aus wässriger Lösung beschichtet. Dies stellte sich als vorteilhaft in Zellkulturstudien heraus. Die Zytotoxizität von Apatit-Nanopartikeln liegt bekannterweise weniger in ihrer Form als vielmehr in der Bildung von reaktiven Sauerstoffspezies auf ihrer Oberfläche.[273] In der biotoxikologischen Beurteilung von Medizinprodukten nach DIN ISO 10993-5 wirkten sich die mit Spinnenseidenprotein

beschichteten Partikel deutlich positiv auf die Zellviabilität aus. In einem simultanen Ansatz wurden daraufhin murine BALB/3T3 Fibroblasten mit Fluorapatit-Partikeln in einem Spinnenseidenhydrogel gegen ein unbeladenes Spinnenseidenhydrogel gedruckt. Das Überleben der Zellen während des Drucks und in Gegenwart von Partikeln im Gradientenmaterial konnte mittels Konfokalmikroskopie der gefärbten Zellen bestätigt werden.

Mit zunehmender Zelldichte im Gradienten nahm ebenfalls die Dichte an anorganischen Fluorapatit-Partikeln zu. Durch den bei der Extrusion herrschenden Druck können vermehrt Zellen mit Partikeln in Kontakt treten, was zum Zellsterben durch auftretende Scherkräfte führen könnte. Es war jedoch zu beobachten, dass sich ebenfalls tote Zellen in ähnlicher Konzentration in der nicht verdruckten Kontrollprobe befanden. Diese Art der Prozessierung erlaubt somit die Herstellung von graduell mineralisierten 3D Biomaterialien mit intrinsischer Zellbesiedelung, eignet sich aber auch für eine Übertragung auf verschiedene Fragestellungen jenseits der Biomedizin.

3.3 Fabrikationsansatz zur Gelherstellung aus Spinnenseiden-proteinen in wässrig-organischen Zwischenphasen

Da die Matrixsteifigkeit einer Substratoberfläche massiv das Differenzierungs- und Mineralisierungsverhalten von Osteoblasten beeinflusst,[234] muss dies beim Materialdesign berücksichtigt werden. eADF4(C16) Spinnenseidenhydrogele, die typischerweise bei 3 Gewichtsprozent in wässriger Lösung zu einem physikalischen Netzwerk gelieren,[43] zeigen eine Steifigkeit von etwa 1 kPa und sind damit weit von der Steifigkeit harter Geweben (im Bereich von MPa-GPa) entfernt. Um die Materialsteifigkeit in eADF4(C16) Spinnenseidengelen zu erhöhen, wurde ein Fabrikationsansatz entwickelt, der auf Selbstassemblierung in mischbaren wässrig-organischen Zwischenphasen beruht (siehe auch Kapitel 5, Teilarbeit V). Die Ausbildung von kristallinen β-Faltblattstrukturen wurde dabei von hydrophoben Effekten der organischen Lösungsmittel beeinflusst und es wurden hoch fibrilläre Strukturen erhalten. Da die Verstrickung von fibrillären Polymerketten in Matrices wie (Hydro-)Gelen direkt seine Mechanik beeinflussen, kann so die Steifigkeit verändert werden.

In dieser Studie wurden die Spinnenseidenproteine eADF4(C16) als negativ geladene Variante, eADF4(κ16) als positive und eADF4(Ω16) als neutrale Variante in ihrer Gelbildung in wässrig-organischen Zwischenphasen aus 10 mM Tris Puffer pH 7,5 und variierender Konzentration an Dimethylsulfoxid untersucht. Dabei wurden Assemblierungskinetiken via Trübungsmessung betrachtet. Die Assemblierungskinetik der negativ geladenen Variante wurde nicht beeinflusst. Für die positiv geladene Variante und die ungeladene Variante wurde ein weniger steiler Anstieg der log-Phase festgestellt, was auf eine langsamer ablaufende Assemblierung hindeutet. Insgesamt wurde unter Zugabe von Dimethylsulfoxid eine Abnahme der Trübung in der Plateauphase für alle Varianten und Konzentrationen beobachtet. Eine optische Betrachtung der Gele zeigte eine unverändert hohe Stabilität für eADF4(C16) (Abbildung 15A), jedoch eine Abnahme der Stabilität für eADF4(κ16) und eine Zunahme der Stabilität für eADF4(Ω16) mit steigendem Anteil an Dimethylsulfoxid.

Um den Anteil der organischen Phase zu erhöhen, wurde das neue Verfahren einer „Organo-Dialyse“ entwickelt. Dabei wurde das Spinnenseidenprotein zunächst in wässriger Phase gelöst und gegen Dimethylsulfoxid dialysiert. Während der Dialyse konnte dann der fortschreitende Prozess der Selbstassemblierung bis zum vollständigen Gelieren beobachtet

werden. Zusätzlich wurden Mischsituationen mit Dimethylsulfoxid und weiteren Lösungsmitteln in einem Volumenanteil von 33 % in Spinnenseidengelen untersucht.

Attenuated Total Internal Reflection Infrarotspektroskopie mit anschließender Fourier-Selbstentfaltung lieferte quantitative Aussagen zu Strukturanteilen in den Gelen. Die jeweiligen Mischungen wurden außerdem transmissions- und rasterelektronenmikroskopisch untersucht. In Spinnenseidengelen mit Dimethylsulfoxid-Anteilen wurden stark fibrilläre Strukturen beobachtet, die die Porenwände zu formen schienen (Abbildung 15B). Spinnenseidengele aus rein wässrigem Puffer zeigten eher lamellare Porenstrukturen, die auf Kollabieren der fibrillären Strukturanteile zurück zu führen sein könnten (Abbildung 15B). Eine weitere Besonderheit zeigte sich in der Zunahme der Speicher- und Verlustmodule der Gele in Gegenwart von Dimethlysulfoxid in rheologischer Charakterisierung. Es wurde allein durch Zugabe von 33 % des Volumenanteils an organischen Lösungsmittel eine höhere Materialsteifigkeit festgestellt. Diese wurde noch in Materialien aus dem Verfahren der „Organo-Dialyse“ gesteigert (Abbildung 15C). Darin zeigte sich zusätzlich eine Verschiebung des Gelpunktes als Kreuzungspunkte des Speicher- und Verlustmoduls hin zu höheren Oszillationsstärken. Dies deutete auf eine höhere Widerstandsfähigkeit des Materials unter Belastung hin, was auf starke Verschlaufung der fibrillären Strukturen im polymeren Gelnetzwerk zurückzuführen ist.

Der hier dargestellte Fabrikationsansatz ermöglicht die Formulierung hydrophober pharmazeutischer Wirkstoffe in Spinnenseidengelen über den Anteil der organischen Phase. Um eine potentielle Wirkstofffreisetzung aus Spinnenseidengelen mit Dimethylsulfoxid zu untersuchen, wurde Fluoreszein als Modellsubstanz eingebracht. In Vergleichsstudien zeigten sich identische Freisetzungsprofile für Gele mit und ohne Dimethylsulfoxid-Anteil. Neben transdermalen Anwendungen sind durch die scherverdünnenden Eigenschaften dieser Gele auch injizierbare oder 3D druckbare Wirkstoffdepots möglich. Die Eigenschaften der Gele können außerdem über die Wahl des Spinnenseidenproteins und des organischen Lösungsmittels kontrolliert werden.

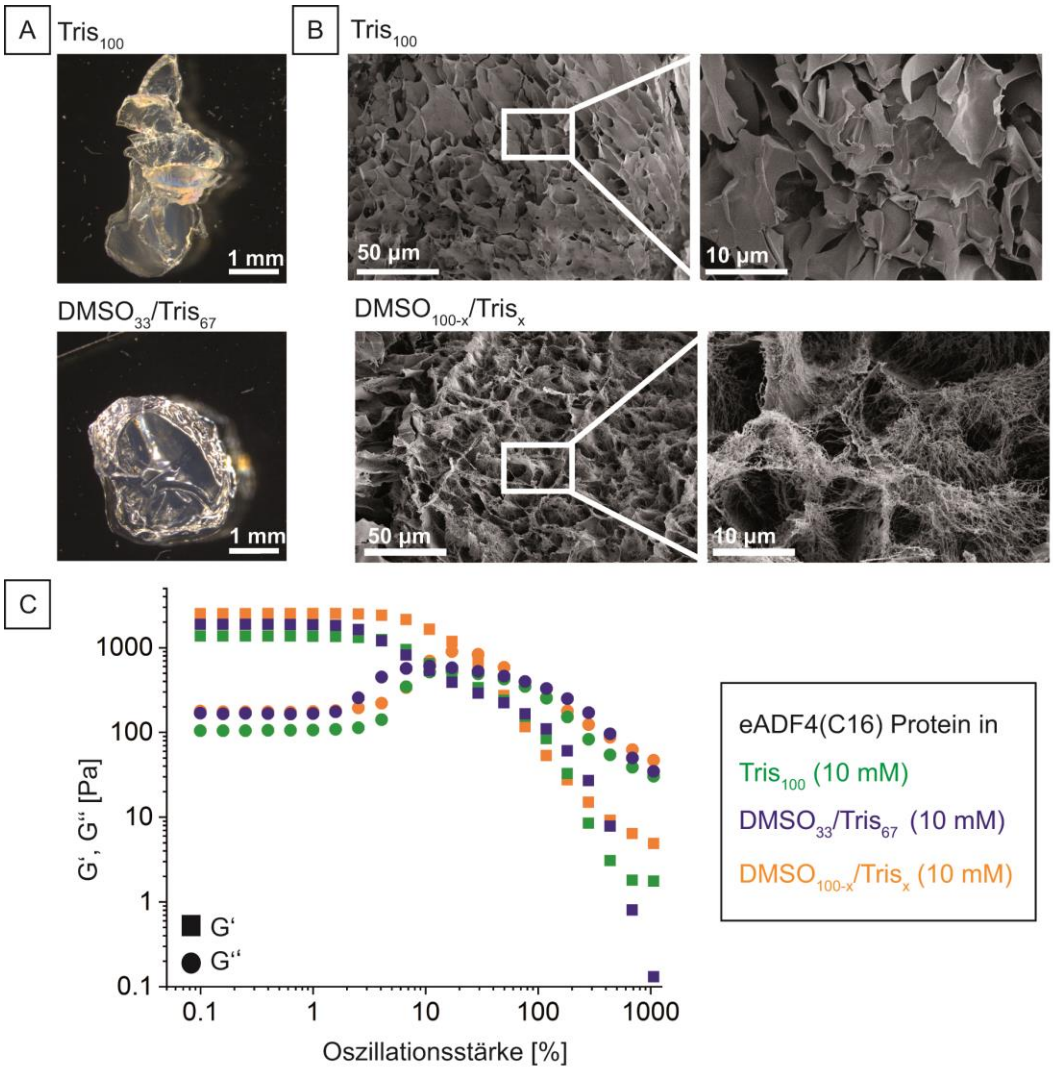

Abbildung 15: Gelstrukturen des Spinnenseidenproteins eADF4(C16), gebildet in einer wässrigen Einzelphase aus 10 mM Tris Puffer pH 7,5 oder Zwischenphasen mit Dimethylsulfoxid unterschiedlichem Anteils. A) Stereomikroskopische Aufnahmen von Gelen in 10 mM Tris ($Tris_{100}$) und mit einem Drittel Volumenanteil Dimethylsulfoxid ($DMSO_{33}/Tris_{67}$). B) Rasterelektronenmikroskopische Aufnahmen gefriergetrockneter Gele in 10 mM Tris ($Tris_{100}$) oder nach „Organo-Dialyse“ gegen Dimethylsulfoxid ($DMSO_{100-x}/Tris_x$) in zwei Vergrößerungen. C) Rheologische Charakterisierung der Gele in 10 mM Tris ($Tris_{100}$), mit einem Drittel Volumenanteil Dimethylsulfoxid ($DMSO_{33}/Tris_{67}$) und nach „Organo-Dialyse“ gegen Dimethylsulfoxid ($DMSO_{100-x}/Tris_x$). Maßstäbe wie angegeben. Nachdruck unter Genehmigung modifiziert nach Neubauer, V. J.; Trossmann, V.T.; Jacobi, S.; Döbl, A.; Scheibel, T. Recombinant Spider Silk Gels Derived from Aqueous–Organic Solvents as Depots for Drugs. *Angew. Chem. Int. Ed.* **2021**, *60*, 11847-11851. Copyright (2021) John Wiley & Sons.

4 Referenzen

1. Lang, G.; Herold, H.; Scheibel, T., Properties of Engineered and Fabricated Silks. In *Fibrous Proteins: Structures and Mechanisms*, Parry, D. A. D.; Squire, J. M., Eds. Springer International Publishing: Cham, 2017; pp 527-573.
2. Andersson, M.; Johansson, J.; Rising, A., Silk Spinning in Silkworms and Spiders. *Int. J. Mol. Sci.* **2016,** *17* (8), 1290.
3. Sutherland, T. D.; Young, J. H.; Weisman, S.; Hayashi, C. Y.; Merritt, D. J., Insect Silk: One Name, Many Materials. *Annu. Rev. Entomol.* **2010,** *55* (1), 171-188.
4. Doblhofer, E.; Heidebrecht, A.; Scheibel, T., To spin or not to spin: spider silk fibers and more. *Appl. Microbiol. Biotechnol.* **2015,** *99* (22), 9361-9380.
5. Heim, M.; Keerl, D.; Scheibel, T., Spider Silk: From Soluble Protein to Extraordinary Fiber. *Angew. Chem. Int. Ed.* **2009,** *48* (20), 3584-3596.
6. Zhou, C.-Z.; Confalonieri, F.; Jacquet, M.; Perasso, R.; Li, Z.-G.; Janin, J., Silk fibroin: Structural implications of a remarkable amino acid sequence. *Proteins* **2001,** *44* (2), 119-122.
7. Guo, C.; Li, C.; Kaplan, D. L., Enzymatic Degradation of *Bombyx mori* Silk Materials: A Review. *Biomacromolecules* **2020,** *21* (5), 1678-1686.
8. Sutherland, T. D.; Weisman, S.; Walker, A. A.; Mudie, S. T., The coiled coil silk of bees, ants, and hornets. *Biopolymers* **2012,** *97* (6), 446-454.
9. Schmidt, M.; Lemke, J.; Frenkler, R.; Kraxenberger, T.; Römer, L.; Böker, A., The biotechnological production of lacewing silk: From gene to protein-based material. *Engineering Reports* **2020,** *2* (4), e12137.
10. Bauer, F.; Scheibel, T., Artificial Egg Stalks Made of a Recombinantly Produced Lacewing Silk Protein. *Angew. Chem. Int. Ed.* **2012,** *51* (26), 6521-6524.
11. Heidebrecht, A.; Scheibel, T., Recombinant Production of Spider Silk Proteins. In *Advances in Applied Microbiology*, Sariaslani, S.; Gadd, G. M., Eds. Elsevier Academic Press Inc: San Diego, 2013; Vol. 82, pp 115-153.
12. Eisoldt, L.; Smith, A.; Scheibel, T., Decoding the secrets of spider silk. *Mater. Today* **2011,** *14* (3), 80-86.
13. Blackledge, T. A.; Hayashi, C. Y., Silken toolkits: biomechanics of silk fibers spun by the orb web spider *Argiope argentata*. *J. Exp. Biol.* **2006,** *209* (13), 2452-2461.
14. Eisoldt, L.; Hardy, J. G.; Heim, M.; Scheibel, T. R., The role of salt and shear on the storage and assembly of spider silk proteins. *J. Struct. Biol.* **2010,** *170* (2), 413-419.
15. Hagn, F.; Thamm, C.; Scheibel, T.; Kessler, H., pH-Dependent Dimerization and Salt-Dependent Stabilization of the N-terminal Domain of Spider Dragline Silk—Implications for Fiber Formation. *Angew. Chem. Int. Ed.* **2011,** *50* (1), 310-313.
16. Eisoldt, L.; Thamm, C.; Scheibel, T., The role of terminal domains during storage and assembly of spider silk proteins. *Biopolymers* **2012,** *97* (6), 355-361.
17. Römer, L.; Scheibel, T., The elaborate structure of spider silk: structure and function of a natural high performance fiber. *Prion* **2008,** *2* (4), 154-161.
18. Guerette, P. A.; Ginzinger, D. G.; Weber, B. H. F.; Gosline, J. M., Silk Properties Determined by Gland-Specific Expression of a Spider Fibroin Gene Family. *Science* **1996,** *272* (5258), 112-115.
19. Vendrely, C.; Scheibel, T., Biotechnological production of spider-silk proteins enables new applications. *Macromol. Biosci.* **2007,** *7* (4), 401-409.

20. Huemmerich, D.; Helsen, C. W.; Quedzuweit, S.; Oschmann, J.; Rudolph, R.; Scheibel, T., Primary Structure Elements of Spider Dragline Silks and Their Contribution to Protein Solubility. *Biochemistry* **2004,** *43* (42), 13604-13612.
21. Huemmerich, D.; Scheibel, T.; Vollrath, F.; Cohen, S.; Gat, U.; Ittah, S., Novel Assembly Properties of Recombinant Spider Dragline Silk Proteins. *Curr. Biol.* **2004,** *14* (22), 2070-2074.
22. Scheibel, T., Spider silks: recombinant synthesis, assembly, spinning, and engineering of synthetic proteins. *Microb. Cell Fact.* **2004,** *3* (14).
23. Heidebrecht, A.; Eisoldt, L.; Diehl, J.; Schmidt, A.; Geffers, M.; Lang, G.; Scheibel, T., Biomimetic Fibers Made of Recombinant Spidroins with the Same Toughness as Natural Spider Silk. *Adv. Mater.* **2015,** *27* (13), 2189-2194.
24. Doblhofer, E.; Scheibel, T., Engineering of Recombinant Spider Silk Proteins Allows Defined Uptake and Release of Substances. *J. Pharm. Sci.* **2015,** *104* (3), 988-994.
25. Kumari, S.; Lang, G.; DeSimone, E.; Spengler, C.; Trossmann, V. T.; Lücker, S.; Hudel, M.; Jacobs, K.; Krämer, N.; Scheibel, T., Engineered spider silk-based 2D and 3D materials prevent microbial infestation. *Data in Brief* **2020,** *32*, 106305.
26. Humenik, M.; Scheibel, T., Nanomaterial Building Blocks Based on Spider Silk–Oligonucleotide Conjugates. *ACS Nano* **2014,** *8* (2), 1342-1349.
27. Wohlrab, S.; Mueller, S.; Schmidt, A.; Neubauer, S.; Kessler, H.; Leal-Egana, A.; Scheibel, T., Cell adhesion and proliferation on RGD-modified recombinant spider silk proteins. *Biomaterials* **2012,** *33* (28), 6650-6659.
28. Weiss, A. C. G.; Herold, H. M.; Lentz, S.; Faria, M.; Besford, Q. A.; Ang, C.-S.; Caruso, F.; Scheibel, T., Surface Modification of Spider Silk Particles to Direct Biomolecular Corona Formation. *ACS Appl. Mater. Interfaces* **2020,** *12* (22), 24635-24643.
29. Herold, H. M.; Aigner, T. B.; Grill, C. E.; Krüger, S.; Taubert, A.; Scheibel, T., SpiderMAEn: recombinant spider silk-based hybrid materials for advanced energy technology. *Bioinspir. Biomim. Nan.* **2019,** *8* (1), 99-108.
30. Humenik, M.; Mohrand, M.; Scheibel, T., Self-Assembly of Spider Silk-Fusion Proteins Comprising Enzymatic and Fluorescence Activity. *Bioconjugate Chem.* **2018,** *29* (4), 898-904.
31. DeSimone, E.; Aigner, T. B.; Humenik, M.; Lang, G.; Scheibel, T., Aqueous electrospinning of recombinant spider silk proteins. *Mater. Sci. Eng. C* **2020,** *106*, 110145.
32. Slotta, U. K.; Rammensee, S.; Gorb, S.; Scheibel, T., An engineered spider silk protein forms microspheres. *Angew. Chem. Int. Ed.* **2008,** *47* (24), 4592-4594.
33. Schierling, M. B.; Doblhofer, E.; Scheibel, T., Cellular uptake of drug loaded spider silk particles. *Biomater. Sci.* **2016,** *4* (10), 1515-1523.
34. Lucke, M.; Mottas, I.; Herbst, T.; Hotz, C.; Römer, L.; Schierling, M.; Herold, H. M.; Slotta, U.; Spinetti, T.; Scheibel, T.; Winter, G.; Bourquin, C.; Engert, J., Engineered hybrid spider silk particles as delivery system for peptide vaccines. *Biomaterials* **2018,** *172*, 105-115.
35. Elsner, M. B.; Herold, H. M.; Müller-Herrmann, S.; Bargel, H.; Scheibel, T., Enhanced cellular uptake of engineered spider silk particles. *Biomater. Sci.* **2015,** *3* (3), 543-551.
36. Hermanson, K. D.; Huemmerich, D.; Scheibel, T.; Bausch, A. R., Engineered Microcapsules Fabricated from Reconstituted Spider Silk. *Adv. Mater.* **2007,** *19* (14), 1810-1815.

37. Hermanson, K. D.; Harasim, M. B.; Scheibel, T.; Bausch, A. R., Permeability of silk microcapsules made by the interfacial adsorption of protein. *Phys. Chem. Chem. Phys.* **2007,** *9* (48), 6442-6446.
38. Lang, G.; Jokisch, S.; Scheibel, T., Air filter devices including nonwoven meshes of electrospun recombinant spider silk proteins. *J. Vis. Exp.* **2013,** *75*, e50492.
39. Jokisch, S.; Neuenfeldt, M.; Scheibel, T., Silk-Based Fine Dust Filters for Air Filtration. *Adv. Sustainable Syst.* **2017,** *1* (10), 1700079.
40. Wohlrab, S.; Spieß, K.; Scheibel, T., Varying surface hydrophobicities of coatings made of recombinant spider silk proteins. *J. Mater. Chem.* **2012,** *22* (41), 22050-22054.
41. Huemmerich, D.; Slotta, U.; Scheibel, T., Processing and modification of films made from recombinant spider silk proteins. *Appl. Phys. A* **2006,** *82* (2), 219-222.
42. Slotta, U.; Tammer, M.; Kremer, F.; Koelsch, P.; Scheibel, T., Structural Analysis of Spider Silk Films. *Supramol. Chem.* **2006,** *18* (5), 465-471.
43. Schacht, K.; Scheibel, T., Controlled Hydrogel Formation of a Recombinant Spider Silk Protein. *Biomacromolecules* **2011,** *12* (7), 2488-2495.
44. Schacht, K.; Juengst, T.; Schweinlin, M.; Ewald, A.; Groll, J.; Scheibel, T., Biofabrication of Cell-Loaded 3D Spider Silk Constructs. *Angew. Chem. Int. Ed.* **2015,** *54* (9), 2816-2820.
45. Schacht, K.; Vogt, J.; Scheibel, T., Foams Made of Engineered Recombinant Spider Silk Proteins as 3D Scaffolds for Cell Growth. *ACS Biomater. Sci. Eng.* **2016,** *2* (4), 517-525.
46. Evans, J. S., Composite Materials Design: Biomineralization Proteins and the Guided Assembly and Organization of Biomineral Nanoparticles. *Materials* **2019,** *12* (4), 581.
47. Evans, J. S., The Biomineralization Proteome: Protein Complexity for a Complex Bioceramic Assembly Process. *Proteomics* **2019,** *19* (16), 1900036.
48. Gower, L. B., Biomimetic Model Systems for Investigating the Amorphous Precursor Pathway and Its Role in Biomineralization. *Chem. Rev.* **2008,** *108* (11), 4551-4627.
49. Combes, C.; Cazalbou, S.; Rey, C., Apatite Biominerals. *Minerals* **2016,** *6* (2), 34.
50. Simmons, D. J., Calcium and Skeletal Tissue Physiology in Teleost Fishes. *Clin. Orthop. Relat. Res.* **1971,** *76*, 244-280.
51. Walther, L. E., Otokonien. *HNO* **2016,** *64* (10), 767-776.
52. Degens, E. T.; Deuser, W. G.; Haedrich, R. L., Molecular structure and composition of fish otoliths. *Mar. Biol.* **1969,** *2* (2), 105-113.
53. Fernández, M. S.; Simoncini, M. S.; Dyke, G., Irregularly calcified eggs and eggshells of *Caiman latirostris* (Alligatoridae: Crocodylia). *Sci. Nat.* **2013,** *100* (5), 451-457.
54. Dhami, N. K.; Reddy, M. S.; Mukherjee, A., Biomineralization of calcium carbonates and their engineered applications: a review. *Front. Microbiol.* **2013,** *4*, 314.
55. Xiao, S.; Zheng, X.; Wang, Z.; Wang, R., A Study of the Organic Matrix of Cuttlebone: Molecular Weights, Characterized Infrared Spectrum and Amino Acid Composition. *Phuket mar. biol. Cent. Res. Bull.* **2005,** *66*, 235-241.
56. Seto, J.; Ma, Y.; Davis, S. A.; Meldrum, F.; Gourrier, A.; Kim, Y.-Y.; Schilde, U.; Sztucki, M.; Burghammer, M.; Maltsev, S.; Jäger, C.; Cölfen, H., Structure-property relationships of a biological mesocrystal in the adult sea urchin spine. *Proc. Natl. Acad. Sci. U. S. A.* **2012,** *109* (10), 3699-3704.

57. Read, B. A.; et.al., Pan genome of the phytoplankton *Emiliania* underpins its global distribution. *Nature* **2013,** *499* (7457), 209-213.
58. Hildebrand, M., Diatoms, Biomineralization Processes, and Genomics. *Chem. Rev.* **2008,** *108* (11), 4855-4874.
59. Uebe, R.; Schüler, D., Magnetosome biogenesis in magnetotactic bacteria. *Nat. Rev. Microbiol.* **2016,** *14* (10), 621-637.
60. Faivre, D.; Schüler, D., Magnetotactic Bacteria and Magnetosomes. *Chem. Rev.* **2008,** *108* (11), 4875-4898.
61. Dorozhkin, S. V.; Epple, M., Biological and Medical Significance of Calcium Phosphates. *Angew. Chem. Int. Ed.* **2002,** *41* (17), 3130-3146.
62. Anderson, H. C., Matrix vesicles and calcification. *Curr. Rheumatol. Rep.* **2003,** *5* (3), 222-226.
63. Cusack, M., Biomineral electron backscatter diffraction for palaeontology. *Palaeontology* **2016,** *59* (2), 171-179.
64. Nassif, N.; Pinna, N.; Gehrke, N.; Antonietti, M.; Jäger, C.; Cölfen, H., Amorphous layer around aragonite platelets in nacre. *Proc. Natl. Acad. Sci. U. S. A.* **2005,** *102* (36), 12653-12655.
65. Feng, Q. L.; Li, H. B.; Pu, G.; Zhang, D. M.; Cui, F. Z.; Li, H. D.; Kim, T. N., Crystallographic alignment of calcite prisms in the oblique prismatic layer of Mytilus edulis shell. *J. Mater. Sci.* **2000,** *35* (13), 3337-3340.
66. Zhang, C.; Zhang, R., Matrix Proteins in the Outer Shells of Molluscs. *Mar. Biotechnol.* **2006,** *8* (6), 572-586.
67. Weiner, S.; Addadi, L., Crystallization Pathways in Biomineralization. In *Annual Review of Materials Research*, Clarke, D. R.; Fratzl, P., Eds. Annual Reviews: Palo Alto, 2011; Vol. 41, pp 21-40.
68. Zhang, Z.; Gao, D.; Zhao, H.; Xie, C.; Guan, G.; Wang, D.; Yu, S.-H., Biomimetic Assembly of Polypeptide-Stabilized CaCO3 Nanoparticles. *J. Phys. Chem. B* **2006,** *110* (17), 8613-8618.
69. Naka, K.; Huang, S.-C.; Chujo, Y., Formation of Stable Vaterite with Poly(acrylic acid) by the Delayed Addition Method. *Langmuir* **2006,** *22* (18), 7760-7767.
70. Sumper, M.; Hett, R.; Lehmann, G.; Wenzl, S., A Code for Lysine Modifications of a Silica Biomineralizing Silaffin Protein. *Angew. Chem. Int. Edit.* **2007,** *46* (44), 8405-8408.
71. Sumper, M., A Phase Separation Model for the Nanopatterning of Diatom Biosilica. *Science* **2002,** *295* (5564), 2430-2433.
72. Komeili, A., Molecular mechanisms of compartmentalization and biomineralization in magnetotactic bacteria. *FEMS Microbiol. Rev.* **2012,** *36* (1), 232-255.
73. Lang, C.; Schüler, D.; Faivre, D., Synthesis of Magnetite Nanoparticles for Bio- and Nanotechnology: Genetic Engineering and Biomimetics of Bacterial Magnetosomes. *Macromol. Biosci.* **2007,** *7* (2), 144-151.
74. Staniland, S.; Ward, B.; Harrison, A.; van der Laan, G.; Telling, N., Rapid magnetosome formation shown by real-time x-ray magnetic circular dichroism. *Proc. Natl. Acad. Sci. U. S. A.* **2007,** *104* (49), 19524-19528.
75. Staniland, Sarah S.; Rawlings, Andrea E., Crystallizing the function of the magnetosome membrane mineralization protein Mms6. *Biochem. Soc. Trans.* **2016,** *44* (3), 883-890.
76. Mickoleit, F.; Borkner, C. B.; Toro-Nahuelpan, M.; Herold, H. M.; Maier, D. S.; Plitzko, J. M.; Scheibel, T.; Schüler, D., In Vivo Coating of Bacterial Magnetic Nanoparticles by Magnetosome Expression of Spider Silk-Inspired Peptides. *Biomacromolecules* **2018,** *19* (3), 962-972.

77. Ito, A.; Shinkai, M.; Honda, H.; Kobayashi, T., Medical application of functionalized magnetic nanoparticles. *J. Biosci. Bioeng.* **2005,** *100* (1), 1-11.
78. Wischmann, J.; Lenze, F.; Thiel, A.; Bookbinder, S.; Querido, W.; Schmidt, O.; Burgkart, R.; von Eisenhart-Rothe, R.; Richter, G. H. S.; Pleshko, N.; Mayer-Kuckuk, P., Matrix mineralization controls gene expression in osteoblastic cells. *Exp. Cell Res.* **2018,** *372* (1), 25-34.
79. Arsenault, A. L.; Frankland, B. W.; Ottensmeyer, F. P., Vectorial sequence of mineralization in the turkey leg tendon determined by electron microscopic imaging. *Calcif. Tissue Int.* **1991,** *48* (1), 46-55.
80. Beniash, E., Biominerals—hierarchical nanocomposites: the example of bone. *WIRES Nanomed. Nanobi.* **2011,** *3* (1), 47-69.
81. Giachelli, C. M.; Steitz, S., Osteopontin: a versatile regulator of inflammation and biomineralization. *Matrix Biol.* **2000,** *19* (7), 615-622.
82. Hoang, Q. Q.; Sicheri, F.; Howard, A. J.; Yang, D. S. C., Bone recognition mechanism of porcine osteocalcin from crystal structure. *Nature* **2003,** *425* (6961), 977-980.
83. Fujisawa, R.; Wada, Y.; Nodasaka, Y.; Kuboki, Y., Acidic amino acid-rich sequences as binding sites of osteonectin to hydroxyapatite crystals. *Biochim. Biophys. Acta-Protein Struct. Molec. Enzym.* **1996,** *1292* (1), 53-60.
84. Ganss, B.; Kim, R. H.; Sodek, J., Bone Sialoprotein. *Crit. Rev. Oral Biol. Med.* **1999,** *10* (1), 79-98.
85. George, A.; Veis, A., Phosphorylated Proteins and Control over Apatite Nucleation, Crystal Growth, and Inhibition. *Chem. Rev.* **2008,** *108* (11), 4670-4693.
86. George, A.; Ravindran, S., Protein templates in hard tissue engineering. *Nano Today* **2010,** *5* (4), 254-266.
87. Gu, L.; Kim, Y. K.; Liu, Y.; Ryou, H.; Wimmer, C. E.; Dai, L.; Arola, D. D.; Looney, S. W.; Pashley, D. H.; Tay, F. R., Biomimetic Analogs for Collagen Biomineralization. *J. Dent. Res.* **2011,** *90* (1), 82-87.
88. Hunter, G. K.; Goldberg, H. A., Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. *Biochem. J.* **1994,** *302*, 175-179.
89. Kretlow, J. D.; Mikos, A. G., Review: Mineralization of Synthetic Polymer Scaffolds for Bone Tissue Engineering. *Tissue Eng.* **2007,** *13* (5), 927-938.
90. Hunter, G. K.; Goldberg, H. A., Nucleation of hydroxyapatite by bone sialoprotein. *Proc. Natl. Acad. Sci. U. S. A.* **1993,** *90* (18), 8562-8565.
91. Jahromi, M. T.; Yao, G.; Cerruti, M., The importance of amino acid interactions in the crystallization of hydroxyapatite. *J. R. Soc. Interface* **2013,** *10* (80), 20120906.
92. Deshpande, A. S.; Beniash, E., Bioinspired Synthesis of Mineralized Collagen Fibrils. *Cryst. Growth Des.* **2008,** *8* (8), 3084-3090.
93. Bleek, K.; Taubert, A., New developments in polymer-controlled, bioinspired calcium phosphate mineralization from aqueous solution. *Acta Biomater.* **2013,** *9* (5), 6283-6321.
94. Addadi, L.; Weiner, S., Interactions between acidic proteins and crystals: stereochemical requirements in biomineralization. *Proc. Natl. Acad. Sci. USA* **1985,** *82* (12), 4110-4114.
95. Hill, A. V., The diffusion of oxygen and lactic acid through tissues. *Proc. R. Soc. B* **1928,** *104* (728), 39-96.
96. Wang, W.; Yeung, K. W. K., Bone grafts and biomaterials substitutes for bone defect repair: A review. *Bioact. Materials* **2017,** *2* (4), 224-247.

97. Zhang, T.; Lin, S.; Shao, X.; Zhang, Q.; Xue, C.; Zhang, S.; Lin, Y.; Zhu, B.; Cai, X., Effect of matrix stiffness on osteoblast functionalization. *Cell Prolif.* **2017,** *50* (3), e12338.
98. Lin, J. T.; Lane, J. M., Osteoporosis: A Review. *Clin. Orthop. Relat. Res.* **2004,** *425*, 126-134.
99. Zioupos, P.; Kirchner, H. O. K.; Peterlik, H., Ageing bone fractures: The case of a ductile to brittle transition that shifts with age. *Bone* **2020,** *131*, 115176.
100. Stewart, V. L.; Herling, P.; Dalinka, M. K., Calcification in Soft Tissues. *JAMA* **1983,** *250* (1), 78-81.
101. Walsh, J. S.; Fairley, J. A., Calcifying disorders of the skin. *J. Am. Acad. Dermatol.* **1995,** *33* (5), 693-706.
102. Kaplan, F. S.; Glaser, D. L.; Hebela, N.; Shore, E. M., Heterotopic Ossification. *J. Am. Acad. Orthop. Surg.* **2004,** *12* (2), 116-125.
103. Baldino, L.; Cardea, S.; Maffulli, N.; Reverchon, E., Regeneration techniques for bone-to-tendon and muscle-to-tendon interfaces reconstruction. *Br. Med. Bull.* **2016,** *117* (1), 25-37.
104. Zhang, Y.; Wang, F.; Tan, H.; Chen, G.; Guo, L.; Yang, L., Analysis of the mineral composition of the human calcified cartilage zone. *Int. J. Med. Sci.* **2012,** *9* (5), 353-360.
105. Oegema, T. R.; Carpenter, R. J.; Hofmeister, F.; Thompson, R. C., The interaction of the zone of calcified cartilage and subchondral bone in osteoarthritis. *Microsc. Res. Tech.* **1997,** *37* (4), 324-332.
106. Jin, L.; Zhao, W.; Ren, B.; Li, L.; Hu, X.; Zhang, X.; Cai, Q.; Ao, Y.; Yang, X., Osteochondral tissue regenerated via a strategy by stacking pre-differentiated BMSC sheet on fibrous mesh in a gradient. *Biomed. Mater.* **2019,** *14* (6), 065017.
107. Ferguson, V. L.; Bushby, A. J.; Boyde, A., Nanomechanical properties and mineral concentration in articular calcified cartilage and subchondral bone. *J. Anat.* **2003,** *203* (2), 191-202.
108. Mente, P. L.; Lewis, J. L., Elastic modulus of calcified cartilage is an order of magnitude less than that of subchondral bone. *J. Orthop. Res.* **1994,** *12* (5), 637-647.
109. Ribeiro, V. P.; Pina, S.; Costa, J. B.; Cengiz, I. F.; García-Fernández, L.; Fernández-Gutiérrez, M. d. M.; Paiva, O. C.; Oliveira, A. L.; San-Román, J.; Oliveira, J. M.; Reis, R. L., Enzymatically Cross-Linked Silk Fibroin-Based Hierarchical Scaffolds for Osteochondral Regeneration. *ACS Appl. Mater. Interfaces* **2019,** *11* (4), 3781-3799.
110. Ding, X.; Zhu, M.; Xu, B.; Zhang, J.; Zhao, Y.; Ji, S.; Wang, L.; Wang, L.; Li, X.; Kong, D.; Ma, X.; Yang, Q., Integrated Trilayered Silk Fibroin Scaffold for Osteochondral Differentiation of Adipose-Derived Stem Cells. *ACS Appl. Mater. Interfaces* **2014,** *6* (19), 16696-16705.
111. Idaszek, J.; Costantini, M.; Karlsen, T. A.; Jaroszewicz, J.; Colosi, C.; Testa, S.; Fornetti, E.; Bernardini, S.; Seta, M.; Kasarełło, K.; Wrzesień, R.; Cannata, S.; Barbetta, A.; Gargioli, C.; Brinchman, J. E.; Święszkowski, W., 3D bioprinting of hydrogel constructs with cell and material gradients for the regeneration of full-thickness chondral defect using a microfluidic printing head. *Biofabrication* **2019,** *11* (4), 044101.
112. Shaw, H. M.; Vázquez, O. T.; McGonagle, D.; Bydder, G.; Santer, R. M.; Benjamin, M., Development of the human Achilles tendon enthesis organ. *J. Anat.* **2008,** *213* (6), 718-724.

113. Thomopoulos, S.; Williams, G. R.; Gimbel, J. A.; Favata, M.; Soslowsky, L. J., Variation of biomechanical, structural, and compositional properties along the tendon to bone insertion site. *J. Orthop. Res.* **2003,** *21* (3), 413-419.
114. Deymier, A. C.; An, Y.; Boyle, J. J.; Schwartz, A. G.; Birman, V.; Genin, G. M.; Thomopoulos, S.; Barber, A. H., Micro-mechanical properties of the tendon-to-bone attachment. *Acta Biomater.* **2017,** *56*, 25-35.
115. Marinovich, R.; Soenjaya, Y.; Wallace, G. Q.; Zuskov, A.; Dunkman, A.; Foster, B. L.; Ao, M.; Bartman, K.; Lam, V.; Rizkalla, A.; Beier, F.; Somerman, M. J.; Holdsworth, D. W.; Soslowsky, L. J.; Lagugne-Labarthet, F.; Goldberg, H. A., The role of bone sialoprotein in the tendon-bone insertion. *Matrix Biol.* **2016,** *52-54*, 325-338.
116. Li, X.; Xie, J.; Lipner, J.; Yuan, X.; Thomopoulos, S.; Xia, Y., Nanofiber Scaffolds with Gradations in Mineral Content for Mimicking the Tendon-to-Bone Insertion Site. *Nano Lett.* **2009,** *9* (7), 2763-2768.
117. Xie, J. W.; Li, X. R.; Lipner, J.; Manning, C. N.; Schwartz, A. G.; Thomopoulos, S.; Xia, Y. N., "Aligned-to-random" nanofiber scaffolds for mimicking the structure of the tendon-to-bone insertion site. *Nanoscale* **2010,** *2* (6), 923-926.
118. Ladd, M. R.; Lee, S. J.; Stitzel, J. D.; Atala, A.; Yoo, J. J., Co-electrospun dual scaffolding system with potential for muscle-tendon junction tissue engineering. *Biomaterials* **2011,** *32* (6), 1549-1559.
119. Frank, C.; Amiel, D.; Woo, S. l.-Y.; Akeson, W., Normal Ligament Properties and Ligament Healing. *Clin. Orthop. Rel. Res.* **1985,** *196*, 15-25.
120. Boyd, S. K.; Matyas, J. R.; Wohl, G. R.; Kantzas, A.; Zernicke, R. F., Early regional adaptation of periarticular bone mineral density after anterior cruciate ligament injury. *J. Appl. Physiol.* **2000,** *89* (6), 2359-2364.
121. Leppälä, J.; Kannus, P.; Natri, A.; Pasanen, M.; Sievänen, H.; Vuori, I.; Järvinen, M., Effect of Anterior Cruciate Ligament Injury of the Knee on Bone Mineral Density of the Spine and Affected Lower Extremity: A Prospective One-Year Follow-Up Study. *Calcif. Tissue Int.* **1999,** *64* (4), 357-363.
122. Pugno, N. M., The commemoration of Leonardo da Vinci. *Meccanica* **2019,** *54* (15), 2317-2324.
123. Mercer, D. M. A., Acoustic design principles. *Rev. Phys. Tech.* **1971,** *2* (3), 146-172.
124. Kennedy, E. B., Biomimicry: Design by Analogy to Biology. *Res.-Technol. Manag.* **2017,** *60* (6), 51-56.
125. Karthick, B.; Maheshwari, R., Lotus-inspired nanotechnology applications. *Resonance* **2008,** *13* (12), 1141-1145.
126. Autumn, K.; Gravish, N., Gecko adhesion: evolutionary nanotechnology. *Phil. Trans. R. Soc. A* **2008,** *366*, 1575-1590.
127. Claussen, K. U.; Scheibel, T.; Schmidt, H. W.; Giesa, R., Polymer Gradient Materials: Can Nature Teach Us New Tricks? *Macromol. Mater. Eng.* **2012,** *297* (10), 938-957.
128. Liu, Z.; Meyers, M. A.; Zhang, Z.; Ritchie, R. O., Functional gradients and heterogeneities in biological materials: Design principles, functions, and bioinspired applications. *Prog. Mater. Sci.* **2017,** *88*, 467-498.
129. Deming, T. J., Mussel byssus and biomolecular materials. *Curr. Opin. Chem. Biol.* **1999,** *3* (1), 100-105.
130. Sun, C.; Waite, J. H., Mapping Chemical Gradients within and along a Fibrous Structural Tissue, Mussel Byssal Threads. *J. Biol. Chem.* **2005,** *280* (47), 39332-39336.

131. Qin, X.-X.; Coyne, K. J.; Waite, J. H., Tough Tendons Mussel Byssus has Collagen with Silk-Like Domains. *J. Biol. Chem.* **1997,** *272* (51), 32623-32627.
132. Miserez, A.; Schneberk, T.; Sun, C.; Zok, F. W.; Waite, J. H., The Transition from Stiff to Compliant Materials in Squid Beaks. *Science* **2008,** *319* (5871), 1816-1819.
133. Miserez, A.; Rubin, D.; Waite, J. H., Cross-linking Chemistry of Squid Beak. *J. Biol. Chem.* **2010,** *285* (49), 38115-38124.
134. Bell, E.; Gosline, J., Mechanical design of mussel byssus: material yield enhances attachment strength. *J. Exp. Biol.* **1996,** *199* (4), 1005-1017.
135. Genin, G. M.; Kent, A.; Birman, V.; Wopenka, B.; Pasteris, J. D.; Marquez, P. J.; Thomopoulos, S., Functional Grading of Mineral and Collagen in the Attachment of Tendon to Bone. *Biophys. J.* **2009,** *97* (4), 976-985.
136. Kawasaki, A.; Watanabe, R., Concept and P/M fabrication of functionally gradient materials. *Ceram. Int.* **1997,** *23* (1), 73-83.
137. Libanori, R.; Erb, R. M.; Reiser, A.; Le Ferrand, H.; Suess, M. J.; Spolenak, R.; Studart, A. R., Stretchable heterogeneous composites with extreme mechanical gradients. *Nat. Commun.* **2012,** *3*, 1265.
138. Bishop, A.; Lin, C. Y.; Navaratnam, M.; Rawlings, R. D.; McShane, H. B., A functionally gradient material produced by a powder metallurgical process. *J. Mater. Sci. Lett.* **1993,** *12* (19), 1516-1518.
139. Chekanov, Y. A.; Pojman, J. A., Preparation of functionally gradient materials via frontal polymerization. *J. Appl. Polym. Sci.* **2000,** *78* (13), 2398-2404.
140. He, J. K.; Du, Y. A.; Guo, Y. Q.; Hancock, M. J.; Wang, B.; Shin, H.; Wu, J. H.; Li, D. C.; Khademhosseini, A., Microfluidic Synthesis of Composite Cross-Gradient Materials for Investigating Cell-Biomaterial Interactions. *Biotechnol. Bioeng.* **2011,** *108* (1), 175-185.
141. Claussen, K. U.; Lintz, E. S.; Giesa, R.; Schmidt, H. W.; Scheibel, T., Protein Gradient Films of Fibroin and Gelatine. *Macromol. Biosci.* **2013,** *13* (10), 1396-1403.
142. Mohan, N.; Gupta, V.; Sridharan, B.; Sutherland, A.; Detamore, M. S., The potential of encapsulating "raw materials" in 3D osteochondral gradient scaffolds. *Biotechnol. Bioeng.* **2014,** *111* (4), 829-841.
143. Wang, S.; Foo, C. W. P.; Warrier, A.; Poo, M. M.; Heilshorn, S. C.; Zhang, X., Gradient lithography of engineered proteins to fabricate 2D and 3D cell culture micro environments. *Biomed. Microdevices* **2009,** *11* (5), 1127-1134.
144. Jin, Z. H.; Batra, R. C., Some basic fracture mechanics concepts in functionally graded materials. *J. Mech. Phys. Solids* **1996,** *44* (8), 1221-1235.
145. Miao, X. G.; Sun, D., Graded/Gradient Porous Biomaterials. *Materials* **2010,** *3* (1), 26-47.
146. Xie, J. W.; Ma, B.; Michael, P. L.; Shuler, F. D., Fabrication of Nanofiber Scaffolds With Gradations in Fiber Organization and Their Potential Applications. *Macromol. Biosci.* **2012,** *12* (10), 1336-1341.
147. Sun, L.; Parker, S. T.; Syoji, D.; Wang, X. L.; Lewis, J. A.; Kaplan, D. L., Direct-Write Assembly of 3D Silk/Hydroxyapatite Scaffolds for Bone Co-Cultures. *Adv. Healthc. Mater.* **2012,** *1* (6), 729-735.
148. Gao, F.; Xu, Z.; Liang, Q.; Liu, B.; Li, H.; Wu, Y.; Zhang, Y.; Lin, Z.; Wu, M.; Ruan, C.; Liu, W., Direct 3D Printing of High Strength Biohybrid Gradient Hydrogel Scaffolds for Efficient Repair of Osteochondral Defect. *Adv. Funct. Mater.* **2018,** *28* (13), 1706644.
149. Kokkinis, D.; Bouville, F.; Studart, A. R., 3D Printing of Materials with Tunable Failure via Bioinspired Mechanical Gradients. *Adv. Mater.* **2018,** *30* (19), 1705808.

150. Lan, H., Active Mixing Nozzle for Multi-Material and Multi-Scale 3D Printing. In *12th International Manufacturing Science and Engineering Conference*, Los Angeles, CA, USA, 2017.
151. Garland, A.; Fadel, G., Manufacturing Functionally Gradient Material Objects With an Off the Shelf 3D Printer: Challenges and Solutions. In *International Design Engineering Technical Conferences & Computers and Information in Engineering Conference*, Boston, Massachusetts, USA, 2015.
152. Mironov, V.; Trusk, T.; Kasyanov, V.; Little, S.; Swaja, R.; Markwald, R., Biofabrication: a 21st century manufacturing paradigm. *Biofabrication* **2009,** *1* (2), 1-16.
153. Zollfrank, C.; Scheibel, T.; Seitz, H.; Travitzky, N., Bioinspired Materials Engineering. In *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, 2014; pp 1-22.
154. Yousaf, S. S.; Houacine, C.; Khan, I.; Ahmed, W.; Jackson, M. J., Chapter 11 - Importance of biomaterials in biomedical engineering. In *Advances in Medical and Surgical Engineering*, Ahmed, W.; Phoenix, D. A.; Jackson, M. J.; Charalambous, C. P., Eds. Academic Press: 2020; pp 149-176.
155. Bodiou, V.; Moutsatsou, P.; Post, M. J., Microcarriers for Upscaling Cultured Meat Production. *Front. Nutr.* **2020,** *7*, 10.
156. Shetti, N. P.; Mishra, A.; Basu, S.; Mascarenhas, R. J.; Kakarla, R. R.; Aminabhavi, T. M., Skin-Patchable Electrodes for Biosensor Applications: A Review. *ACS Biomater. Sci. Eng.* **2020,** *6* (4), 1823-1835.
157. Wu, Q.; Liu, J.; Wang, X.; Feng, L.; Wu, J.; Zhu, X.; Wen, W.; Gong, X., Organ-on-a-chip: recent breakthroughs and future prospects. *Biomed. Eng. Online* **2020,** *19* (1), 9.
158. Borkner, C. B.; Wohlrab, S.; Möller, E.; Lang, G.; Scheibel, T., Surface Modification of Polymeric Biomaterials Using Recombinant Spider Silk Proteins. *ACS Biomater. Sci. Eng.* **2017,** *3* (5), 767-775.
159. Zeplin, P. H.; Maksimovikj, N. C.; Jordan, M. C.; Nickel, J.; Lang, G.; Leimer, A. H.; Römer, L.; Scheibel, T., Spider Silk Coatings as a Bioshield to Reduce Periprosthetic Fibrous Capsule Formation. *Adv. Funct. Mater.* **2014,** *24* (18), 2658-2666.
160. Saha, S.; Pramanik, K.; Biswas, A., Silk fibroin coated TiO2 nanotubes for improved osteogenic property of Ti6Al4V bone implants. *Mater. Sci. Eng. C* **2019,** *105*, 109982.
161. Chisti, Y., Biodiesel from microalgae beats bioethanol. *Trends Biotechnol.* **2008,** *26* (3), 126-131.
162. Das, S.; Dhar, B. B., Green synthesis of noble metal nanoparticles using cysteine-modified silk fibroin: catalysis and antibacterial activity. *RSC Advances* **2014,** *4* (86), 46285-46292.
163. Groll, J.; Boland, T.; Blunk, T.; Burdick, J. A.; Cho, D.-W.; Dalton, P. D.; Derby, B.; Forgacs, G.; Li, Q.; Mironov, V. A.; Moroni, L.; Nakamura, M.; Shu, W.; Takeuchi, S.; Vozzi, G.; Woodfield, T. B. F.; Xu, T.; Yoo, J. J.; Malda, J., Biofabrication: reappraising the definition of an evolving field. *Biofabrication* **2016,** *8* (1), 013001.
164. Moroni, L.; Boland, T.; Burdick, J. A.; De Maria, C.; Derby, B.; Forgacs, G.; Groll, J.; Li, Q.; Malda, J.; Mironov, V. A.; Mota, C.; Nakamura, M.; Shu, W.; Takeuchi, S.; Woodfield, T. B. F.; Xu, T.; Yoo, J. J.; Vozzi, G., Biofabrication: A Guide to Technology and Terminology. *Trends Biotechnol.* **2018,** *36* (4), 384-402.

165. Malda, J.; Visser, J.; Melchels, F. P.; Jungst, T.; Hennink, W. E.; Dhert, W. J. A.; Groll, J.; Hutmacher, D. W., 25th Anniversary Article: Engineering Hydrogels for Biofabrication. *Adv. Mater.* **2013,** *25* (36), 5011-5028.
166. Langer, R.; Vacanti, J., Tissue engineering. *Science* **1993,** *260* (5110), 920-926.
167. Njoo, M. D.; Westerhof, W.; Bos, J. D.; Bossuyt, P. M. M., A Systematic Review of Autologous Transplantation Methods in Vitiligo. *Arch. Dermatol.* **1998,** *134* (12), 1543-1549.
168. Bajaj, P.; Schweller, R. M.; Khademhosseini, A.; West, J. L.; Bashir, R., 3D Biofabrication Strategies for Tissue Engineering and Regenerative Medicine. *Annu. Rev. Biomed. Eng.* **2014,** *16* (1), 247-276.
169. Andrews, P. A.; Burnapp, L.; Manas, D.; Bradley, J. A.; Dudley, C., Summary of the British Transplantation Society/Renal Association UK Guidelines for Living Donor Kidney Transplantation. *Transplantation* **2012,** *93* (7), 666-673.
170. Miyagawa, S.; Hirose, H.; Shirakura, R.; Naka, Y.; Nakata, S.; Kawashima, Y.; Seya, T.; Matsumoto, M.; Uenaka, A.; Kitamura, H., The Mechanism of Discordant Xenograft Rejection. *Transplantation* **1988,** *46* (6), 825-830.
171. Sachs, D. H., The pig as a potential xenograft donor. *Vet. Immunol. Immunopathol.* **1994,** *43* (1), 185-191.
172. Alsberg, E.; Anderson, K. W.; Albeiruti, A.; Franceschi, R. T.; Mooney, D. J., Cell-interactive Alginate Hydrogels for Bone Tissue Engineering. *J. Dent. Res.* **2001,** *80* (11), 2025-2029.
173. Leblond, C. P.; Walker, B. E., Renewal of cell populations. *Physiol. Rev.* **1956,** *36* (2), 255-276.
174. Bracaglia, L. G.; Smith, B. T.; Watson, E.; Arumugasaamy, N.; Mikos, A. G.; Fisher, J. P., 3D printing for the design and fabrication of polymer-based gradient scaffolds. *Acta Biomater.* **2017,** *56*, 3-13.
175. Venkatesan, J.; Bhatnagar, I.; Manivasagan, P.; Kang, K.-H.; Kim, S.-K., Alginate composites for bone tissue engineering: A review. *Int. J. Biol. Macromol.* **2015,** *72*, 269-281.
176. Stevens, M. M.; Qanadilo, H. F.; Langer, R.; Prasad Shastri, V., A rapid-curing alginate gel system: utility in periosteum-derived cartilage tissue engineering. *Biomaterials* **2004,** *25* (5), 887-894.
177. Tabriz, A. G.; Hermida, M. A.; Leslie, N. R.; Shu, W., Three-dimensional bioprinting of complex cell laden alginate hydrogel structures. *Biofabrication* **2015,** *7* (4), 045012.
178. Dash, M.; Chiellini, F.; Ottenbrite, R. M.; Chiellini, E., Chitosan—A versatile semi-synthetic polymer in biomedical applications. *Prog. Polym. Sci.* **2011,** *36* (8), 981-1014.
179. Hayes, M.; Carney, B.; Slater, J.; Brück, W., Mining marine shellfish wastes for bioactive molecules: Chitin and chitosan – Part B: Applications. *Biotechnol. J.* **2008,** *3* (7), 878-889.
180. Aigner, T. B.; DeSimone, E.; Scheibel, T., Biomedical Applications of Recombinant Silk-Based Materials. *Adv. Mater.* **2018,** *30* (19), 1704636.
181. Peng, Y. Y.; Stoichevska, V.; Howell, L.; Madsen, S.; Werkmeister, J. A.; Dumsday, G. J.; Ramshaw, J. A. M., Preparation and characterization of monomers to tetramers of a collagen-like domain from *Streptococcus pyogenes*. *Bioengineered* **2014,** *5* (6), 378-385.
182. Chouhan, D.; Thatikonda, N.; Nilebäck, L.; Widhe, M.; Hedhammar, M.; Mandal, B. B., Recombinant Spider Silk Functionalized Silkworm Silk Matrices as Potential

Bioactive Wound Dressings and Skin Grafts. *ACS Appl. Mater. Interfaces* **2018,** *10* (28), 23560-23572.
183. Mironov, V.; Visconti, R. P.; Kasyanov, V.; Forgacs, G.; Drake, C. J.; Markwald, R. R., Organ printing: Tissue spheroids as building blocks. *Biomaterials* **2009,** *30* (12), 2164-2174.
184. Mertgen, A.-S.; Trossmann, V. T.; Guex, A. G.; Maniura-Weber, K.; Scheibel, T.; Rottmar, M., Multifunctional Biomaterials: Combining Material Modification Strategies for Engineering of Cell-Contacting Surfaces. *ACS Appl. Mater. Interfaces* **2020,** *12* (19), 21342-21367.
185. Le Saux, G.; Magenau, A.; Böcking, T.; Gaus, K.; Gooding, J. J., The Relative Importance of Topography and RGD Ligand Density for Endothelial Cell Adhesion. *PLOS ONE* **2011,** *6* (7), e21869.
186. Wozniak, M. A.; Modzelewska, K.; Kwong, L.; Keely, P. J., Focal adhesion regulation of cell behavior. *Biochim. Biophys. Acta* **2004,** *1692* (2), 103-119.
187. Melchels, F.; SeverinWiggenhauser, P.; DavidWarne; Barry, M.; Ong, F. R.; Chong, W. S.; Hutmacher, D. W.; Schantz, J.-T., CAD/CAM-assisted breast reconstruction. *Biofabrication* **2011,** *3*, 034114.
188. Aguado, B. A.; Mulyasasmita, W.; Su, J.; Lampe, K. J.; Heilshorn, S. C., Improving viability of stem cells during syringe needle flow through the design of hydrogel cell carriers. *Tissue Eng. Part A* **2012,** *18* (7-8), 806-815.
189. Snyder, J.; Rin Son, A.; Hamid, Q.; Wang, C.; Lui, Y.; Sun, W., Mesenchymal stem cell printing and process regulated cell properties. *Biofabrication* **2015,** *7* (4), 044106.
190. Blaeser, A.; Duarte Campos, D. F.; Puster, U.; Richtering, W.; Stevens, M. M.; Fischer, H., Controlling Shear Stress in 3D Bioprinting is a Key Factor to Balance Printing Resolution and Stem Cell Integrity. *Adv. Healthc. Mater.* **2016,** *5* (3), 326-333.
191. Neubauer, J. W.; Hauck, N.; Männel, M. J.; Seuss, M.; Fery, A.; Thiele, J., Mechanoresponsive Hydrogel Particles as a Platform for Three-Dimensional Force Sensing. *ACS Appl. Mater. Interfaces* **2019,** *11* (29), 26307-26313.
192. Leal-Egana, A.; Scheibel, T., Silk-based materials for biomedical applications. *Biotechnol. Appl. Biochem.* **2010,** *55*, 155-167.
193. Umuhoza, D.; Yang, F.; Long, D.; Hao, Z.; Dai, J.; Zhao, A., Strategies for Tuning the Biodegradation of Silk Fibroin-Based Materials for Tissue Engineering Applications. *ACS Biomater. Sci. Eng.* **2020,** *6* (3), 1290-1310.
194. Petzold, J.; Aigner, T. B.; Touska, F.; Zimmermann, K.; Scheibel, T.; Engel, F. B., Surface Features of Recombinant Spider Silk Protein eADF4(κ16)-Made Materials are Well-Suited for Cardiac Tissue Engineering. *Adv. Funct. Mater.* **2017,** *27*, 1701427.
195. Kramer, J. P. M.; Aigner, T. B.; Petzold, J.; Roshanbinfar, K.; Scheibel, T.; Engel, F. B., Recombinant spider silk protein eADF4(C16)-RGD coatings are suitable for cardiac tissue engineering. *Sci. Rep.* **2020,** *10* (1), 8789-8789.
196. Steiner, D.; Lang, G.; Fischer, L.; Winkler, S.; Fey, T.; Greil, P.; Scheibel, T.; Horch, R. E.; Arkudas, A., Intrinsic Vascularization of Recombinant eADF4(C16) Spider Silk Matrices in the Arteriovenous Loop Model. *Tissue Eng. Part A* **2019,** *25* (21-22), 1504-1513.
197. Hardy, J. G.; Torres-Rendon, J. G.; Leal-Egana, A.; Walther, A.; Schlaad, H.; Coelfen, H.; Scheibel, T. R., Biomineralization of Engineered Spider Silk Protein-Based Composite Materials for Bone Tissue Engineering. *Materials* **2016,** *9* (7), 560.

198. Dinjaski, N.; Plowright, R.; Zhou, S.; Belton, D. J.; Perry, C. C.; Kaplan, D. L., Osteoinductive recombinant silk fusion proteins for bone regeneration. *Acta Biomater.* **2017,** *49*, 127-139.
199. Gomes, S.; Leonor, I. B.; Mano, J. F.; Reis, R. L.; Kaplan, D. L., Spider silk-bone sialoprotein fusion proteins for bone tissue engineering. *Soft Matter* **2011,** *7* (10), 4964-4973.
200. Neubauer, V. J.; Scheibel, T., Spider Silk Fusion Proteins for Controlled Collagen Binding and Biomineralization. *ACS Biomater. Sci. Eng.* **2020,** *6* (10), 5599–5608.
201. Pawar, K.; Welzel, G.; Haynl, C.; Schuster, S.; Scheibel, T., Recombinant Spider Silk and Collagen-Based Nerve Guidance Conduits Support Neuronal Cell Differentiation and Functionality in Vitro. *ACS Appl. Bio Mater.* **2019,** *2* (11), 4872-4880.
202. Aigner, T. B.; Haynl, C.; Salehi, S.; O'Connor, A.; Scheibel, T., Nerve guidance conduit design based on self-rolling tubes. *Mater. Today Bio* **2020,** *5*, 100042.
203. Baoyong, L.; Jian, Z.; Denglong, C.; Min, L., Evaluation of a new type of wound dressing made from recombinant spider silk protein using rat models. *Burns* **2010,** *36* (6), 891-896.
204. MacGregor-Ramiasa, M.; Hopp, I.; Bachhuka, A.; Murray, P.; Vasilev, K., Surface nanotopography guides kidney-derived stem cell differentiation into podocytes. *Acta Biomater.* **2017,** *56*, 171-180.
205. Zouani, O. F.; Chanseau, C.; Brouillaud, B.; Bareille, R.; Deliane, F.; Foulc, M.-P.; Mehdi, A.; Durrieu, M.-C., Altered nanofeature size dictates stem cell differentiation. *J. Cell Sci.* **2012,** *125* (5), 1217-1224.
206. Dolatshahi-Pirouz, A.; Jensen, T.; Kraft, D. C.; Foss, M.; Kingshott, P.; Hansen, J. L.; Larsen, A. N.; Chevallier, J.; Besenbacher, F., Fibronectin Adsorption, Cell Adhesion, and Proliferation on Nanostructured Tantalum Surfaces. *ACS Nano* **2010,** *4* (5), 2874-2882.
207. Wang, P.-Y.; Wu, T.-H.; Tsai, W.-B.; Kuo, W.-H.; Wang, M.-J., Grooved PLGA films incorporated with RGD/YIGSR peptides for potential application on skeletal muscle tissue engineering. *Colloids Surf. B* **2013,** *110*, 88-95.
208. Saino, E.; Focarete, M. L.; Gualandi, C.; Emanuele, E.; Cornaglia, A. I.; Imbriani, M.; Visai, L., Effect of Electrospun Fiber Diameter and Alignment on Macrophage Activation and Secretion of Proinflammatory Cytokines and Chemokines. *Biomacromolecules* **2011,** *12* (5), 1900-1911.
209. Zhao, L.; Chen, D.; Yao, Q.; Li, M., Studies on the use of recombinant spider silk protein/polyvinyl alcohol electrospinning membrane as wound dressing. *Int. J. Nanomed.* **2017,** *12*, 8103-8114.
210. Zhang, X.; Reagan, M. R.; Kaplan, D. L., Electrospun silk biomaterial scaffolds for regenerative medicine. *Adv. Drug Deliv. Rev.* **2009,** *61* (12), 988-1006.
211. Reimers, K.; Liebsch, C.; Radtke, C.; Kuhbier, J. W.; Vogt, P. M., Silks as scaffolds for skin reconstruction. *Biotechnol. Bioeng.* **2015,** *112* (11), 2201-2205.
212. Zebrowski, D. C.; Becker, R.; Engel, F. B., Towards regenerating the mammalian heart: challenges in evaluating experimentally induced adult mammalian cardiomyocyte proliferation. *Am. J. Physiol. Heart Circ. Physiol.* **2016,** *310* (9), H1045-H1054.
213. Hong, K. U.; Bolli, R., Cardiac stem cell therapy for cardiac repair. *Curr. Treat. Options Cardiovasc. Med.* **2014,** *16*, 324.
214. Hou, D.; Youssef, E. A.; Brinton, T. J.; Zhang, P.; Rogers, P.; Price, E. T.; Yeung, A. C.; Johnstone, B. H.; Yock, P. G.; March, K. L., Radiolabeled cell distribution

after intramyocardial, intracoronary, and interstitial retrograde coronary venous delivery: implications for current clinical trials. *Circulation* **2005,** *112* (9), I150-I156.
215. Ashammakhi, N.; Hasan, A.; Kaarela, O.; Byambaa, B.; Sheikhi, A.; Gaharwar, A. K.; Khademhosseini, A., Advancing Frontiers in Bone Bioprinting. *Adv. Healthc. Mater.* **2019,** *8* (7), 1801048.
216. Bose, S.; Vahabzadeh, S.; Bandyopadhyay, A., Bone tissue engineering using 3D printing. *Mater. Today* **2013,** *16* (12), 496-504.
217. Albrektsson, T.; Johansson, C., Osteoinduction, osteoconduction and osseointegration. *Eur. Spine J.* **2001,** *10* (2), S96-S101.
218. Lee, J. Y.; Choo, J. E.; Choi, Y. S.; Park, J. B.; Min, D. S.; Lee, S. J.; Rhyu, H. K.; Jo, I. H.; Chung, C. P.; Park, Y. J., Assembly of collagen-binding peptide with collagen as a bioactive scaffold for osteogenesis in vitro and in vivo. *Biomaterials* **2007,** *28* (29), 4257-4267.
219. Gilbert, M.; Shaw, W. J.; Long, J. R.; Nelson, K.; Drobny, G. P.; Giachelli, C. M.; Stayton, P. S., Chimeric peptides of statherin and osteopontin that bind hydroxyapatite and mediate cell adhesion. *J. Biol. Chem.* **2000,** *275* (21), 16213-16218.
220. Harris, N. L.; Rattray, K. R.; Tye, C. E.; Underhill, T. M.; Somerman, M. J.; D'Errico, J. A.; Chambers, A. F.; Hunter, G. K.; Goldberg, H. A., Functional analysis of bone sialoprotein: Identification of the hydroxyapatite-nucleating and cell-binding domains by recombinant peptide expression and site-directed mutagenesis. *Bone* **2000,** *27* (6), 795-802.
221. Nagano, A.; Sato, H.; Tanioka, Y.; Nakazawa, Y.; Knight, D.; Asakura, T., Characterization of a Ca binding-amphipathic silk-like protein and peptide with the sequence (Glu)8(Ala-Gly-Ser-Gly-Ala-Gly)4 with potential for bone repair. *Soft Matter* **2012,** *8* (3), 741-748.
222. Dellaquila, A.; Greco, G.; Campodoni, E.; Mazzocchi, M.; Mazzolai, B.; Tampieri, A.; Pugno, N. M.; Sandri, M., Optimized production of a high-performance hybrid biomaterial: biomineralized spider silk for bone tissue engineering. *J. Appl. Polym. Sci.* **2019,** *137*, 48739.
223. Zhang, W.; Zheng, K.; Ren, J.; Fan, Y.; Ling, S., Strong, ductile and lightweight bionanocomposites constructed by bioinspired hierarchical assembly. *Compos. Commun.* **2020,** *17*, 97-103.
224. Kokubo, T.; Takadama, H., How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* **2006,** *27* (15), 2907-2915.
225. Oyane, A.; Kim, H. M.; Furuya, T.; Kokubo, T.; Miyazaki, T.; Nakamura, T., Preparation and assessment of revised simulated body fluids. *J. Biomed. Mater. Res. A* **2003,** *65A* (2), 188-195.
226. Griffanti, G.; Jiang, W.; Nazhat, S. N., Bioinspired mineralization of a functionalized injectable dense collagen hydrogel through silk sericin incorporation. *Biomater. Sci.* **2019,** *7* (3), 1064-1077.
227. Zafar, B.; Mottaghitalab, F.; Shahosseini, Z.; Negahdari, B.; Farokhi, M., Silk fibroin/alumina nanoparticle scaffold using for osteogenic differentiation of rabbit adipose-derived stem cells. *Materialia* **2020,** *9*, 100518.
228. Johari, N.; Madaah Hosseini, H. R.; Samadikuchaksaraei, A., Mechanical modeling of silk fibroin/TiO2 and silk fibroin/fluoridated TiO2 nanocomposite scaffolds for bone tissue engineering. *Iran. Polym. J.* **2020,** *29* (3), 219-224.
229. Xing, X.; Cheng, G.; Yin, C.; Cheng, X.; Cheng, Y.; Ni, Y.; Zhou, X.; Deng, H.; Li, Z., Magnesium-containing silk fibroin/polycaprolactone electrospun nanofibrous

scaffolds for accelerating bone regeneration. *Arab. J. Chem.* **2020,** *13* (5), 5526-5538.

230. Tanasa, E.; Zaharia, C.; Hudita, A.; Radu, I.-C.; Costache, M.; Galateanu, B., Impact of the magnetic field on 3T3-E1 preosteoblasts inside SMART silk fibroin-based scaffolds decorated with magnetic nanoparticles. *Mater. Sci. Eng. C* **2020,** *110*, 110714.
231. Narimani, M.; Teimouri, A.; Shahbazarab, Z., Synthesis, characterization and biocompatible properties of novel silk fibroin/graphene oxide nanocomposite scaffolds for bone tissue engineering application. *Polym. Bull.* **2019,** *76* (2), 725-745.
232. Liu, F.; Liu, C.; Zheng, B.; He, J.; Liu, J.; Chen, C.; Lee, I.-S.; Wang, X.; Liu, Y., Synergistic Effects on Incorporation of β-Tricalcium Phosphate and Graphene Oxide Nanoparticles to Silk Fibroin/Soy Protein Isolate Scaffolds for Bone Tissue Engineering. *Polymers* **2020,** *12* (1), 69.
233. Nie, L.; Zhang, H.; Ren, A.; Li, Y.; Fu, G.; Cannon, R. D.; Ji, P.; Wu, X.; Yang, S., Nano-hydroxyapatite mineralized silk fibroin porous scaffold for tooth extraction site preservation. *Dent. Mater.* **2019,** *35* (10), 1397-1407.
234. Jin, R.; Wang, Y.; Liu, Y.; Ding, C.; Xie, J.; Li, J., Biomineralization and osteogenic differentiation modulated by substrate stiffness. *Eur. Polym. J.* **2020,** *122*, 109395.
235. Wang, D.; Christensen, K.; Chawla, K.; Xiao, G.; Krebsbach, P. H.; Franceschi, R. T., Isolation and Characterization of MC3T3-E1 Preosteoblast Subclones with Distinct In Vitro and In Vivo Differentiation/Mineralization Potential. *J. Bone Miner. Res.* **1999,** *14* (6), 893-903.
236. Franceschi, R. T.; Iyer, B. S.; Cui, Y., Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J. Bone Miner. Res.* **1994,** *9* (6), 843-854.
237. Valenti, M. T.; Zanatta, M.; Donatelli, L.; Vivano, G.; Cavallini, C.; Scupoli, M. T.; Carbonare, L. D., Ascorbic Acid Induces either Differentiation or Apoptosis in MG-63 Osteosarcoma Lineage. *Anticancer Res.* **2014,** *34* (4), 1617-1627.
238. Shams, N.; Ghasemi, M.; Sadatmansouri, S.; Bonakdar, S., Morphology and Differentiation of MG63 Osteoblast Cells on Saliva Contaminated Implant Surfaces. *J. Dent. (Tehran)* **2015,** *12* (6), 424-429.
239. Qian, S.; Wang, Z.; Zheng, Z.; Ran, J.; Zhu, J.; Chen, W., A Collagen and Silk Scaffold for Improved Healing of the Tendon and Bone Interface in a Rabbit Model. *Med. Sci. Monit.* **2019,** *25*, 269-278.
240. Sun, S.; Zeng, H., Size-Controlled Synthesis of Magnetite Nanoparticles. *J. Am. Chem. Soc.* **2002,** *124* (28), 8204-8205.
241. Daou, T. J.; Pourroy, G.; Bégin-Colin, S.; Grenèche, J. M.; Ulhaq-Bouillet, C.; Legaré, P.; Bernhardt, P.; Leuvrey, C.; Rogez, G., Hydrothermal Synthesis of Monodisperse Magnetite Nanoparticles. *Chem. Mater.* **2006,** *18* (18), 4399-4404.
242. Cuevas, R.; Durán, N.; Diez, M. C.; Tortella, G. R.; Rubilar, O., Extracellular Biosynthesis of Copper and Copper Oxide Nanoparticles by *Stereum hirsutum*, a Native White-Rot Fungus from Chilean Forests. *J. Nanomater.* **2015,** *2015*, 789089.
243. Narasaiah, P.; Mandal, B. K.; Sarada, N. C., Biosynthesis of Copper Oxide nanoparticles from *Drypetes sepiaria* Leaf extract and their catalytic activity to dye degradation. *IOP Conf. Ser.-Mat. Sci* **2017,** *263*, 022012.
244. Karuppaiah, M.; Akilan, R.; Sakthivel, P.; Asaithambi, S.; Shankar, R.; Yuvakkumar, R.; Hayakawa, Y.; Ravi, G., Synthesis of self-assembled micro/nano structured manganese carbonate for high performance, long lifespan asymmetric

supercapacitors and investigation of atomic-level intercalation properties of OH− ions via first principle calculation. *J. Energy Storage* **2020,** *27*, 101138.

245. Xiao, L.; Wang, S.; Wang, Y.; Meng, W.; Deng, B.; Qu, D.; Xie, Z.; Liu, J., High-Capacity and Self-Stabilized Manganese Carbonate Microspheres as Anode Material for Lithium-Ion Batteries. *ACS Appl. Mater. Interfaces* **2016,** *8* (38), 25369-25378.
246. Yoshida, N.; Higashimura, E.; Saeki, Y., Catalytic Biomineralization of Fluorescent Calcite by the Thermophilic Bacterium *Geobacillus thermoglucosidasius*. *Appl Environ Microbiol* **2010,** *76* (21), 7322-7327.
247. Sharma, A.; Bhattacharya, A., Enhanced biomimetic sequestration of CO2 into CaCO3 using purified carbonic anhydrase from indigenous bacterial strains. *J. Mol. Catal., B Enzym.* **2010,** *67* (1), 122-128.
248. Chow, L. C.; Takagi, S., A Natural Bone Cement-A Laboratory Novelty Led to the Development of Revolutionary New Biomaterials. *J. Res. Natl. Inst. Stand. Technol.* **2001,** *106* (6), 1029-1033.
249. Fei, J. B.; Cui, Y.; Yan, X. H.; Qi, W.; Yang, Y.; Wang, K. W.; He, Q.; Li, J. B., Controlled Preparation of MnO2 Hierarchical Hollow Nanostructures and Their Application in Water Treatment. *Adv. Mater.* **2008,** *20* (3), 452-456.
250. Hu, A.; Liu, S.; Lin, W., Immobilization of chiral catalysts on magnetite nanoparticles for highly enantioselective asymmetric hydrogenation of aromatic ketones. *RSC Advances* **2012,** *2* (6), 2576-2580.
251. Yang, D.; Yang, F.; Hu, J.; Long, J.; Wang, C.; Fu, D.; Ni, Q., Hydrophilic multi-walled carbon nanotubes decorated with magnetite nanoparticles as lymphatic targeted drug delivery vehicles. *Chem. Comm.* **2009,** (29), 4447-4449.
252. Das, M.; Mishra, D.; Maiti, T. K.; Basak, A.; Pramanik, P., Bio-functionalization of magnetite nanoparticles using an aminophosphonic acid coupling agent: new, ultradispersed, iron-oxide folate nanoconjugates for cancer-specific targeting. *Nanotechnology* **2008,** *19* (41), 415101.
253. Brif, A.; Ankonina, G.; Drathen, C.; Pokroy, B., Bio-Inspired Band Gap Engineering of Zinc Oxide by Intracrystalline Incorporation of Amino Acids. *Adv. Mater.* **2014,** *26* (3), 477-481.
254. Rajiv, P.; Rajeshwari, S.; Venckatesh, R., Bio-Fabrication of zinc oxide nanoparticles using leaf extract of *Parthenium hysterophorus* L. and its size-dependent antifungal activity against plant fungal pathogens. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **2013,** *112*, 384-387.
255. Zare, E.; Pourseyedi, S.; Khatami, M.; Darezereshki, E., Simple biosynthesis of zinc oxide nanoparticles using nature's source, and it's in vitro bio-activity. *J. Mol. Struct.* **2017,** *1146*, 96-103.
256. Elumalai, K.; Velmurugan, S.; Ravi, S.; Kathiravan, V.; Ashokkumar, S., Bio-fabrication of zinc oxide nanoparticles using leaf extract of curry leaf (*Murraya koenigii*) and its antimicrobial activities. *Materials Mater. Sci. Semicond. Process.* **2015,** *34*, 365-372.
257. Azizi, S.; Mohamad, R.; Bahadoran, A.; Bayat, S.; Rahim, R. A.; Ariff, A.; Saad, W. Z., Effect of annealing temperature on antimicrobial and structural properties of bio-synthesized zinc oxide nanoparticles using flower extract of *Anchusa italica*. *J. Photochem. Photobiol. B, Biol.* **2016,** *161*, 441-449.
258. Yan, C.; Zhu, Y.; Fang, Z.; Lv, C.; Zhou, X.; Chen, G.; Yu, G., Heterogeneous Molten Salt Design Strategy toward Coupling Cobalt–Cobalt Oxide and Carbon for Efficient Energy Conversion and Storage. *Adv. Energy Mater.* **2018,** *8* (23), 1800762.

259. Kim, J.-W.; Choi, S. H.; Lillehei, P. T.; Chu, S.-H.; King, G. C.; Watt, G. D., Cobalt oxide hollow nanoparticles derived by bio-templating. *Chem. Comm.* **2005,** *32*, 4101-4103.
260. Schenk, A. S.; Zope, H.; Kim, Y.-Y.; Kros, A.; Sommerdijk, N. A. J. M.; Meldrum, F. C., Polymer-induced liquid precursor (PILP) phases of calcium carbonate formed in the presence of synthetic acidic polypeptides—relevance to biomineralization. *Faraday Discuss.* **2012,** *159*, 327-344.
261. Kim, Y.-Y.; Hetherington, N. B. J.; Noel, E. H.; Kröger, R.; Charnock, J. M.; Christenson, H. K.; Meldrum, F. C., Capillarity Creates Single-Crystal Calcite Nanowires from Amorphous Calcium Carbonate. *Angew. Chem. Int. Ed.* **2011,** *50* (52), 12572-12577.
262. Schenk, A. S.; Zlotnikov, I.; Pokroy, B.; Gierlinger, N.; Masic, A.; Zaslansky, P.; Fitch, A. N.; Paris, O.; Metzger, T. H.; Cölfen, H.; Fratzl, P.; Aichmayer, B., Hierarchical Calcite Crystals with Occlusions of a Simple Polyelectrolyte Mimic Complex Biomineral Structures. *Adv. Funct. Mater.* **2012,** *22* (22), 4668-4676.
263. Evans, J. S., "Tuning in" to Mollusk Shell Nacre- and Prismatic-Associated Protein Terminal Sequences. Implications for Biomineralization and the Construction of High Performance Inorganic−Organic Composites. *Chem. Rev.* **2008,** *108* (11), 4455-4462.
264. Schenk, A. S.; Cantaert, B.; Kim, Y.-Y.; Li, Y.; Read, E. S.; Semsarilar, M.; Armes, S. P.; Meldrum, F. C., Systematic Study of the Effects of Polyamines on Calcium Carbonate Precipitation. *Chem. Mater.* **2014,** *26* (8), 2703-2711.
265. Schenk, A. S.; Eiben, S.; Goll, M.; Reith, L.; Kulak, A. N.; Meldrum, F. C.; Jeske, H.; Wege, C.; Ludwigs, S., Virus-directed formation of electrocatalytically active nanoparticle-based Co3O4 tubes. *Nanoscale* **2017,** *9* (19), 6334-6345.
266. Shaheen, W. M.; Selim, M. M., Thermal Decompositions of Pure and Mixed Manganese Carbonate and Ammonium Molybdate Tetrahydrate. *J. Therm. Anal. Calorim.* **2000,** *59* (3), 961-970.
267. Gebauer, D.; Cölfen, H.; Verch, A.; Antonietti, M., The Multiple Roles of Additives in CaCO3 Crystallization: A Quantitative Case Study. *Adv. Mater.* **2009,** *21* (4), 435-439.
268. Mao, L.-B.; Gao, H.-L.; Yao, H.-B.; Liu, L.; Cölfen, H.; Liu, G.; Chen, S.-M.; Li, S.-K.; Yan, Y.-X.; Liu, Y.-Y.; Yu, S.-H., Synthetic nacre by predesigned matrix-directed mineralization. *Science* **2016,** *354* (6308), 107-110.
269. Prewitz, M. C.; Stißel, A.; Friedrichs, J.; Träber, N.; Vogler, S.; Bornhäuser, M.; Werner, C., Extracellular matrix deposition of bone marrow stroma enhanced by macromolecular crowding. *Biomaterials* **2015,** *73*, 60-69.
270. Willigeroth, S. F. Strategien zur Synthese kolloidaler Fluorapatitpartikel und Nanokomposite-Dissertation. Christian-Albrechts-Universität Kiel, 2002.
271. Liu, J.; Wang, X.; Jin, Q.; Jin, T.; Chang, S.; Zhang, Z.; Czajka-Jakubowska, A.; Giannobile, W. V.; Nör, J. E.; Clarkson, B. H., The stimulation of adipose-derived stem cell differentiation and mineralization by ordered rod-like fluorapatite coatings. *Biomaterials* **2012,** *33* (20), 5036-5046.
272. Niu, Y.; Cao, L.; Wei, J.; Ma, Y.; Song, S.; Weng, W.; Li, H.; Liu, C.; Su, J., Development of a bioactive composite of nanofluorapatite and poly(butylene succinate) for bone tissue regeneration. *J. Mat. Chem. B* **2014,** *2*, 1174-1181.
273. Montazeri, N.; Jahandideh, R.; Biazar, E., Synthesis of fluorapatite-hydroxyapatite nanoparticles and toxicity investigations. *Int. J. Nanomedicine* **2011,** *6*, 197-201.

5 Publikationen und Manuskripte

I **Neubauer, V. J.**[†]; Kellner, C.[†]; Gruen, V.; Schenk, A. S.[*]; Scheibel, T.[*] Recombinant major ampullate spidroin-particles as biotemplates for manganese carbonate mineralization. *Multifunctional Materials* **2021**, *4*, 014002. doi.org/10.1088/2399-7532/abddc4

II **Neubauer, V. J.**[†]; Döbl, A.[†]; Scheibel, T. Silk-Based Materials for Hard Tissue Engineering. *Materials* **2021**, *14* (3), 674. doi.org/10.3390/ma14030674

III **Neubauer, V. J.**; Scheibel, T. Spider Silk Fusion Proteins for Controlled Collagen Binding and Biomineralization. *ACS Biomaterials Sciene and Engineering*, **2020**, *6* (10), 5599-5608. doi.org/10.1021/acsbiomaterials.0c00818

IV **Neubauer, V. J.**; Hüter, F.; Wittmann, J.; Trossmann, V. T.; Kleinschrodt, C.; Alber-Laukant, B.; Rieg, F.; Scheibel, T. (2021) Flow Simulation and Gradient Printing of Fluorapatite loaded Recombinant Spider Silk Hydrogels.
Manuskript in der Vorbereitung

V **Neubauer, V. J.**; Trossmann, V. T.; Jacobi, S.; Döbl, A.; Scheibel, T. Recombinant Spider Silk Gels Derived from Aqueous–Organic Solvents as Depots for Drugs. *Angewandte Chemie International Edition* **2021**, *60*, 11847-11851. doi.org/10.1002/anie.202103147

Deutsche Version:
Neubauer, V. J.; Trossmann, V. T.; Jacobi, S.; Döbl, A.; Scheibel, T. Rekombinante Spinnenseidengele aus wässrig-organischen Mischphasen als Wirkstoffdepots. *Angewandte Chemie* **2021**, *133*, 11953 –11958. doi.org/10.1002/ange.202103147

Nicht Teil dieser Abhandlung:

VI Trossmann, V. T.[†]; **Neubauer, V. J.**[†]; Ng, X. J.; Peng, Y. Y.; Scheibel, T.[*]; Glattauer, V.[*] (2021) Recombinant collagen with enhanced 3D printability in spider silk blend hydrogels.
Manuskript in der Vorbereitung

VII Haynl, C.; Vongsvivut, J.; Mayer, K. R. H.; Bargel, H.; **Neubauer, V. J.**; Tobin, M. J.; Elgar, M. A.; Scheibel, T. Free-standing spider silk webs of the thomisid *Saccodomus formivorus* are made of composites comprising micro- and submicron fibers. *Scientific Reports*, **2020**, *10*, 17624. doi.org/10.1038/s41598-020-74469-z

VIII **Wicklein, V. J.**; Singer, B. B.; Scheibel, T.; Salehi, S. Chapter 17 - Nanoengineered biomaterials for corneal regeneration. In *Nanoengineered Biomaterials for Regenerative Medicine*. Mozafari, M.; Rajadas, J.; Kaplan, D., Hrsg. Elsevier Amsterdam, Niederlande, 2020, pp. 379-415. doi.org/10.1016/B978-0-12-813355-2.00017-X

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6 Darstellung des Eigenanteils

Diese Dissertation besteht aus fünf Teilarbeiten, die teilweise unter Zusammenarbeit mit KooperationspartnerInnen entstanden sind. Im Folgenden wird der Beitrag der einzelnen Autoren für jede Teilarbeit gesondert dargestellt.

I **Neubauer, V. J.**[†]; Kellner, C.[†]; Gruen, V.; Schenk, A. S.[*]; Scheibel, T.[*] Recombinant major ampullate spidroin-particles as biotemplates for manganese carbonate mineralization. *Multifunctional Materials* **2021**, *4*, 014002. doi.org/10.1088/2399-7532/abddc4

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Das Konzept dieser Studie wurde von Anna Schenk, Thomas Scheibel, Christine Kellner und mir erstellt. Die biotechnologische Herstellung der rekombinanten Proteine wurde von Johannes Diehl und mir und die Reinigung durch meinen Forschungsstudenten Ugonna Fredrick und mich durchgeführt. Die Proteine wurden von mir in Mikropartikel prozessiert. Eine Charakterisierung der Seidenmorphologien mit DLS, TGA und ATR-FTIR wurde von mir durchgeführt und REM Messungen mit Hendrik Bargel koordiniert. Christine Kellner hat die Mineralisierungsexperimente durchgeführt und die entstandenen Spezies mittels ATR-FTIR, REM und REM-EDX Messungen charakterisiert, sowie XRD Messungen für Florian Puchtler angewiesen. Viktoria Grün half ihr bei der experimentellen Umsetzung und brachte sich bei wissenschaftlichen Diskussionen ein. Das Manuskript wurde von mir erstellt, von allen Autoren wissenschaftlich editiert und von mir mit Anna Schenk und Thomas Scheibel fertig gestellt.

II **Neubauer, V. J.**[†]; Döbl, A.[†]; Scheibel, T. Silk-Based Materials for Hard Tissue Engineering. *Materials* **2021**, *14* (3), 674. doi.org/10.3390/ma14030674

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Der Übersichtsartikel wurde von Thomas Scheibel und mir konzipiert. Annika Döbl und ich führten Literaturrecherchen durch und gestalteten Abbildungen. Annika Döbl

fokussierte sich auf Seide und Tissue Engineering im Allgemeinen. Alle Passagen zu hartem Gewebeersatz und alle Abbildungen wurden von mir erstellt. Das Manuskript wurde von allen Autoren editiert und durch mich und Thomas Scheibel finalisiert.

III **Neubauer, V. J.**; Scheibel, T. Spider Silk Fusion Proteins for Controlled Collagen Binding and Biomineralization. *ACS Biomaterials Sciene and Engineering*, **2020**, *6* (10), 5599-5608. doi.org/10.1021/acsbiomaterials.0c00818

Das Projekt wurde von Thomas Scheibel und mir konzipiert. Die rekombinanten Spinnenseidenproteine wurden von mir designt, mit Hilfe von Johannes Diehl und Andreas Schmidt hergestellt und von mir mit meiner wissenschaftlichen Hilfskraft Razieh Moeini gereinigt. Alle experimentellen Arbeiten, falls nicht anders erwähnt, wurden von mir durchgeführt und REM und REM-EDX Messungen mit Hendrik Bargel koordiniert. Alexandra Pellert unterstützte bei Zellkulturstudien. XRD Messungen wurden von Florian Puchtler durchgeführt und mit Theresa Dörres bewertet. Das Manuskript wurde von mir vorbereitet und mit Thomas Scheibel abgeschlossen.

IV **Neubauer, V. J.**; Hüter, F.; Wittmann, J.; Trossmann, V. T.; Kleinschrodt, C.; Alber-Laukant, B.; Rieg, F.; Scheibel, T. (2021) Flow Simulation and Gradient Printing of Fluorapatite loaded Recombinant Spider Silk Hydrogels.
Manuskript in der Vorbereitung

Das Konzept dieses Kooperationsprojekts wurde von Florian Hüter, Claudia Kleinschrodt, Frank Rieg, Thomas Scheibel und mir erstellt. Bettina Alber-Laukant und Claudia Kleinschrodt unterstützten die wissenschaftliche Diskussion zur Strömungssimulation, die von Florian Hüter, Johannes Wittmann und der wissenschaftlichen Hilfskraft Ugonna Fredrick erstellt wurde. Das Prinzip des Gradientendrucks wurde von mir und meinem Forschungsstudenten Ugonna Fredrick erdacht. Alle Experimente wurden von mir durchgeführt, bis auf die Aufnahmen mittels REM von Hendrik Bargel und Claudia Stemmann sowie Konfokalmikroskopie durch Vanessa Troßmann. Das Manuskript wurde von mir verfasst, von allen Autoren editiert und von mir und Thomas Scheibel finalisiert.

V **Neubauer, V. J.**; Trossmann, V. T.; Jacobi, S.; Döbl, A.; Scheibel, T. Recombinant Spider Silk Gels Derived from Aqueous–Organic Solvents as Depots for Drugs. *Angewandte Chemie International Edition* **2021**, *60*, 11847.
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Die Studie wurde von Thomas Scheibel und mir konzipiert. Die experimentelle Durchführung wurde wie folgt aufgeteilt: Meine Forschungsstudentin Sofia Jacobi unterstützte mit rheologischen Messungen und Erstellung des Wirkstofffreisetzungsprofils. Sie koordinierte REM Messungen mit Hendrik Bargel und TEM Messungen mit Anika Winkler. REM Messungen mit Hendrik Bargel, rheologische und ATR-FTIR Messungen mit Sekundärstrukturbestimmung sowie das Konzept der Organo-Dialyse wurden von mir erstellt. Die Assemblierungskinetik mit mikroskopischen Aufnahmen wurde von Vanessa Troßmann durchgeführt. Sie fermentierte und reinigte das eADF4(Ω16) Protein und trug ebenfalls zur wissenschaftlichen Diskussion bei. Das Manuskript wurde von mir formuliert, von allen Autoren editiert und von Thomas Scheibel und mir vollendet.

7 Teilarbeiten

7.1 Teilarbeit I

Teilarbeit I erschien am 09.02.2021 unter dem Titel *„Recombinant major ampullate spidroin-particles as biotemplates for manganese carbonate mineralization“* in *Multifunctional Materials*.

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Multifunctional Materials

PAPER

Recombinant major ampullate spidroin-particles as biotemplates for manganese carbonate mineralization

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Abstract

Mineral deposition in biological systems is often templated by organic matrices including proteins directing the nucleation and growth of bioceramics by interacting with early stage species of the mineralization process or coordinating specific facets of the forming crystal. Structurally, charged surface patches are a characteristic motif of biomineralization-associated proteins, which are able to accumulate and bind ions from the surrounding media and, therefore, initiate, promote or inhibit mineralization. Controlled protein engineering enables the manipulation and control of bioinspired *in vitro* precipitation systems, and thus not only opens prospects for the design of environmentally benign synthetic strategies towards hierarchically structured functional materials, but also enhances the understanding of fundamental interaction mechanisms in biomineralization processes. Here, two recombinant variants of the spider silk protein ADF4 were engineered with oppositely charged peptide tags. Both were processed into micrometer-sized particles and investigated for their influence on manganese carbonate mineralization. Micro- and nano-structured manganese carbonate represents an attractive material for diverse applications including catalysis and wastewater treatment. While both types of spider silk particles were incorporated into the mineral structure, the positively tagged proteins appeared to interact more strongly with the formed manganese carbonate crystals than their negatively charged counterparts. Combination of the spider silk particles and poly(acrylic acid) (PAA), a water-soluble structure-directing agent associated with the stabilization of amorphous precursor phases in carbonates, resulted in the formation of film-like non-equilibrium structures of $MnCO_3$ entrapping the spider silk particles. With the aim to gain mechanistic insights and to elucidate the interaction between the different components involved in the mineralization process, we studied the interplay between PAA, positively or negatively tagged spider silk particles, and Mn(II) ions by time-resolved dynamic light scattering. The here used set-up affords the possibility to identify control strategies for the template-mediated mineralization of manganese carbonate.

1. Introduction

Biogenic minerals such as bone or sea shells combine light weight architectures with dedicated structural and functional properties (mechanical and optical) and are often unrivalled by man-made materials concerning their complex composition and function, thus endowing them with impressive performance e.g. as an

intra- or extra-skeletal support or protective tissue [1–4]. Relying on only a limited selection of available elements, living organisms have developed elaborate mechanisms to construct sophisticated, hierarchically organized mineral structures, often based on inherently brittle but abundant materials such as calcium carbonates, calcium phosphates, and amorphous silica, grown under mild conditions. However, to less extent, also transition metal oxides such as Fe_3O_4 [5] and MnO_2 [6] are employed by chitons, bacteria, and fungi amongst others. In comparison to geological and synthetic precipitation and crystallization processes, the degree of control over crystal morphology, polymorphism, and texture achieved in biomineralization is astonishing.

Biological mineral deposition is characteristically influenced by charged moieties of water-soluble or insoluble organic compounds such as proteins [7] and carbohydrates [8] attracting and interacting with ionic species from the surroundings, thus initiating and directing crystallization, often resulting in structural complexity over several hierarchical levels and the formation of organic-inorganic composites [8, 9]. In the case of bone, for example, collagen type I comprises the major component of the bone-associated organic matrix and acts as a scaffold for mineral deposition [10, 11], while small amounts of intrinsically disordered non-collagenous proteins with localized negative charges [10, 12] are involved in the formation and organisation of hydroxyapatite nanoplatelets. Specifically, members of the small integrin binding ligand N-linked glycoprotein protein family [10] are secreted at different stages of bone mineralization during cell differentiation, with the acidic side chains of glutamic or aspartic acid residues enabling them to bind polyvalent ions and interfere with calcium phosphate nucleation and growth [7, 13]. In diatoms, a class of unicellular microalgae representing the dominant group of phytoplankton in the ocean, silaffin proteins, functionalized with high proportions of modified lysine and serine residues, are involved in the formation of the intriguingly complex patterns seen in silica-based cell walls [14, 15]. Similarly, $CaCO_3$ mineralization in the nacre layer of mollusc shells is directed by proteins with a conspicuously high proportion of up to 50% basic or acidic amino acid moieties acting as 'inhibitors' or 'promoters' of nucleation and growth [8]. While the specific mineralization mechanisms underlying the formation of nacre are not yet fully understood [10, 13], the process seems to critically depend on the presence of charged moieties, their position and accumulation on the protein's surface.

In view of technological applications, bioinspired mineralization approaches translate the design and constructing principles of natural blueprints into artificial materials and tailor-made solutions. Over the last decades, controlled *in vitro* precipitation with elaborate control over crystal polymorphism, morphology, and texture has been achieved in calcium carbonate, a material ubiquitous in biogenic materials, by using soluble [16] polymer matrices including polyelectrolytes such as poly(acrylic acid) (PAA) [17] and insoluble organic templates [18, 19]. Another setup uses confinement for the mineralization control [20, 21]. Remarkably, the successful occlusion of a wide range of entities in different size regimes ranging from small molecules (~1 nm) [22, 23] over micelle-forming block copolymers (~20 nm) [24] to gel fibres [25, 26] and latex spheres (~250 nm) [27] into single crystalline calcite hosts has resulted in organic-inorganic composite crystals exhibiting composition-dependent mechanical properties, partially resembling the toughening and hardening mechanisms observed in biogenic calcite. More recently, bioinspired concepts of crystallization control have also been transferred to the deposition of technologically relevant materials including iron oxide [28], copper oxide [29, 30] and zinc oxide [31] as well as cobalt hydroxide carbonate and spinel-type cobalt oxide [32] to fabricate functional materials with complex morphologies and nanocomposite character, resulting in materials with structure-dependent and tunable emerging optical, magnetic, and catalytic properties.

In this context manganese carbonate ($MnCO_3$) is attracting great interest as an auspicious material because of its crystallographic similarity to calcium carbonate (usually isostructural to calcite), its chemical composition based on earth-abundant, non-toxic elements, and its potential use as a supercapacitor [33] or as an anode material in lithium-ion batteries [34]. Most importantly, $MnCO_3$ represents a thermally unstable precursor for manganese oxide species, which find broad applications in catalysis and water treatment [35]. Therefore, there is a demand for establishing synthetic protocols towards the production of manganese carbonates and oxides with well-defined, purpose-optimized architectures proceeding under mild reaction conditions [36, 37].

In order to control bioinspired mineralization, proteins are well suited as mineralization triggers. As easily engineerable tuneable biopolymers, which can be produced in high yield and processed into a variety of morphologies, recombinant spider silk proteins with dedicated functionalization allowed to mimic of biomineralization-associated proteins and thus are well established in tissue engineering and template-directed crystallization [38–42].

Here, we address the effects of functionalized spider silk particles as biotemplates on room temperature crystallization of manganese carbonate. For this purpose, we used an engineered polyanionic 48 kDa variant of the *Araneus diadematus* fibroin-4 (eADF4) as a starting material and designed two novel silk protein

variants carrying differently charged peptide tags in order to selectively modify the surface properties of particles made thereof. Recently, it has been shown that other spider silk variants processed into structured films comprising peptide tags with specific binding sequences for TiO_2 and Au allowed for the fabrication of hybrid photo-catalyst materials with promising properties in water-hydrolysis [43]. Here, we generated $MnCO_3$-based organic-inorganic composite materials templated by spider silk particles. By combining a range of diverse characterization methods including vibration spectroscopy, powder x-ray diffraction, electron microscopy and time-resolved dynamic light scattering, our study reveals insights into the nature of the template-directed inorganic phases as well as the underlying mechanisms of spider silk-mediated mineralization.

2. Materials and methods

2.1. Recombinant spider silk production and purification

A seamless cloning technique was used to ligate the oligonucleotide sequences encoding the peptide tags to the 3′ end of the engineered spider silk gene encoding eADF4(C16) (*A. diadematus* fibroin) comprising 16 repeats of a consensus motif (C-module) of fibroin-4 of the European garden spider *A. diadematus* [40]. The production of variants comprising an E_8G-tag (pI = 3.3) and a K_8G-tag (pI = 4.6) was performed according to a protocol developed for RGD-tagged eADF4(C16) as published previously [39].

2.2. Spider silk processing

Spider silk solutions were prepared by dissolving lyophilized proteins in 6 M guanidinium thiocyanate (Carl Roth, Karlsruhe, Germany) and dialysis against 10 mM Tris buffer (Carl Roth, Karlsruhe, Germany) at pH 7.5 overnight. Spider silk particles were precipitated out of a 1 mg ml^{-1} solution in 10 mM Tris buffer, pH 7.5 upon addition of potassium phosphate, pH 8 (Carl Roth, Karlsruhe, Germany) at a final concentration of 1 M and rotation for 30 min at RT. The final (submicron) particles were dialysed against de-ionized MQ water for 3 d to remove residing potassium phosphateand stored in water until further use.

2.3. Precipitation of $MnCO_3$

Sliced microscopy glass slides (Thermo Scientific, Waltham, MA, USA) were used as substrates for the deposition of manganese carbonate. In order to remove organic residues, the substrates were cleaned by treatment with Piranha solution, a 4:1 mixture of sulfuric acid (Sigma-Aldrich, Taufkirchen, Germany) and hydrogen peroxide (Sigma-Aldrich, Taufkirchen, Germany), followed by rinsing in copious amounts of de-ionized MQ water and drying in a dust-free fume hood.

A gas diffusion technique, based on the slow decomposition of ammonium carbonate, well-established for the precipitation of calcium carbonate [44], was adapted for the RT deposition of manganese carbonate. In brief, aqueous solutions of $MnCl_2$ (Sigma-Aldrich, Taufkirchen, Germany) in suspension with spider silk particles, and poly(acrylic acid) (PAA; M_W = 100 000 g mol^{-1}, Sigma-Aldrich, Taufkirchen, Germany) were adjusted to final concentrations of (Mn(II)) = 25 mM pH 6.3, (spider silk particles) = 0.2 mg ml^{-1} pH 5.5, and (PAA) = 0.02 mg ml^{-1} pH 5.8, and pipetted into glass vials. After homogenization of the reaction media in an Ika Vortex 3 shaker (Sigma-Aldrich, Taufkirchen, Germany), the vials were covered with a snap-cap into which three holes were punched with a fine needle and subsequently placed inside a sealed desiccator together with two glass vials filled with solid ammonium carbonate (Grüssing, Filsum, Germany) as a carbon dioxide source upon decomposition. After exposure to NH_3 and CO_2 vapour for 24–48 h, the glass substrates, which supported $MnCO_3$ nucleation, were removed from the reactant solution, rinsed with copious amounts of de-ionized MQ water and left to air dry at RT.

2.4. Scanning electron microscopy (SEM) and energy-dispersive spectroscopy (EDS)

Recombinant spider silk particles were studied using SEM after platinum sputter coating (1.3 nm) using a Sputter Coater 208 h from Cressington Scientific Instruments (Watford, United Kingdom) to prevent the accumulation of electrostatic charging during imaging, which was performed with a ZEISS Sigma 300 VP and Sigma 500 chamber (Zeiss, Oberkochen, Germany) at an acceleration voltage of 3 kV. Further, glass substrates supporting $MnCO_3$ deposits and mineralized silk particles were mounted on a standard sample holder using conductive adhesion graphite-pads (Plano, Wetzlar, Germany) and subsequently platinum sputter coated (1 nm). Mineralized spider silk particles were examined using a Zeiss LEO 1530 VP Gemini instrument (FE-SEM equipped with a Schottky-field-emission cathode and secondary electron as well as backscattered electron detectors). The elemental composition of the samples was studied using EDS with an UltraDry silicon drift detector Noran system from Thermo Fisher Scientific. The instrument was operated at an accelerating voltage of 3 kV for SEM imaging and 20 kV for EDS measurements.

2.5. Attenuated total reflectance-fourier transformat infrared spectroscopy (ATR-FTIR)

In order to confirm protein integrity and to exclude the presence of phosphate ions co-precipitated during particle formation, the chemical composition of the samples was studied using ATR–FTIR spectroscopy. Single spectra were recorded using a Bruker Tensor 27 (Ettlingen, Germany) and a Specac golden gate single reflection ATR and a germanium crystal and a spectral resolution of 2 cm^{-1} with 100 scans. An atmospheric compensation algorithm was applied in OPUS 8.0 software to correct for water vapour and carbon dioxide fluctuations during the measurement.

2.6. Powder x-ray diffraction (PXRD)

X-ray diffractograms of finely ground powders were recorded to study the crystallinity of the mineralized precipitates and to identify the deposited mineral phases. All measurements were performed using monochromatic Cu-Kα1 radiation on a PANalytical XPert Pro diffractometer (Malvern Panalytical B.V., Malvern, United Kingdom) operated at 40 kV and 30 mA in Bragg-Brentano geometry. Data were recorded in an angular range between 10° and 70° with a total exposure time of 600 s and analysed using X'Pert HighScore Plus Version 3.0 (Malvern Panalytical B.V., Malvern, United Kingdom). Diffraction peaks were assigned according to reference data for synthetic rhodochrosite (ICDD PDF# 00-044-1472).

2.7. Thermogravimetric analysis (TGA)

To determine the content of organic material occluded within mineralized species, TGA was carried out using a TGA2 instrument (Mettler-Toledo AG, Schwerzenbach, Switzerland). Samples were transferred into alumina vessels and heated from 25 to 1000 °C at a rate of 10° min^{-1} under nitrogen flow. The mass difference between 250 and 500 °C was determined based on the horizontal onset and offset of the curve in the STARe Software (Mettler-Toledo AG, Schwerzenbach, Switzerland) and used to calculate the content of the components based on dry weight.

2.8. Dynamic light scattering (DLS)

DLS was performed using an Anton Paar LiteSizerTM500 (Graz, Austria) in order to study the aggregation behaviour of spider silk particles in Mn(II) solutions in the absence and presence of PAA.

For this purpose, aqueous stock solutions of Mn(II) ions, PAA and recombinant spider silk particle suspensions were prepared in de-ionized MQ water and mixed according to the ratios present in the considered mineralization assays. The mean hydrodynamic radii of species, formed upon the interaction between the individual components, were then determined after different incubation times ($t = 5$ min, 30 min, 120 min).

Specimens investigated at each incubation time were degassed under nitrogen flow to avoid CO_2 intake. In between the DLS experiments, the solutions were stored at RT. Prior to the measurements, the samples were freshly mixed, and 50 μl of sample were diluted with de-ionized MQ water to give a final volume of 1 ml. Hydrodynamic diameters were determined as a mean value obtained from triplicate sample measurements and from automated 10–20 runs.

3. Results and discussion

3.1. Recombinant spider silk particles induce different mineralized composites depending on their surface charge and the interaction with poly(acrylic acid)

The effect of particle surface charge on manganese carbonate mineralization was investigated using two engineered spider silk variants with oppositely charged peptide tags. The spider silk core protein is based on 16 repeats of a consensus motif (C-module) derived from the repetitive core domain of the European garden spider *A. diadematus* fibroin-4, which has previously been described by Scheibel and coworkers as eADF4(C16) [40]. Peptide tags were C-terminally engineered comprising 8 glutamic acid residues yielding carboxyl-functionalized eADF4(C16)-E_8G or 8 lysine residues yielding amine-functionalized eADF4(C16)-K_8G (figure 1(A)) [40]. Particles formed by a salting-out process of both variants appeared roughly spherical and homogenous when analysed using SEM, exhibiting smooth surfaces and average diameters for dehydrated particles of 498 ± 253 nm for eADF4(C16)-E_8G and 549 ± 320 nm for eADF4(C16)-K_8G (figures 1(B) and (C)). Spider silk particles were previously reported to provide surface-exposed charges in accordance with their theoretical net charge resulting from amino acid residues [45]. The obtained protein particles were then dispersed in an Mn(II)-containing aqueous crystallization medium in order to investigate possible modes of interaction with divalent metal cations and forming mineral crystals based on the size and shape as well as the charge of the water-insoluble spider silk templates.

Mineralization was induced by slow CO_2 and NH_3 vapour diffusion resulting from the decomposition of ammonium carbonate (figure 2(A)), and this procedure was adapted from a previously reported method for

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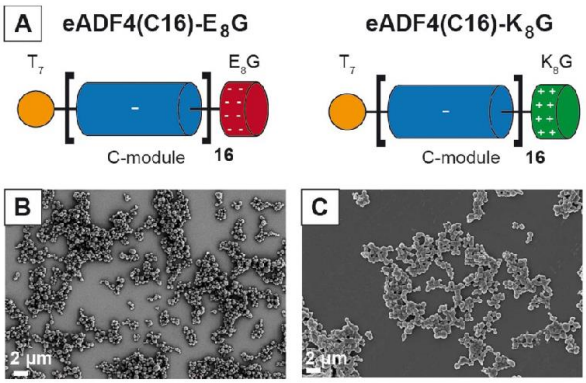

Figure 1. (A) Schematic illustration of the two recombinant spider silk proteins used in this study: eADF4(C16)-E_8G with a negatively charged and eADF4(C16)-K_8G with a positively charged C-terminal tag at physiological pH values; scanning electron micrographs of (B) eADF4(C16)-E_8G and (C) eADF4(C16)-K_8G spider silk particles produced upon precipitation with potassium phosphate at pH 8.

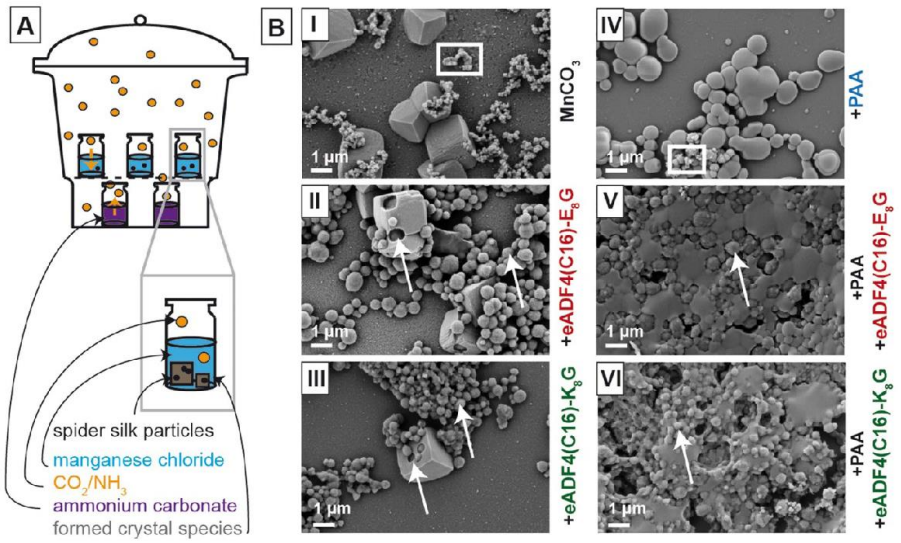

Figure 2. (A) Schematic illustration of the mineralization setup in which the precipitation of $MnCO_3$ particles from $MnCl_2$ solutions is induced by $(NH_4)_2CO_3$ vapour diffusion in a sealed desiccator; (B) scanning electron micrographs showing precipitates of $MnCO_3$ deposited after 24 h (I) in the absence of additives; (II) in the presence of eADF4(C16)-E_8G particles; (III) in the presence of eADF4(C16)-K_8G particles; (IV) in the presence of poly(acrylic acid) (PAA) as a structure-directing additive; (V) in the presence of PAA and eADF4(C16)-E_8G particles; (VI) in the presence of PAA and eADF4(C16)-K_8G particles; arrows indicate spider silk particles, boxes indicate secondary spherical mineralization by-products.

the virus-directed deposition of basic cobalt carbonate [32]. $MnCO_3$ precipitation was studied under different experimental conditions (a) in the absence of additives, (b) in the presence of spider silk particles carrying peptide tags with either positive or negative net charge (insoluble template), (c) mediated by PAA as a structure-directing additive (flexible, water-soluble matrix) and (d) in the presence of spider silk particles as well as PAA (combination of insoluble and soluble matrix). Preliminary studies employing particles made of non-functionalized eADF4(C16) without peptide tags as templates in the mineralization setup, both in the absence and presence of PAA, indicated a tendency to interfere with $MnCO_3$ crystallization such that poorly defined crystal structures with irregular size and shape were formed. For this reason, the new engineered protein variants were used for the following experiments exclusively.

Pure manganese carbonate precipitates obtained after 24 h consisted of cubic particles (~1.5 μm) coexisting with a minority phase of smaller spheres (~0.2 μm) showing a pronounced tendency towards particle fusion (figures 2(B)-(I)). Manganese carbonate morphology was reported earlier to be highly dependent on the preparation conditions [34]. Both, eADF4(C16)-E_8G and eADF4(C16)-K_8G particles, in contrast, appeared to become entrapped in the templated manganese carbonate mineral phase while largely preserving the cubic morphology of the crystals. The different functionalization of the tagged silk particles, however, seemed to affect the binding affinity between the organic templates and the inorganic phase. While eADF4(C16)-E_8G particles were located in larger voids exceeding the silk particle diameter after 24 h, thus being only in loose contact with the mineral (figure 2(B)-(II)), very regular manganese carbonate cubes with tightly incorporated spider silk particles were formed in the presence of eADF4(C16)-K_8G particles (figure 2(B)-(III)).

With the aim to achieve enhanced incorporation of spider silk particles into the mineral phase and to further mediate the interaction between the organic and inorganic components, we introduced PAA as polymer additive into the precipitation system. PAA, a polycarboxylate, is known to be water-soluble and can influence calcium carbonate mineralization due to its high interaction capacity with the mineral [46, 47]. This results in a strong structure- and process-directing effect, stabilizing amorphous colloidal nanoparticles [48] as well as hydrogels [49], thus enabling the formation of non-equilibrium mineral morphologies via assembly of highly hydrated amorphous 'droplets' followed by dehydration, solidification and crystallization processes [50]. Therefore, we used PAA as an additional trigger in order to unravel the mode of interaction between spider silk particles and manganese carbonate in more detail. In similarity with non-equilibrium carbonate structures reported in previous studies, $MnCO_3$ obtained in the presence of PAA precipitated in the form of smooth droplet-like objects or film-like structures (figure 2(B)-(IV)) both of which have been identified as characteristic fingerprints of a polymer-induced liquid precursor mechanism in calcium carbonate [51] and other mineral systems [52]. The formation of a transient hydrated mineral phase with liquid-like properties (e.g. coalescence, spreading on substrates) has also been suggested for manganese carbonate even in the absence of PAA [53]. These unusual mineralization pathways based on emulsion-like intermediates stabilized by weak electrostatic interactions have attracted great interest in view of synthetic strategies, as the isotropic precursor phase can be moulded into any shape, which is then preserved in the final crystal [54].

Upon combination of spider silk particles and the soluble polymer additive, film-like PAA/mineral patches incorporating a large number of spherical objects with uniform size in the range expected for eADF4(C16)-E_8G and eADF4(C16)-K_8G particles were observed (figures 2(B)-(V)–(VI)). Therefore, homogeneous blending between the mineral and the template phase suggested that physical entrapment of silk particles within an amorphous emulsion-like phase followed by solidification seemed to be the dominant effect under these reaction conditions. However, the comparison of figures 2(B)-(V) and (VI) showed different degrees of interaction for both particles types. Therefore, an opposite charge-dependent mineralization effect was achieved upon addition of PAA to the different spider silk particles, indicating that physical entrapment of silk particles in the amorphous liquid-like material was the predominant effect at these conditions.

In a next step, the chemical composition and crystallographic phase of the mineralized species were studied using vibrational spectroscopy, powder x-ray diffraction and thermogravimetric analysis (figure 3). The ATR-FTIR spectrum of pure $MnCO_3$ (figure 3(A), left panel, black curve) served as a reference and showed distinct bands centred at ~850 cm^{-1} and ~1400 cm^{-1}, which are attributable to the ν_2 and ν_3 vibrations of CO_3^{2-} ions in divalent metal carbonate minerals, respectively [55, 56]. In agreement with previous data, the pristine (as precipitated) silk particles showed characteristic signals at 1650 cm^{-1} and 1550 cm^{-1} reflecting the amide I and amide II regions of the recombinant spider silk proteins (data not shown) [57]. While these amide bands could not be clearly identified in the spider silk/mineral composite crystals (figure 3(A), left panel, red and green curves), an additional signal, which was not representative of the inorganic carbonate mineral is observed at 1600 cm^{-1}. As bands in this spectral region could be assigned to vibrations in carboxylic acid groups, we assume that the signal originated from protein side chains.

The ATR–FTIR spectra of PAA-mediated mineralization products again confirmed the presence of signals associated with vibrations of the carbonate ion (figure 3(A), right panel). An additional band observed at 1456 cm^{-1} was particularly pronounced in the samples containing silk particles and could be attributed to δCH_2 deformation vibrations, thus presumably originating from protein structures or the PAA backbone. A broadened band at 1542 cm^{-1} in combination with a significant shoulder at 1638 cm^{-1} seen in the negatively tagged eADF4(C16)-E_8G/PAA mineral composites could be assigned to the amide II and amide I regions of proteins, respectively. In comparison, the positively tagged eADF(C16)-K_8G/PAA did not show any signal at 1638 cm^{-1}. In summary, the IR spectra are in agreement with the formation of a manganese carbonate mineral phase incorporating organic material.

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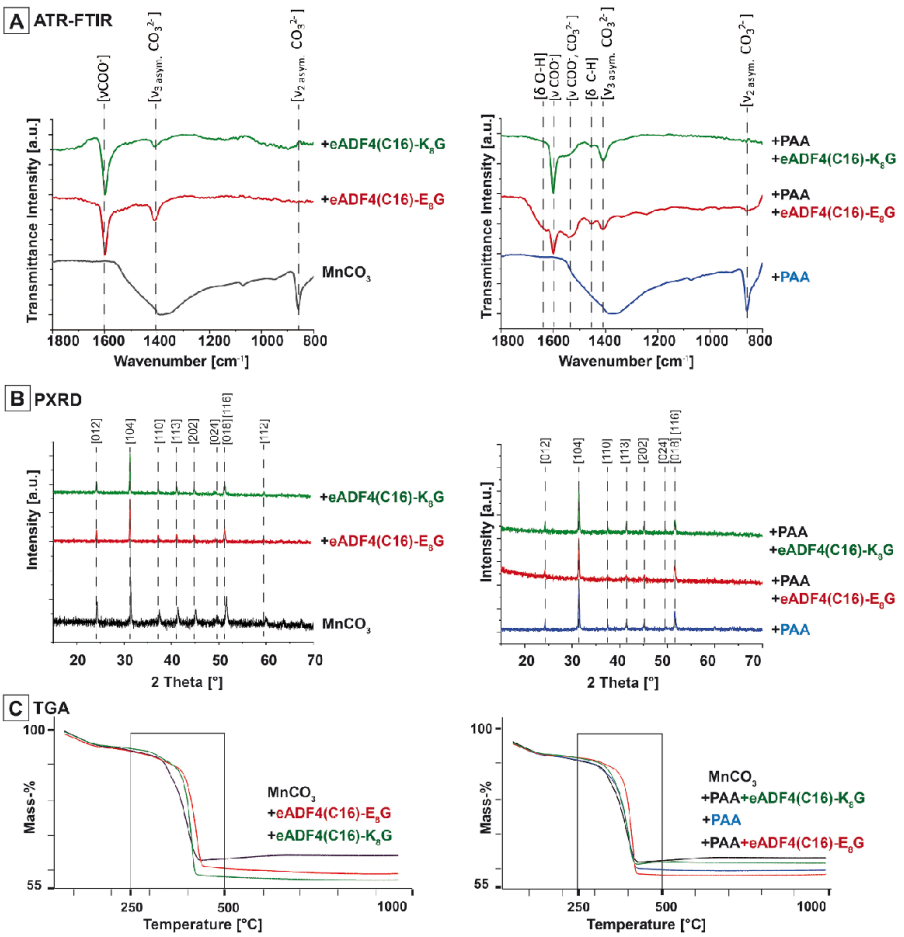

Figure 3. Chemical and structural analysis of $MnCO_3$/spider silk precipitates obtained in the absence (left panel) and in the presence of PAA (right panel) after 24 h. (A) ATR-FTIR spectra; (B) Powder x-ray diffraction (P-XRD) patterns of formed crystal species for polymorph identification; (C) Thermogravimetric analysis (TGA) of mineralized species. The framed area represents the temperature range for decomposition of the mineral phase and concomitant combustion of organic additives. (A-C, left panel) Black line: $MnCO_3$ deposited in the absence of additives; green line: $MnCO_3$ deposited in the presence of eADF4(C16)-K_8G particles; red line: $MnCO_3$ deposited in the presence of eADF4(C16)-E_8G particles. (A_C, right panel) Black line: $MnCO_3$ deposited in the absence of additives; blue line: $MnCO_3$ deposited in the presence of PAA; green line: $MnCO_3$ deposited in the presence of eADF4(C16)-K_8G particles and PAA; red line: $MnCO_3$ deposited in the presence of eADF4(C16)-E_8G particles and PAA.

P-XRD revealed that both, spider silk particles and PAA did not exert a substantial effect on the crystallographic phase of the precipitated mineral (figure 3(B)). All diffractograms recorded for solids isolated after 24 h showed regular peak patterns, which could be assigned to rhodochrosite (syn) (according to R. Sailer and G. McCarthy, ICDD Grant-in-Aid, 1992, North Dakota State University, Fargo, North Dakota), a crystalline $MnCO_3$ mineral isostructural to calcite [58]. Rhodochrosite occurs geologically and has also been identified in biogenic minerals e.g. in protective extracellular crusts of the haloarchaea organism *Haloferax alexandrinus* [59] or as a myogenic crystalline phase produced by fungi [60].

Next, the thermal stability of the different samples was analysed using TGA (figure 3(C)). A weight loss event at low temperatures up to 150 °C was seen in all curves and indicated the evaporation of surface-absorbed or intra-structural water. The decomposition of $MnCO_3$ releases CO_2 and proceeds almost simultaneously with the combustion of organic components in a temperature window between 250 °C and 500 °C (335 °C for recombinant spider silk [61] and 450 °C for PAA [62] in literature). With the aim to further analyse the amounts of occluded or associated organic material, the mass loss data were quantified based on the nominal molar mass of Mn_2O_3 as the general calcination product. A CO_2-related mass loss of

Table 1. Evolution of hydrodynamic radii of all occurring species upon incubation for 120 min.

Sample composition	Hydrodynamic radius (μm) after		
	5 min	30 min	120 min
$MnCl_2$ + PAA	0.2 ± 0.02	0.3 ± 0.02	0.2 ± 0.1
eADF4(C16)-E_8G	0.9 ± 0.4 and 9.4 ± 4.8	0.6 ± 0.1	0.8 ± 0.1
eADF4(C16)-E_8G + PAA	1.3 ± 0.7	1.0 ± 0.2	1.1 ± 0.8
Mn(II) + eADF4(C16)-E_8G	0.9 ± 0.2 and 7.3 ± 1.4	0.6 ± 0.1 and 6.2 ± 2.7	0.8 ± 0.3
Mn(II) + eADF4(C16)-E_8G + PAA	1.1 ± 0.9 and 3.7 ± 2.9	1.1 ± 0.1	1.4 ± 0.2
eADF4(C16)-K_8G	0.3 ± 0.02 and 4.0 ± 3.9	0.3 ± 0.1 and 2.7 ± 2.0	0.4 ± 0.1 and 2.9 ± 1.6
eADF4(C16)-K_8G + PAA	0.3 ± 0.2 and 1.9 ± 0.4	0.8 ± 0.3	0.6 ± 0.2 and 4.8 ± 4.8
Mn(II) + eADF4(C16)-K_8G	2.1 ± 1.4	0.4 ± 0.3 and 4.0 ± 3.2	1.1 ± 0.5
Mn(II) + eADF4(C16)-K_8G + PAA	2.5 ± 1.0 and 0.3 ± 0.1	3.0 ± 1.4	2.3 ± 2.5

31% was calculated for pure manganese carbonate (based on dry weight after water release), which was in good agreement with the theoretical value expected for the transformation from $MnCO_3$ to Mn_2O_3 [63]. The mineralization products obtained in the presence of silk templates and PAA showed an additional contribution to the mass loss due to the presence of intra-crystalline and surface adsorbed organic material. Specifically, an excess mass loss of −2.7% was detected for $MnCO_3$/eADF4(C16)-E_8G composite materials and −6.2% for $MnCO_3$/eADF4(C16)-K_8G. These results added further evidence to the assumption that the positively tagged eADF4(C16)-K_8G particles were integrated in and/or associated with manganese carbonate crystals to a higher degree. For PAA-mediated $MnCO_3$ deposits, an additional mass loss of −3% was recorded as compared to additive-free manganese carbonate, whereas −5.4% excess weight loss was detected for the mineralized PAA + eADF4(C16)-E_8G species, and −1.3% weight loss for the PAA + eADF4(C16)-K_8G-directed product, thus suggesting an increased tendency for the occlusion of negatively tagged silk particles in the presence of PAA.

3.2. Time-resolved component interactions between spider silk particles, PAA and Mn(II)

As the pendant side chains of recombinant spider silk proteins as well as the carboxylate groups of PAA provide binding sites for metal coordination, the template-mediated mineralization of $MnCO_3$ may proceed via the initial formation of association complexes between the different species present within the reactant solution. In order to elucidate the interaction and aggregation between spider silk particles, PAA, and Mn(II) cations prior to mineralization (i.e. under CO_2 deprivation), DLS studies were performed providing a quantitative estimate of the hydrodynamic radii of single components and aggregated species (table 1).

Both, manganese chloride and PAA were not detectable individually using DLS, but upon combination they formed complexes of ∼200 nm radius [64–66]. Hydrated spider silk particles showed a constant hydrodynamic radius of ∼900 nm and ∼400 nm when they were made of negatively and positively tagged variants, respectively, with only little fluctuations over time. Larger, less stable species were also detected with μm dimensions (table 1), and this observation can be explained by particle aggregation due the low overall colloidal stability of the protein particles in pure water [67]. The addition of PAA led to a subtle increase in the hydrodynamic radius for both particle types. In case of the amine-functionalized eADF4(C16)-K_8G particles, aggregated micronscaled species were additionally detected, which suggests that interaction with the oppositely charged polyelectrolyte leads to charge screening resulting in reduced colloidal stability. Indeed, PAA has been reported to form coacervates with charged proteins [68]. In this context, it is important to consider that the negatively tagged variant is carrying 24 negative net charges and 1 positive one in total, whereas the positively tagged variant exhibits 16 negative net charges and 9 positive ones.

Upon interaction with Mn(II) cations, particles from both spider silk variants showed markedly different behaviour in the presence or absence of PAA in particular with respect to the temporal evolution of the system. While the size of PAA in water was not affected in the presence of $MnCl_2$ over time, larger micronscale species were detectable after some time, presumably formed via accumulation of manganese cations at the surface of the negatively tagged spider silk particles due to electrostatic interaction. However, after 120 min equilibration time, only one distinct colloidally stabilized particle fraction with 0.8 μm radius was observed, which was then involved in subsequent mineralization processes. The disappearance of aggregated species in progressing time may result from precipitation of destabilized Mn(II)/eADF4(C16)-E_8G complexes upon increasing saturation with Mn(II). In the presence of dissolved PAA, which exhibits additional carboxyl

moieties and, therefore, enhances the number of the negative charges available for metal coordination, the stable colloid size increased to 1.4 μm, but equilibration seemed to be reached faster than in the absence of PAA, as only one species of 1.1 μm radius was observed already after 30 min (table 1).

In contrast, eADF4(C16)-K_8G particles in manganese solution showed comparably large hydrodynamic radii from the beginning, but no significant fluctuations with respect to aggregate formation or disintegration over time. We assume that the positively tagged peptide tag and the metal cations repulse each other, whereas attractive interactions may result between the eADF4(C16)-K_8G protein particles and chloride as well as carbonate ions present within the crystallization solution. Upon mixing with PAA, the equilibrium size after 120 min increased from 1.1 μm to 2.3 μm. In such complex three-component system, PAA molecules may interact with both Mn(II) and the spider silk protein, eventually facilitating the formation of micronscale associated species.

From these findings, a mechanistic interaction hypothesis could be derived: Upon providing positive charges by Mn(II) and amine groups by eADF4(C16)-K_8G in the suspension, when CO_3^{2-} is present, mineralization starts from both templates in a suction effect. With ongoing mineralization of both species, strong interaction with the forming mineral is achieved. When additionally introducing negatively charged PAA in the system, Mn(II) ions are accumulating around the soluble polymer due to electrostatic interactions and induce the formation of an amorphous phase rich in Mn(II), CO_3^{2-} and PAA. As physical trapping of secondary species within this emulsion-like mineral phase then becomes the predominant mode of particle incorporation, specific interaction with nucleation sites provided at solid particle surfaces are then rendered less important in determining the mineralization product. In case of two oppositely charged species with Mn(II) and eADF4(C16)-E_8G in suspension, colloidally stable particles evolved with manganese ion saturation at the particle surface, and, consequently, particles did not strongly interact with the mineral phase during crystal growth. With PAA present, the soluble polymer represented the dominating structure-directing additive and leads to a simultaneous nucleation and emulsion formation along with eADF4(C16)-E_8G in a suction effect. It can be concluded that dissolved PAA dominated over the solid spider silk particle templating effect due to its favourable electrostatic interaction with Mn(II) ions. Therefore, spider silk particles can template mineral formation when they present the same charge as the main soluble mineralization-driving agent.

3.3. Time-resolved crystal growth and composition

In order to obtain a clearer picture of the spatial distribution of inorganic and organic species within the composite crystals, EDS mapping of the elemental composition was performed. Specifically, we imaged the intensity of characteristic x-ray signals associated with the relative abundance of manganese and chloride. SEM images recorded after 48 h revealed continuous crystal growth for all species compared to samples taken at 24 h (figure 4). Pure manganese carbonate continued to grow into even larger fused cubic shapes. Cubic structures were also found in the presence of eADF4(C16)-E_8G particles over time with little apparent interactions between particles and minerals, and ratherstrong particle occulsion was observed in the minerals in case of eADF4(C16)-K_8G. While chloride was not a principle component of the mineral phase, the chloride peak can be regarded as an indirect measure for the presence of silk particles. EDS analysis on pure $MnCO_3$ and protein control samples indicated that spider silk particles tended to accumulate chloride ions from the precursor solution, as they showed distinct chloride but no Mn-related signals (data not shown). Cubic $MnCO_3$ crystals were associated with spider silk particles located in distinct regions (figures 4(A) and (B)). Subtle differences between the positively and negatively charged particles were indicated by chloride signals associated with eADF4(C16)-E_8G preferentially detected as segregated entities adjacent to the inorganic cubes (figure 4(A)), whereas the eADF4(C16)-K_8G-mediated crystals showed a higher tendency towards inbound occluded silk particles (figure 4(B)).

Upon PAA addition, mainly droplet- and film-like mineral structures were formed, which did not substantially gain in size when monitored over time up to 48 h. Elemental analysis using EDS demonstrated a homogenous distribution of manganese and chloride signals (figures 4(C) and (D)), thus suggesting that both particle variants were incorporated within the mineral phases.

Manganese carbonate can be deposited in various morphologies and sizes and, therefore, offers a plethora of possibilities for applications. Minerals in the shape of micropeanuts or nanoshuttles can be used, for example, as electrode materials in lithium ion batteries [37]. When applied as lithium ion battery anodes, manganese carbonate microspheres enable high capacity and enhanced cycling performance, even exceeding their nanoscale counterparts [34]. Further, manganese carbonate micro- and nano-structures can be used for high performance asymmetric supercapacitors [33]. Moreover, as manganese carbonate can act as a precursor and allows the formation of desired structures, these can be transferred into manganese oxides upon oxidation, opening up a new field of applications e.g. in the form of hierarchical hollow managanese oxide nanospheres for water treatment [35]. Perspectively, similarly hollow/porous structures could be

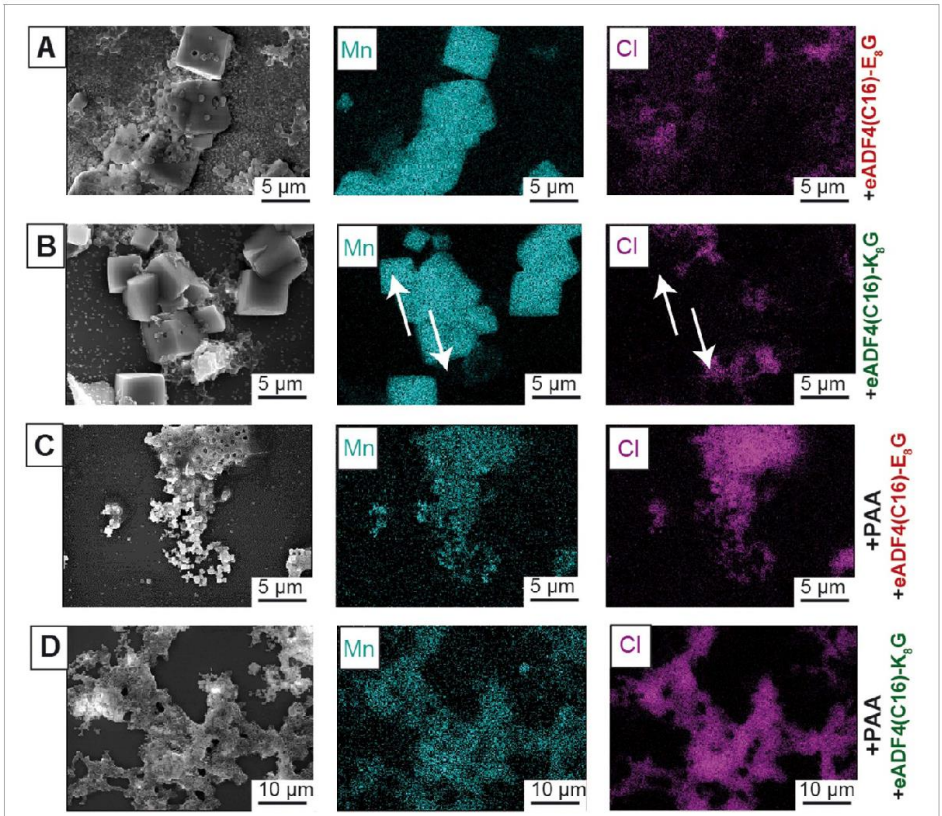

Figure 4. SEM-EDS maps of mineralized species extracted from reactant solution after 48 h illustrating the spatial distribution of manganese (turquoise) and chloride (violet): (A) $MnCO_3$ precipitates obtained in the presence of eADF4(C16)-E_8G particles; (B) $MnCO_3$ precipitates obtained in the presence of eADF4(C16)-K_8G particles, arrows indicate regions with distinct signals for Mn or Cl; (C) $MnCO_3$ precipitation in the presence of PAA and eADF4(C16)-E_8G particles; (D) $MnCO_3$ precipitation in the presence of PAA and eADF4(C16)-K_8G particles.

potentially obtained upon sintering manganese carbonate cubes or amorphous PAA-stabilized phases with entrapped spider silk particles yielded in this study. Comparing our results to previous studies, hollow cubes were reported for hydrothermal preparation methods [69], whilst room temperature deposition so far typically leads to spherical particles [34].

4. Conclusions

Particles made of engineered spider silk variants were used to template manganese carbonate mineralization in the absence and presence of PAA at RT. Based thereon, an interaction hypothesis could be proposed: Solid spider silk particles can template mineral formation when they present the same charge as the main soluble mineralization-driving agent. The resulting degree of interaction between silk particles and mineral could be confirmed with various characterization methods. In this respect, the overall manganese carbonate phase was identified as rhodochrosite, a mineral which is also typically associated with biogenic deposition processes of manganese carbonate e.g. in protective extracellular crusts of the haloarchaea organism *H. alexandrinus* [59]. For prospective technical applications of these materials, calcination can yield manganese oxides as hollow microporous structures for catalytic applications. The various structures obtained in our study open interesting perspectives for the room temperature deposition of bio-inorganic manganese carbonate composites based on tuning the spider silk variant acting as template. These results are relevant for the design and fabrication of functional inorganic materials such as mesostructured porous oxides.

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Author contributions

The concept for this study was conceived by TS and ASS; the manuscript was written by VJN, edited by TS and ASS, and proofread by all authors; protein production, purification and processing of particles, DLS and TGA were performed by VJN; mineralisation studies and according SEM and SEM-EDS were carried out by CK; ATR-FTIR was conducted by VJN of spider silk particles and by CK of mineral species; VG supported the implementation and analysis of mineralization experiments, and provided scientific input; TS and ASS acquired funding.

Conflict of interest

The authors declare no conflict of interests.

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References

[1] Seto J *et al* 2012 *Proc. Natl Acad. Sci. USA* **109** 3699–704
[2] Dorozhkin S V and Epple M 2002 *Angew. Chem., Int. Ed.* **41** 3130–46
[3] Liu B, Cao Y, Huang Z, Duan Y and Che S 2015 *Adv. Mater.* **27** 479–97
[4] Lohsse A, Ullrich S, Katzmann E, Borg S, Wanner G, Richter M, Voigt B, Schweder T and Schüler D 2011 *PLoS One* **6** e25561–e
[5] Faivre D and Schüler D 2008 *Chem. Rev.* **108** 4875–98
[6] Moura H M and Unterlass M M 2020 *Biomimetics* **5** 29
[7] Ganss B, Kim R H and Sodek J 1999 *Crit. Rev. Oral Biol. Med.* **10** 79–98
[8] Evans J S 2008 *Chem. Rev.* **108** 4455–62
[9] Gower L B 2008 *Chem. Rev.* **108** 4551–627
[10] Beniash E 2011 *WIREs Nanomed. Nanobiotechnol.* **3** 47–69
[11] George A and Veis A 2008 *Chem. Rev.* **108** 4670–93
[12] Tavafoghi M and Cerruti M 2016 *J. R. Soc. Interface* **13** 20160462
[13] Anderson H C 2003 *Curr. Rheumatol. Rep.* **5** 222–6
[14] Sumper M, Hett R, Lehmann G and Wenzl S 2007 *Angew. Chem., Int. Ed.* **46** 8405–8
[15] Hildebrand M 2008 *Chem. Rev.* **108** 4855–74
[16] Schenk A S *et al* 2012 *Adv. Funct. Mater.* **22** 4668–76
[17] Oaki Y, Kajiyama S, Nishimura T, Imai H and Kato T 2008 *Adv. Mater.* **20** 3633–7
[18] Aizenberg J, Black A J and Whitesides G M 1999 *Nature* **398** 495–8
[19] Aizenberg J, Muller D A, Grazul J L and Hamann D R 2003 *Science* **299** 1205–8
[20] Kim -Y-Y, Hetherington N B J, Noel E H, Kröger R, Charnock J M, Christenson H K and Meldrum F C 2011 *Angew. Chem., Int. Ed.* **50** 12572–7
[21] Schenk A S, Albarracin E J, Kim Y-Y, Ihli J and Meldrum F C 2014 *Chem. Commun.* **50** 4729–32
[22] Borukhin S, Bloch L, Radlauer T, Hill A H, Fitch A N and Pokroy B 2012 *Adv. Funct. Mater.* **22** 4216–24
[23] Kim Y Y *et al* 2016 *Nat. Mater.* **8** 903–10
[24] Kim Y Y *et al* 2011 *Nat. Mater.* **10** 890–6
[25] Li H, Xin H L, Muller D A and Estroff L A 2009 *Science* **326** 1244–7
[26] Greiner M, Yin X, Fernández-Díaz L, Griesshaber E, Weitzel F, Ziegler A, Veintemillas-Verdaguer S and Schmahl W W 2018 *Cryst. Growth Des.* **18** 1401–14
[27] Kim -Y-Y, Ribeiro L, Maillot F, Ward O, Eichhorn S J and Meldrum F C 2010 *Adv. Mater.* **22** 2082–6
[28] Mirabello G, Lenders J J M and Sommerdijk N A J M 2016 *Chem. Soc. Rev.* **45** 5085–106
[29] Zhou H and Wong S S 2008 *ACS Nano* **2** 944–58
[30] Asenath-Smith E, Noble J M, Hovden R, Uhl A M, DiCorato A, Kim Y-Y, Kulak A N, Meldrum F C, Kourkoutis L F and Estroff L A 2017 *Chem. Mater.* **29** 555–63
[31] Brif A, Ankonina G, Drathen C and Pokroy B 2014 *Adv. Mater.* **26** 477–81
[32] Schenk A S, Eiben S, Goll M, Reith L, Kulak A N, Meldrum F C, Jeske H, Wege C and Ludwigs S 2017 *Nanoscale* **9** 6334–45
[33] Karuppaiah M, Akilan R, Sakthivel P, Asaithambi S, Shankar R, Yuvakkumar R, Hayakawa Y and Ravi G 2020 *J. Energy Storage* **27** 101138

[34] Xiao L, Wang S, Wang Y, Meng W, Deng B, Qu D, Xie Z and Liu J 2016 *ACS Appl. Mater. Interfaces* **8** 25369–78
[35] Fei J B, Cui Y, Yan X H, Qi W, Yang Y, Wang K W, He Q and Li J 2008 *Adv. Mater.* **20** 452–6
[36] Oaki Y and Imai H 2007 *Angew. Chem., Int. Ed.* **46** 4951–5
[37] Wang L, Sun Y, Zeng S, Cui C, Li H, Xu S and Wang H 2016 *Cryst. Eng. Commun.* **18** 8072–9
[38] Doblhofer E and Scheibel T 2015 *J. Pharm. Sci.* **104** 988–94
[39] Wohlrab S, Müller S, Schmidt A, Neubauer S, Kessler H, Leal-Egana A and Scheibel T 2012 *Biomaterials* **33** 6650–9
[40] Huemmerich D, Helsen C W, Quedzuweit S, Oschmann J, Rudolph R and Scheibel T 2004 *Biochemistry* **43** 13604–12
[41] Leal-Egana A and Scheibel T 2010 *Biotechnol. Appl. Biochem.* **55** 155–67
[42] Hardy J G, Torres-Rendon J G, Leal-Egana A, Walther A, Schlaad H, Coelfen H and Scheibel T 2016 *Materials* **9** 560
[43] Herold H M, Aigner T B, Grill C E, Krüger S, Taubert A and Scheibel T 2019 *Bioinspir. Biomim. Nanobiomater.* **8** 99–108
[44] Ihli J, Bots P, Kulak A, Benning L G and Meldrum F C 2013 *Adv. Funct. Mater.* **23** 1965–73
[45] Weiss A C G, Herold H M, Lentz S, Faria M, Besford Q A, Ang C-S, Caruso F and Scheibel T 2020 *ACS Appl. Mater. Interfaces* **12** 24635–43
[46] Gebauer D, Cölfen H, Verch A and Antonietti M 2009 *Adv. Mater.* **21** 435–9
[47] Mao L-B *et al* 2016 *Science* **354** 107–10
[48] Sagara Y, Yamane S, Mitani M, Weder C and Kato T 2016 *Adv. Mater.* **28** 1073–95
[49] Cölfen H and Antonietti M 2005 *Angew. Chem., Int. Ed.* **44** 5576–91
[50] Wolf S E and Gower L B 2017 Challenges and perspectives of the polymer-induced liquid-precursor process: the pathway from liquid-condensed mineral precursors to mesocrystalline products *New Perspectives on Mineral Nucleation and Growth: From Solution Precursors to Solid Materials* ed A E S Van Driessche, M Kellermeier, L G Benning and D Gebauer (Cham: Springer) pp 43–75
[51] Schenk A S, Zope H, Kim Y-Y, Kros A, Sommerdijk N A J M and Meldrum F C 2012 *Faraday Discuss.* **159** 327–44
[52] Homeijer S J, Barrett R A and Gower L B 2010 *Cryst. Growth Des.* **10** 1040–52
[53] Wolf S E, Müller L, Barrea R, Kampf C J, Leiterer J, Panne U, Hoffmann T, Emmerling F and Tremel W 2011 *Nanoscale* **3** 1158–65
[54] Ruiz-Agudo C, Ruiz-Agudo E, Burgos-Cara A, Putnis C V, Ibáñez-Velasco A, Rodriguez-Navarro C and Putnis A 2016 *Cryst. Eng. Commun.* **18** 2830–42
[55] Zhang Q, Liu X, Fan W and Wang Y 2011 *Appl. Catal.* B **102** 207–14
[56] Boulard E, Goncharov A F, Blanchard M and Mao L W 2015 *J. Geophys. Res. Solid Earth* **120** 4069–79
[57] Slotta U, Tammer M, Kremer F, Koelsch P and Scheibel T 2006 *Supramol. Chem.* **18** 465–71
[58] Lapraz D and Iacconi P 1976 *Phys. Status Solidi* c **36** 603–16
[59] Naik-Samant S and Furtado I 2019 *J. Clust Sci.* **30** 1435–41
[60] Gadd G M, Rhee Y J, Stephenson K and Wei Z 2012 *Environ. Microbiol. Rep.* **4** 270–96
[61] Spiess K, Ene R, Keenan C D, Senker J, Kremer F and Scheibel T 2011 *J. Mater. Chem.* **21** 13594–604
[62] Kitahara Y, Okuyama K, Ozawa K, Suga T, Takahashi S and Fujii T 2012 *J. Therm. Anal. Calorim.* **110** 423–9
[63] Shaheen W M and Selim M M 2000 *J. Therm. Anal. Calorim.* **59** 961–70
[64] Tomida T, Hamaguchi K, Tunashima S, Katoh M and Masuda S 2001 *Ind. Eng. Chem. Res.* **40** 3557–62
[65] Roma-Luciow R, Sarraf L and Morcellet M 2001 *Eur. Polym. J.* **37** 1741–5
[66] Gustafson R L and Lirio J A 1968 *J. Phys. Chem.* **72** 1502–5
[67] Helfricht N, Doblhofer E, Duval J F L, Scheibel T and Papastavrou G 2016 *J. Phys. Chem.* C **120** 18015–27
[68] Zhao M and Zacharia N S 2018 *J. Chem. Phys.* **149** 163326
[69] Liu L, Zhang X, Wang R and Liu J 2014 *Superlattices Microstruct.* **72** 219–29

7.2 Teilarbeit II

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Review

Silk-Based Materials for Hard Tissue Engineering

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Abstract: Hard tissues, e.g., bone, are mechanically stiff and, most typically, mineralized. To design scaffolds for hard tissue regeneration, mechanical, physico-chemical and biological cues must align with those found in the natural tissue. Combining these aspects poses challenges for material and construct design. Silk-based materials are promising for bone tissue regeneration as they fulfill several of such necessary requirements, and they are non-toxic and biodegradable. They can be processed into a variety of morphologies such as hydrogels, particles and fibers and can be mineralized. Therefore, silk-based materials are versatile candidates for biomedical applications in the field of hard tissue engineering. This review summarizes silk-based approaches for mineralized tissue replacements, and how to find the balance between sufficient material stiffness upon mineralization and cell survival upon attachment as well as nutrient supply.

Keywords: silk fibroin; silk spidroin; biomineralization; composite materials; bone; teeth; cartilage; tendon

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1. Introduction

The development of hard tissue in the human body is a process of mineral formation by cellular metabolism, named biomineralization, yielding support structures of the skeleton and neighboring tissues such as tendon and cartilage or functional tissues such as teeth [1]. There are several different mineralization pathways, but they are not yet fully explored [2]. Generally, mineral formation in tissues needs to be highly controlled to prevent local over-mineralization, which could be pathogenic [2]. The high process control of biomineralization is provided by tissue-specific cells and biopolymers such as proteins, which are templating and nucleating mineral formation [3]. Therefore, biogenic crystals often exhibit a different morphology than their geogenic counterpart [4].

Tissue-specific cells are taking a crucial role in biomineralization as they trigger mineral nucleation and growth upon secretion of so-called non-collagenous proteins [2,5]. The main proteinous material (90 wt.%) of hard tissues is collagen type I as flexible filler in this composite material, while the non-collagenous proteins cover the remaining 10 wt.% [2]. Collagen is not mineralized on its own, but collagen fibrils can interact with non-collagenous proteins, which induce mineralization from saturated media at the gap regions of the stacked triple-helical collagen fibrils [2,5]. The phosphorylated, non-collagenous proteins of the so-called SIBLING family (Small Integrin-Binding Ligand, *N*-Linked Glycoprotein) include bone sialoprotein and osteopontin in bone-related tissues, whereas in teeth dentin and cementum, dentin matrix protein 1 and dentin phosphoryn

are present. These proteins provide two functions, as on the one hand, they can bind at specific locations to the structural collagen scaffold and on the other hand, they can bind ions due to their, in most cases, highly charged nature with repetitive motifs of glutamic or aspartic acid residues [5]. This local charge density allows to accumulate mineral ions and, thereby, to initiate crystal nucleation, when the ion density reaches a critical concentration, which then triggers the further mineralization processes in mineralized tissues, such as bone, teeth, cartilage and tendon [5].

Further, mineralization is driven by tissue-related osteoblasts (in bone and tendon), odontoblasts (in teeth) and chondrocytes (in cartilage) upon the accumulation of ions from the surrounding environment in mostly separated membrane vesicles [6]. With ongoing mineralization, the extracellular matrix around these cells densifies, and nutrients and oxygen are increasingly provided only passively by diffusion until easy nutrient supply is finally prevented. In the case of bone and neighboring tissue, osteoblasts differentiate into osteocytes [1]. Osteoclasts, on the other hand, are constantly remodeling fully mineralized tissue to guarantee healthy and reconstructed bone [7].

For traditional and engineering approaches to reconstruct hard tissues, natural processes have to be understood. Further, as bone represents the most abundant fully mineralized tissue, a majority of tissue engineering approaches focus on respective reconstructive solutions. Bone defects such as fractures easily occur, for example due to critical non-physiologically high loads. Shortly after fracture, inflammatory responses are initiated at the defect site, followed by a cell-induced regeneration cascade for initial callus formation, which is then remodeled to form new bone. With progressing age, bones become increasingly brittle due to changes in the cellular metabolism of osteoblast cells, which is indicated by 10–40 times lower strain rates until breakage. One possible reason might be remodeling cycles, which affect the mineral phase and allow more micro-cracking, finally leading to bone failure [8]. Once fractured, bone defects can be detected by x-raying of the defect site. New techniques such as ultrasonography for detecting bone fractures are more sensitive than classical radiographs, which are typically used to trace fractures of long bones. Sonographic methods provide the advantage of no radiation exposure, lower cost and wider availability in non-hospitals. A study among German general practitioners showed that most articulated sono-methods are inferior to classical x-ray [9]. In clinical procedures, the defect site is often bridged and stabilized with bone platelets or screws to stabilize material in place during the regeneration process [10]. In order to further support bone healing or large defects with bone loss, hard tissue engineering methods are increasingly used. In contrast to the self-regenerating ability of bone tissue, other mineralized tissues rely more on artificial replacement than supportive healing.

In the following, approaches for hard tissue engineering, with the focus on bone, and some examples including teeth, cartilage and tendon, based on silk scaffolds are discussed in detail.

2. Tissue Engineering Approaches

Defined in 1993, tissue engineering is the combination of principles of engineering and life sciences with the goal of developing biological substitutes that are able to restore, maintain or improve biological tissue function or a whole organ [11,12]. While one part of this interdisciplinary field deals with the generation of 3D models for the development of therapies, its main potential lies in regenerative medicine. With the goal of replacing tissues and organs damaged by trauma, disease or age, the ever-extending field of TERM (tissue engineering and regenerative medicine) includes basic and advanced cell and stem cell biology, scaffold material research and various fabrication and processing techniques [13,14]. The classical TERM approach to overcome drawbacks of autografts or allografts consists of scaffold-based top-down strategies (Figure 1). Such engineered tissues are typically created by manufacturing biodegradable polymeric scaffolds on which cells are seeded. During cultivation, and in some cases stimulated by perfusion, growth factors or

mechanical cues, cells dynamically remodel and replace the scaffold through degradation and new extracellular matrix (ECM) deposition [15]. Traditionally, three-dimensional scaffolds are manufactured by employing techniques such as freeze-drying, leaching of particles or salt, chemical or gas foaming and thermally induced phase separation [16–19]. While these processes allow partial control over the scaffold properties, there are severe restrictions to generate precise micro-architectures, including pore size, geometry and connectivity.

With the rise of additive manufacturing, more techniques are available to create scaffolds for tissue engineering, overcoming previous restrictions. With an emphasis on the regeneration of bone tissue, four major layer-by-layer methods have been reviewed recently by Madrid et al. [20]. A variety of natural and synthetic polymers, as well as ceramics and bioceramics and even metals can be processed using stereolithography (SLA), selective laser sintering (SLS), fused deposition modeling (FDM) and three-dimensional printing (3DP). These techniques generally allow for more accurate scaffolds with better resolution. The specific processes, including laser and heat treatment, nevertheless tremendously restrict the choice of material [20]. Since additive manufacturing is based on computer-aided-design, structures that are more sophisticated can be created, including patient-specific scaffolds with the help of computer tomography. While these top-down approaches allow for good reproduction of the macroscopic structures of desired tissues and organs, the complexity, micro-arrangement and heterogeneity of natural tissues, including different cell types and materials, is far beyond what is found in such artificial acellular scaffolds [15,21].

To overcome this limitation, a multitude of bottom-up approaches has been developed in recent years. In contrast to traditional top-down approaches, where first the scaffold is produced, followed by seeding with cells, in bottom-up approaches, cells are used from the very beginning in combination with materials to build up tissue constructs step-by-step (i.e., bottom-up) (Figure 1). Biocompatible materials in various morphologies, like particles, one-dimensional fibers, two-dimensional films and three-dimensional hydrogels, have been used, alongside cells, as building blocks to generate assemblies at the nano- or micro-scale. Further self- or directed-assembly leads to engineered macroscopic three-dimensional tissue constructs. A comprehensive review examining these advanced bottom-up approaches has recently been published by Gaspar et al. [21,22].

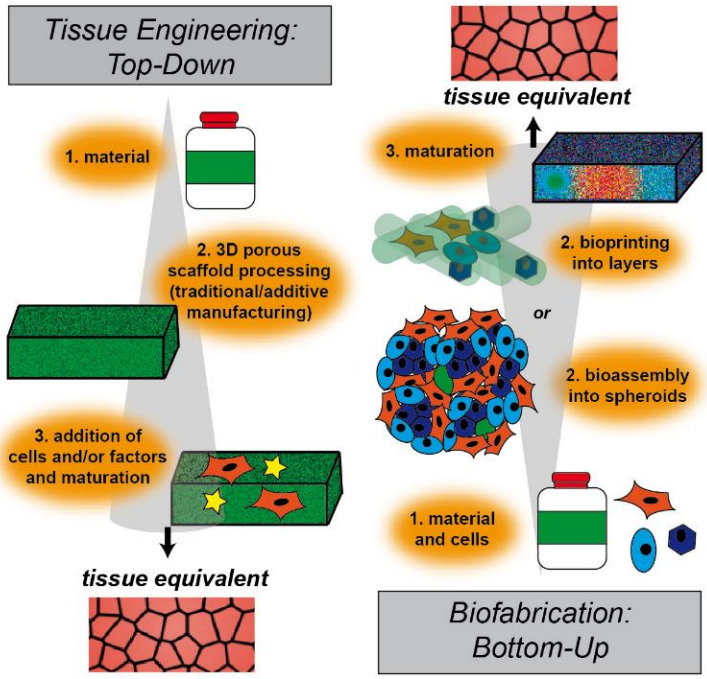

Figure 1. Schematic illustration of engineering approaches to fabricate tissue. In top-down strategies, a scaffold is produced, followed by cell seeding and/or addition of factors for cellular stimulation. This technique is called tissue engineering. Bottom-up approaches use cells and raw materials simultaneously to build larger constructs, which are then maturated. This technique is called biofabrication.

Benefits of these strategies include the involvement of cells in the development of the tissue right from the beginning, as well as the possibility to generate constructs made from different types of assemblies, leading to various cell types and materials organized hierarchically within the resulting construct. Different assemblies can be divided into two main categories, mostly scaffold-free cell-rich and cell-biomaterial assemblies. Adhesive surfaces, possibly in combination with functionalized and/or non-adhesive surfaces, are used to generate monolayer cell sheets by cultivation and subsequent detachment of the grown layer. Stacking, rolling and folding of these monolayer sheets is the basis to create combined three-dimensional assemblies, including multicellular and pre-vascularized constructs [23,24]. By using cellular spheroids, often made of mesenchymal stem cells, as scaffold-free building blocks, processes like cell-cell and cell-ECM interactions, differentiation and fusion are recapitulated [25,26]. In addition, genetic or chemical engineering of the cell surface allows control over cellular behavior and assembly into higher-order structures [27,28]. Inclusion of biological materials is a crucial part of bottom-up tissue engineering strategies, such as the addition of biocompatible layers within cellular sheets, functionalized with nucleic acids, viruses, enzymes and structural proteins, as well as peptides and polymers. To increase structural assembly within cellular spheroids or hydrogels, fibers and particles can be incorporated. These materials can add structural support and guidance, promote and/or control the assembly of building blocks and stimulate cellular behavior in general [21,29,30]. For example, a silk fibroin derived hydrogel was used as a scaffold for articular cartilage tissue engineering, and integrated poly(lactid-co-glycolid) nanoparticles were used to simultaneously deliver two growth factors, resulting in beneficial effects on proliferation and differentiation of dental pulp

stem cells [31]. On the way to tissue or organ replacement, such multicellular and multimaterial assemblies are used to generate vascularized multicomponent constructs or spatially organized multiblock hydrogels [21].

In the context of advanced bottom-up tissue engineering approaches, a new field called biofabrication has been reviewed recently by Groll et al. [32]. Biofabrication mainly, but not solely, uses additive manufacturing techniques to process bottom-up building blocks into hierarchically structured cell-biomaterial constructs. Biofabrication describes the automated generation of biologically functional constructs through bioprinting, meaning the direct spatial arrangement of cells, materials and/or factors, and through the automated assembly of cell-containing building blocks, so-called bioassembly. In both cases, in vitro maturation and or fusion of the products is a crucial step before obtaining a tissue equivalent for implantation or pharmaceutical screening [32]. Relevant technologies within biofabrication have been recently reviewed by Moroni et al. [33]. With the possibility of simultaneous deposition of cells and material in an additive manufacturing process, bioplotting, ink-jet bioprinting and valve-jet bioprinting are major biofabrication tools for bottom-up tissue engineering and regenerative medicine. Formulations of materials, cells and biological molecules, so-called bioinks, are processed using these technologies. Bioplotting, also called robotic dispensing or extrusion bioprinting, dispenses continuous filaments of hydrogel materials or bioinks through a nozzle (piston-, screw-, or pneumatic-driven). Droplets are ejected over a nozzle head, controlled either by piezo- and thermal-actuators (ink-jet) or by solenoid micro-valves (valve-jet) [32,33].

All approaches, whether they include manufacturing a scaffold followed by cell-seeding or bioprinting/bioassembly, have strict requirements on the used material. Physical and mechanical properties need to be suitable for processing using the respective technology on the one hand and ensure cellular survival and proliferation on the other. The material also plays an important role in guiding specific cellular development and maturation, for example, by surface functionalization, the inclusion of biological molecules or the tuning of degradation behavior. With the goal of implantation of constructs, biocompatibility, meaning the performance of intended purpose without evoking an immune response, is absolutely required and can be enhanced e.g., upon introduction of nanoparticles [34–36]. Due to their inherent biological and chemical similarities to native tissue, natural polymers, natural polymer-based composites and bioceramics are of great interest for tissue engineering applications. Due to the high load-bearing requirement, hard tissue engineering approaches so far mainly focus on top-down strategies using porous scaffolds for cell seeding [37,38].

3. Hard Tissue Engineering

3.1. State of the Art

After diagnosis of a bone defect, the respective site is commonly deprived from extensive movement as both bone sides need to reconnect during regeneration in a correct manner, otherwise malfunction might be the result of improper healing. The origin of the cells, which are taking part in bone repair, were found to influence the healing progress. The cells present in bone encompass, for example, stem cells during bone healing or endothelial cells building vasculature, but also pre-osteoblasts, which differentiate into osteocytes during bone formation and maturation as described above. Osteoclasts are undertaking the function of degradation, which is a continuously ongoing process to maintain healthy bone and allow for expansion of the skeleton during the development of children [39]. When artificially delivered into bone defects, neural crest-derived frontal bone and mesoderm-derived parietal bone cells from newborn rats were found to exhibit both similar bone regeneration ability, although the mesodermal cells showed a potentially higher bone regeneration efficiency in vitro [40]. MC3T3 E1 pre-osteoblast cells were posed in hydroxyapatite microcracks similar to bone fractures and found to underlie

initial apoptosis at a region of 200 nm around the cracks [41]. Besides fixation, flexoelectricity, meaning the ability to generate electricity under pressure, was found crucial for bone healing [41]. Exposed to strain such as physical activity during bone healing, bone regeneration was increased, and so rehabilitation measures actively contributed to tissue regeneration [42]. With near-infrared fluorescent probes,[43] bone repair could be imaged concerningin vitro differentiation of human mesenchymal stem cells into osteoblasts. A cyclic peptide coupled with a fluorophore was used to bind to $\alpha 5\beta 1$ integrin as an osteoblast-specific marker. A second probe was coupled with the drug pamidronate to a fluorescent gold nanocluster, where the drug bound specifically to hydroxyapatite and allowed for monitoring osteogenesis [43].

Loss of bone material due to cancer or other pathogenic relations such as osteoporosis is often not recovered spontaneously and needs tissue replacement. Autologous bone grafts are still considered as the gold standard transplant due to facilitated integration at the defect site. As concerns about donor availability, healing and disease transmission arise, artificial bone substitutes become increasingly attractive to overcome these obstacles [39]. Therefore, titanium implants are state of the art as they are biologically inert materials, which offer high load transmission. Unfortunately, these foreign body materials are rarely fully integrated into the surrounding tissue and might become loose; therefore, surgical rearrangement might be necessary. One major reason for this issue is a bacterial infection, especially concerning dental implants with extensive biofilm formation [34]. To improve integration, for example titanium alloy (Ti6Al4V) implants with TiO_2 nanotubes were coated with silk fibroin, which was found to enhance osteoconductive and osteogenic properties in case of bone implant performance [44]. Bone, cell and implant interaction was found to be enhanced for MG63 bone cells and human mesenchymal stem cells, which is beneficial for implant applicability. Biomimetic minerals for hard tissue engineering, which enhance osseointegration, can rely on biosimilars such as calcium sulfate or phosphate ceramics as synthetic and hydroxyapatite as a naturally occurring form of bone mineral [39]. Building scaffolds out of these materials can be realized upon melting and fusing individual ceramic particles using laser sintering at temperatures above 1000 °C [45–47]. Utilizing this rapid prototyping technique, also polymeric carrier materials can be fused at lower temperatures (about 70–200 °C) whilst molding and binding ceramic particles into bionanocomposites and simultaneously removing the binder [48,49]. With such polymeric binders, 3D extrusion and additional sintering of the composite materials is possible, yielding solely the remaining solid ceramic structures (Figure 2) [50,51].

Further, injectable calcium phosphate cements including ceramics and a curing agent were invented by Brown and Cho in the 1980ies to fill dental cavities in the first place [52]. As state of the art, synthetic polymeric materials are widely used as matrix materials in hard tissue engineering, however, they often cannot complement features of biomaterials such as non-toxic degradation products and bioactive surfaces for cell adhesion [53].

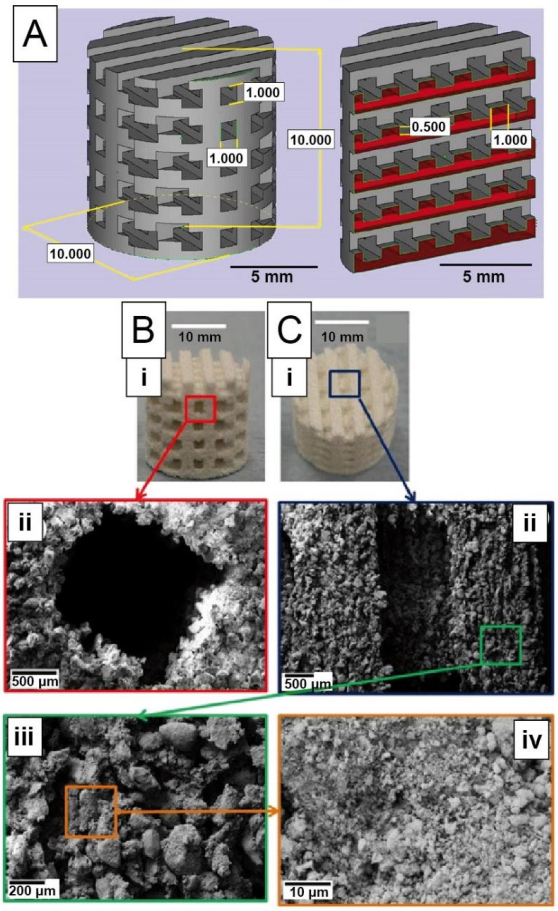

Figure 2. Scaffolds containing 50 wt.% hydroxyapatite nanoparticles suspended in poly(vinyl alcohol) as matrix material. (**A**) CAD design of a layered scaffold showing porous structures in the cylinder. Extrusion printed scaffolds after drying and thermal curing in side-view (**B**(**i**)) with higher magnification of a channel pore (**B**(**ii**)), and in top view (**C**(**i**)) with higher magnification of a channel (**C**(**ii**)), showing individual hydroxyapatite particle agglomerates (**C**(**iii**),**C**(**iv**)). Reprinted and adopted with permission from Cox, S. C. et al., 3D printing of porous hydroxyapatite scaffolds intended for use in bone tissue engineering applications. *Mater. Sci. Eng. C* **2015**, 47, 237–247. Copyright (2015) Elsevier.

3.2. Design Criteria and Challenges

It is important that various design criteria and factors have to be taken into account in tissue engineering approaches to fulfill the requirements of a successful tissue engineering construct (Figure 3). In the case of hard tissues, besides biological and physico-chemical cues, also the appropriate mechanics play an important role. In the following, these aspects are discussed in more detail and illuminated why they can be challenging during scaffold preparation.

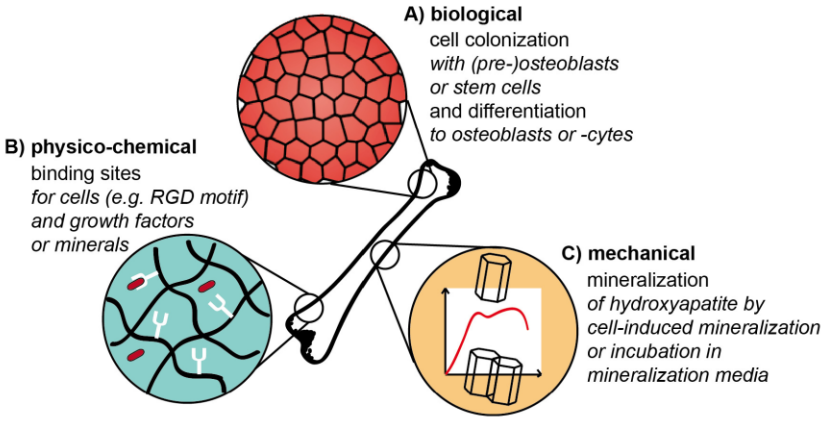

Figure 3. Illustration of biological, mechanical and physico-chemical factors of scaffold materials relevant for hard tissue engineering. (**A**) Media, growth factors, etc. are needed for tissue-specific cell colonization and differentiation on artificial scaffolds. (**B**) Materials/scaffolds have to provide binding sites for cells, factors and minerals. (**C**) Biomineralization is necessary to gain composite materials with adopted mechanical properties (such as stiffness, etc.).

Concerning the mechanical design, it has to be taken into account that mature bone has compressive strengths in the order of up to 20 GPa [54], whereas they are far lower for immature bone, as the mineralization process is still ongoing [55,56]. However, not only high strength but also flexibility must be provided. Therefore, mostly brittle materials are not suitable for bone regeneration applications, as the risk of failure is high [8]. It can be challenging to combine high load-bearing materials with high flexibility, but these mechanical requirements can be fulfilled in biomaterial matrices applying reinforcing filler materials such as ceramic particles into composite materials. To gain homogenous mineralization, it is important that filler and matrix material interact well with each other to avoid phase separation, which is an additional criterion. Practical hints can be found when taking a closer look at the natural blueprint: Bone is a composite material [57] with collagen fibrils (20–30 wt.%) and ceramic particles made of hydroxyapatite (60–70 wt.%) [2]. Besides composite materials, biomineralization of protein precursor materials can be triggered in vitro upon immersion in mineralization agents forming calcium phosphate species. These can for example be single aqueous salt solutions, which are subsequently applied to the materials [58–60]. More complex mineralization is provided by Simulated Body Fluid, a model solution at pH 7.4, which was designed to simulate mineralization processes found during bone formation. Its ion composition and concentration are proximately close to human blood plasma [61].

Tailoring mechanical properties upon controlled mineralization is highly interconnected with the scaffold's biological function and vice versa. During mineralization of tissue, cells play an important role as they secrete non-collagenous proteins with highly located charge [2]. Especially SIBLING proteins [5] are to be mentioned among others, as they coordinate nucleation, growth and inhibition phase during mineral formation as they accumulate ions from the surrounding intestinal fluids [62]. Further, hydroxyapatite precursor phases can be accumulated in cell membrane-bound vesicles and released at mineralization sites [6]. As a result, tissue-specific cell colonization is an additional design cue to mimic natural tissue in engineered constructs. Its respective challenge is posed not only by cell adhesion to the surface or in the construct but also to trigger osteoblast lineage in osteoblast precursor cells or stem cells. Biomineralization and osteogenic differentiation were found to be highly dependent on matrix stiffness [63]. 2D surfaces of different controllable substrate stiffness showed the

best results for medium stiffness (50–100 kPa), as mineralization was completed after three weeks. Osteoblast differentiation was directly related to the formed mineral layer and only indirectly regulated by matrix stiffness [63]. The release of ions from the material, which is sensed by cells, can also lead to differentiation responses. One example for such materials is 45S5 Bioglass embedded in silk fibroin/gelatine scaffolds [64]. The bioglass composition comprises SiO_2, CaO, Na_2O and P_2O_5, and the ion release profile triggers osteogenic cell differentiation [65]. For this functionalization, it is important to control the osmotic balance of the media for cell survival.

Moreover, not only mechanical but also physico-chemical properties of the scaffold can lead to desired cell differentiation. The design of such cues can be related to binding sites for cells, growth factors or minerals. Besides cell-specific binding motifs [66], the integrin binding peptide motif arginyl glycyl aspartic acid (RGD) [67] is universally applied. The incorporation of this motif can be a challenge when it is not intrinsically provided by the biomaterial. This can be solved upon genetic engineering of proteins used in the material or chemical coupling of the motif to the material [68]. Related to the natural tissue, growth factors such as the most important one, the Transforming Growth Factor beta (TGF-beta), as well as bone morphogenetic proteins are agreed to have a beneficial impact on the success of hard tissue engineering scaffolds [69]. The factors can be delivered via the construct and trigger stem cells towards osteo-differentiation [70]. As a challenge, their concentration must be maintained [69] at levels confirmed to be active (nM) during cell cultivation by specific binding, otherwise, scaffolds become depleted fast by diffusion. Binding sites for ions were discussed above to be provided by non-collagenous proteins with located charges. Mimics of these proteins can be designed and incorporated into the scaffold. However, the preparation of hybrid proteins from silk and non-collagenous proteins can be challenging to gain functional mineral binding sites [71,72].

Taking all these complex requirements into regard, scaffolds must comply not only with mechanical but also physico-chemical and biological demands to build a successful hard tissue engineering construct. One crucial role plays the material choice. The named requirements can be met, for example, with synthetic or natural materials [14]. However, synthetic materials pose the risk of toxic degradation products during tissue regeneration, and their biocompatibility is limited [73]. Naturally derived materials avoid these obstacles. Further, they naturally can provide biological and/or mineral binding sites. However, disease transmission from donor animal sources and material heterogeneity must be avoided. Collagen and gelatin are common materials due to their native occurrence in bone and the presence of biomineralization nucleation sites. Moreover, bone takes a long time to develop, and the collagen will often degrade before it can be remodeled. Among artificial natural biomaterials, silk appears to be an attractive material, as it provides non-toxicity and biodegradability. Further, silk proteins can be produced biotechnologically, modified and processed into a variety of morphologies [73]. The upcoming sections will shed light on how silk materials can be used, for example, as matrix materials for bone tissue engineering.

4. Silk

4.1. Naturally Derived Silk

Silks are a class of protein fibers spun by arthropods such as fleas, mites, spiders and silkworms, amongst others. They are based on fibrous proteins containing highly repetitive amino acid sequences stored in the animal as liquid and transformed into fibers once shear stress is applied during spinning [74].

Fibers produced by the silkworm *Bombyx mori* consist of two silk fibroins (SF) and glue-like (non-silk) proteins named sericins. The fibroin heavy chain consists of a highly repetitive (12 times) glycine and alanine-rich region and two hydrophilic N- and C-terminal domains. The fibroin light chain is an arginine- and lysine-rich non-repetitive

protein [75]. Upon secretion, both fibroins and a third small glycoprotein, p25, assemble into twin filaments that represent the inner part of the core-shell structure typical for *B. mori* silk. Sericins coat and stick these fibroin filaments together. The structure is completed by an additional coating with various proteins for the protection of the cocoon [76].

The most commonly studied spider silk is dragline silk made of proteins secreted from the major ampullate gland, and it consists of multiple proteins, called spidroins. The overall layout and amino acid composition of these major ampullate spidroins (MaSps) are similar to the architecture of the fibroin heavy chain. The primary structure (i.e., amino acid composition) of the core domains, however, is quite different. One spidroin filament is coated with a thin shell containing other silk proteins, lipids and glycoproteins constituting a core-shell-structure [77]. While most silk fibers have a high toughness compared to man-made fibers, spider silk outperforms the others concerning its mechanical properties [75].

Natural spider silk fibers, mostly from female adult *Nephila* spiders, have been used as suture threads or processed into scaffolds for neuron guidance, skin repair and bladder reconstruction [78]. While most spiders exhibit cannibalistic behavior, silkworms can easily be farmed to harvest their silk in large quantities [79]. Consequently, silk from the domesticated silkworm *B. mori* has been extensively characterized and is the main silk material used in biomedical applications, for example, as sutures and in tissue engineering and regenerative medicine approaches [38]. To extract fibroin from the harvested cocoons, a thermochemical treatment is applied, called *degumming*. This step is particularly important since it also removes the sericin component from the fibroin fibers, which has been shown to be problematic by causing immune reactions [80].

4.2. Bioengineered Silk

Advanced tissue engineering approaches cannot only take the physical properties of the fabricated scaffold into account. Apart from biocompatibility, the degradation rate of specific scaffolds is highly important. In the best case, the degradation should be identical to the rate at which new tissue is formed by the cells. When working with *B. mori* silk fibroin materials, the degradation behavior can be tuned by the choice of fabrication strategy, for example, the use of different solvents during processing, or by the incorporation of enzyme-sensitive peptides or degradation-promoting supplements [81]. To mimic the complexity of natural tissue, engineering approaches are destined to use multiple materials, fabricated in various morphology, together with cells and biologicals to carry out specific functions. Genetic engineering is further used to extend the availability and functionality of different silks for tissue engineering applications. Nagano et al. added a poly(glutamic acid) domain to the repetitive amino acid sequence of *B. mori* fibroin to incorporate calcium-binding sites for mineralization [82]. In another study, Saotome et al. improved revascularization by introducing the vascular endothelial growth factor and the RGD-cell adhesion motif into the silk fibroin heavy chain of transgenic *B. mori* silkworms [83].

The majority of recombinant spider silk proteins for biomedical applications is produced in the heterologous expression system *Escherichia coli*. Therefore, the natural silk sequence is determined first and then engineered to be produced in the host organism. After transformation and production, protein purification yields recombinant spider silk proteins [78]. Most recombinant sequences are based on proteins of the major ampullate silks from either *Nephila clavipes* or *Araneus diadematus* spiders [84]. For example, a chimeric protein was genetically engineered containing a consensus sequence from *N. clavipes'* dragline silk fused to the carboxyl-terminal domain of the dentin matrix protein 1 [85]. In another work, Gomes et al. created a fusion protein by combining a consensus sequence from *N. clavipes'* dragline silk with the complete sequence of bone sialoprotein for improved cell attachment and deposition of calcium phosphate [71]. Cellular adhesion and proliferation were enhanced on materials made of an *A. diadematus* derived

recombinant spider silk protein fused with the cell adhesion motif RGD [68]. Alternatively, mineral- and collagen-binding motifs were introduced to this protein for materials applications at the tendon-bone interface [72].

4.3. Silk-Based Morphologies

Naturally derived or bioengineered silk proteins can be processed into various morphologies. *B. mori* fibroin films are obtained by dissolving the proteins in aqueous lithium bromide solutions and dialysis against water, followed by film casting. To obtain insoluble films, they can be treated with a mixture of water and methanol [86,87]. Similarly, recombinant spider silk proteins are processed into films by dissolving them in the organic solvent hexafluoroisopropanol (HFIP), followed by casting and post-treatment with isopropanol or methanol [88,89].

To imitate the natural fibrous ECM more closely for tissue engineering applications, non-woven mats containing fibroin or spider silk fibers have been produced using electrospinning. The technique constantly evolved to gain more control over the process and outcome. Traditionally, *B. mori* fibroin is dissolved in organic solvents like HFIP, hexafluoroacetone (HFA) or formic acid and spun by applying voltages between 2 kV to 30 kV. Non-woven mats containing fibers with diameters in the low nanometer range up to one micrometer were generated with this set-up [90,91]. In a recent work by Keirouz et al. [92], composite fibers were spun using nozzle-free electrospinning. DeSimone et al. [93] developed an all aqueous electrospinning process for recombinant spider silk proteins. The elimination of harsh processing conditions led to the conformational stability of biological components throughout spinning and posttreatment, promising the inclusion of sensitive biological components for tissue engineering applications [93]. Upon blending poly(caprolactone) (PCL) with poly(glycerol sebacate) and silk fibroin, also the hydrophilicity of the non-woven mats could be increased, which is beneficial for tissue engineering applications [94].

Nano- and microparticles are used in tissue engineering within three-dimensional scaffolds, e.g., to introduce biologically or chemically active factors in constructs or to increase the mechanical stability. Silk particles can be produced by salting-out with potassium phosphate. Tuning protein concentration and mixing intensity, particles in the size range between 150 nm and 10 μm can be fabricated [95,96]. Fibroin particles with a diameter of around 6 μm can also be produced by chopping and wet-milling *B. mori* fibers, while even smaller particles, down to 200 nm, were produced using ethanol precipitation and freezing [97,98]. Through well-suited loading and release properties for various substances, silk particles can be applied as drug carriers. For example, a human recombinant bone morphogenic protein has been successfully encapsulated in silk fibroin particles allowing sustained delivery thereof in bone tissue engineering approaches [69]. Furthermore, the recombinant nature of spider silk proteins allowed genetic modification for covalent, triggerable substance delivery systems [99].

For top-down tissue engineering approaches, porous three-dimensional structures offer adherence points and mechanical stability for cells, and pores facilitate nutrient, oxygen and waste transport. Foaming of silk fibroin solutions with varying nitrous oxide pressure and protein concentration led to scaffolds with pore sizes in the range between 100 and 400 μm [100]. Pore sizes below 100 μm were generated by increasing the protein concentration from 5 wt.% up to 12 wt.%, followed by freeze-drying and immersion in methanol [101]. Recombinant spider silk proteins have been processed into scaffolds with pores sizes of around 100 μm by dissolving the protein in HFIP and using different sized salt crystals as porogens [102].

Hydrogels are hydrophilic polymer networks, physically or chemically cross-linked, that can absorb water up to thousands of times their dry weight [103]. Recombinant spider silk proteins form hydrogels through chain entanglement, which can be printed using dispense plotting at room temperature while supporting encapsulated cells [104]. Due to unfavorable physical properties, like low viscosity, hydrogels made of *B. mori* silk fibroin

are less suitable for bioprinting applications without additives. Here, strategies to blend the material with other (bio)polymers to enhance printability have been applied. For example, Chameettachal et al. successfully bioprinted fibroin-gelatin-blends using dispense plotting at room temperature [105].

5. Silk-Based Hard Tissue Engineering

The following chapter summarizes recent approaches in silk-based hard tissue engineering, and in Table 1, various examples are listed. Upon providing multiple examples for hard tissue engineering approaches based on silk, the adaptability and compatibility of silk materials are shown.

Table 1. Overview of silk-based hard tissue engineering approaches.

Mineralization	Silk Source	Filler Materials/Additives	Morphology/Fabrication Technique	Cell Types	Biocompatibility Study	Target Tissue
non-mineralized	*Bombyx mori* silk fibroin [106]	glycerol, PEG	2D film casting	human dermal fibroblasts	in vitro	bone
–	*Bombyx mori* silk fibroin [107]	–	3D porous scaffold/lyophilization	human adipose mesenchymal stem cells	in vitro and in vivo in rat calvarial bone model	bone
–	*Bombyx mori* silk fibroin [108]	bacterial nanocellulose; photo-crosslinker	3D hydrogels/3D printing	mouse lung fibroblasts	in vitro	bone
–	*Bombyx mori* silk fibroin [109]	collagen I	3D scaffold with aligned or knitted fibers/lyophilization	rabbit bone marrow stem cells	in vitro and in vivo in rotator cuff rabbit model	tendon-to-bone transition
biomineralized	recombinant spider silk [72]	–	2D film casting	mouse pre-osteoblasts	in vitro	tendon-to-bone transition
–	*Cupiennius salei* spider silk fibers [110]	–	2.5D fibers/naturally harvested	–	–	bone
pre-mineralized materials	*Bombyx mori* silk fibroin [111]	alumina nanoparticles	3D porous scaffold/lyophilization	rabbit adipose-derived stem cells	in vitro	bone
–	*Bombyx mori* silk fibroin, soy protein [112]	graphene oxide, β-tricalcium phosphate	3D porous scaffold/lyophilization	rat bone marrow stem cells	in vitro	bone
–	*Bombyx mori* silk fibroin [113]	graphene oxide, nano-hydroxyapatite	3D porous scaffold/lyophilization	bone marrow stem cells, human umbilical vein endothelial cells (HUVECs)	in vitro	bone, vasculature

–	*Bombyx mori* silk fibroin [114]	doped β-tricalcium phosphate, crosslinker	3D porous scaffold/ lyophilization	human osteoblasts, human articular chondrocytes	in vitro	bone, cartilage
–	*Bombyx mori* silk fibroin [115]	–	3D porous sponges/salt leaching	stem cells from human exfoliated deciduous teeth	in vitro	teeth

5.1. Bone Tissue Engineering

Among the recent approaches in hard tissue engineering for various tissue types (bone, tendon, cartilage), three types of scaffold materials are used. On the one hand, there are studies based on non-mineralized scaffolds, which were examined concerning their biocompatibility and properties for bone repair without pre-mineralization. Others were mineralized upon incubation with cells or mineralization agents. In the third set-up, inorganic components such as bioceramics or minerals were directly added to the fabrication process to yield composite scaffolds. The following section is describing these three types in more detail, as well as the morphology of the underlying silk scaffolds.

5.1.1. Non-Mineralized Scaffolds

Scaffolds can be fabricated in different dimensions, from 1D fibers, to 2D films to 3D printed scaffolds. Different processing methods have been used to influence the mechanical properties of the silk scaffolds to be more bone-like and to adopt its performance in cell culture. Silk fibroin films as 2D structures were blended with glycerol and poly(ethylene glycol) (PEG) to improve ductility and porosity, beneficial for cell adhesion. Different film properties were obtained upon adjusting the casting temperature. For example, the ultimate tensile strength was increased when films were cast at 60 °C in comparison to 4 °C, indicating higher crystallinity in the films. Moreover, pore sizes decreased in the same manner when film casting was conducted at elevated temperatures. Further, double blends with PEG and glycerol showed the best results, as both additives might interact, yielding stable constructs [106].

Hard tissue repair has high demands regarding the form of the construct, as defect sites are individual and often complex and, therefore, 3D structures such as foams, sponges, injectable or printable hydrogels are of most interest. Silk scaffolds with no chemical crosslinker were fabricated using a solvent exchange method. Silk fibroin as well as spider silk proteins were dissolved in formic acid or HFIP and blended with sodium chloride crystals as porogens to control pore sizes between 200–300 μm. The obtained structures showed high content of β-sheet structures resulting in stable constructs [116]. 3D spongy silk/sericin scaffolds were fabricated using freeze-drying in order to investigate the influence of sericin addition to the material. Structural, biological and immunological properties were investigated with different weight ratios of sericin (0–4.7 wt.%). Further, scaffolds were chemically crosslinked using glutaraldehyde vapor. Structural transition towards β-sheets was induced upon immersion in ethanol. These highly porous structures with more than 90% porosity showed a decrease in pore size in the presence of increasing amounts of sericin. Similar trends were observed for mechanical properties, which were significantly higher upon increasing sericin content. As a result, cell culture studies with human osteoblast MG63 cells revealed no enhanced cytotoxic effect of the sericin present in the scaffolds. Further, macrophage adhesion was not highly pronounced, and inflammatory marker genes were not upregulated with increasing sericin content [117]. Another scaffold was fabricated using lyophilization to investigate the effect of pre-seeding of human adipose-derived mesenchymal stem cells for bone regeneration in vitro and in vivo [107]. Harsh crosslinking agents could be avoided, and constructs were solely post-treated in ethanol. In cell studies,

cytocompatibility was confirmed, and a mineralized matrix formation was found after two weeks as a sign of osteogenic differentiation [107]. Rat calvaria models served to evaluate the in vivo performance of cell-seeded scaffolds in comparison to that of non-seeded silk scaffolds. Micro-CT showed no significant impact on the amount of regenerated bone after 12 weeks [107]. However, looking deeper into the composition of the newly formed tissue, new bone with a higher amount of collagen and vasculature was formed in pre-seeded scaffolds [107].

When using hydrogels for hard tissue engineering, mechanical properties must be investigated and adapted. Different crosslinking methods were used in the following three approaches: Long et al. [118] mixed silk fibroin and elastin to assemble hybrid hydrogels using a physical heat crosslinking method for higher β-sheet content in the silk material and chemical crosslinking with glutaraldehyde between silk and elastin. In this case, silk was used due to its easy processing into different morphologies and elastin to add biochemical cues to the material. Hydrogels exhibited 4–70 kPa in compressive modulus and shear compressive moduli up to 40 kPa. A proliferation assay using L929 lung fibroblasts showed no negative effects of the chemical crosslinking. Wu et al. [119] also combined two crosslinking methods for silk fibroin hydrogels, but in this case, ethanol for physical and γ-ray for chemical crosslinking of the solutions were applied. Crosslinking through irradiation occurred with the assumed mechanism of radical formation as a result of high energy transfer to silk fibroin molecules. Such treated hydrogels could cover several orders of magnitude in elastic moduli from the Pa range up to hard hydrogels in the MPa range upon different irradiation times (Figure 4). Reflecting the possible variation of mechanical properties, irradiation did also alter pore structure, resulting in denser gels with smaller pores upon increasing crosslinking. Interestingly, biodegradation in the presence of protease XIV was not influenced upon irradiation but upon ethanol physical crosslinking, as the latter is related to silk crystallinity. Cell toxicity of rat bone marrow mesenchymal stem cells was studied using supernatants from scaffolds, as irradiation might cause toxic radicals in solution, but no significant changes compared to the control group were observed.

Laomeephol et al. [120] used a phospholipid (1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol) sodium salt) as a gelling additive to accelerate hydrogel formation of silk fibroin solutions. Changing the concentration of the lipid resulted in different gelation rates, and, thereby, gelation could be controlled. The mechanism is based on the amphiphilic nature of the lipid, which forms electrostatic and hydrophobic interactions with silk fibroin. Cytocompatibility was confirmed using ISO 109931:2009 cell tests and cell lines such as L929 lung fibroblast and NIH/3T3 fibroblasts [120]. While proliferation was found in the gels using these cell lines, cancer-derived cells such as SaOS-2 did not proliferate after 21 days and stayed in round shape. In this case, the described constructs seemed to be rather unfavorable substrates while showing inhibition of growth of cancer-derived cell lines [120].

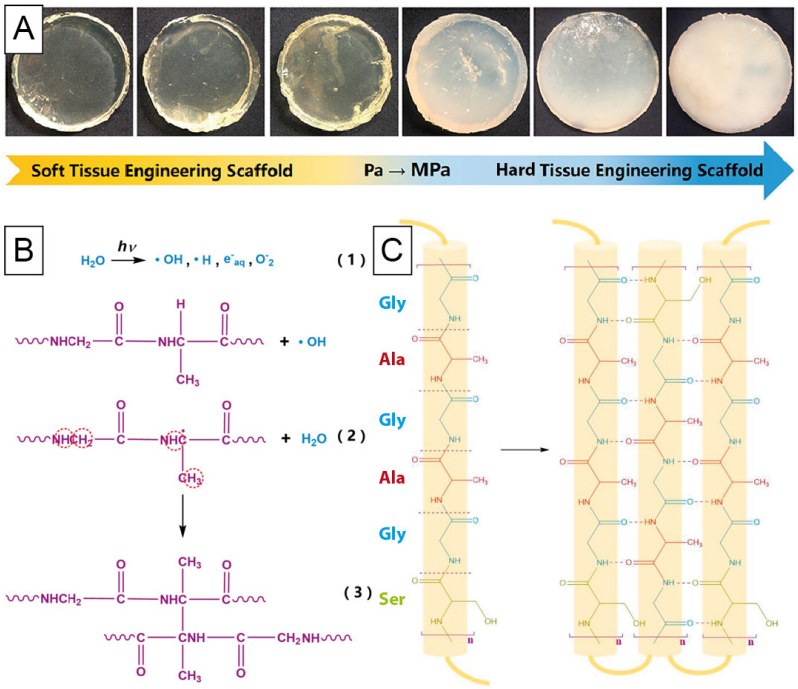

Figure 4. (**A**) Photographs of *Bombyx mori* silk fibroin hydrogels with a controlled degree of crosslinking using γ-radiation (yellow) and ethanol treatment (blue) to cover six orders of magnitude of material stiffness. Crosslinking mechanism upon (**B**) γ-radiation, triggering radical water splitting and the evolution of free radicals, leading to combinational events between polymer chains and (**C**) ethanol treatment, inducing hydrogen bonding and intermolecular interaction. Reprinted and adopted with permission from Wu, N. et al., Investigation on the Structure and Mechanical Properties of Highly Tunable Elastomeric Silk Fibroin Hydrogels Cross-Linked by γ-Ray Radiation. *ACS Appl. Mater. Interfaces* **2020**, 3(1), 721–734. Copyright (2020) American Chemical Society.

To use silk hydrogels in 3D printing applications, one blend was examined using silk fibroin and alginate [121]. Since bioinks further contain cells for 3D printing applications, they must be cytocompatible and printable to enable cell survival and, in the best case, proliferation. As shear forces are acting on the material during printing, cells have to be protected from damaging shear stress. In this study, 1 wt.% sodium alginate with 2 wt.% silk fibroin underwent rapid gelation upon addition of calcium chloride, which crosslinks alginate [121]. The addition of 1 wt.% rather than 0.5 wt.% alginate resulted in higher strand fidelity during printing. Encapsulated osteosarcoma cells were loaded into the hydrogel blends and manually printed from syringes, which represents a first printability evaluation of a material. Extruded strands were cultured, and cell viability was evaluated in live/dead staining after print-induced shearing. Besides few dead cells in one sample, cells survived the process. Cell viability was confirmed using the metabolic PrestoBlue assay. However, it was found that at higher amounts of silk fibroin, less signal could be detected, although viable cells were found. This led to the assumption that the dye, but also nutrients and waste products can only hardly travel through the construct upon increasing silk fibroin content.

For the adjustment of mechanical properties and printability, filler materials such as nanocellulose can be added to silk fibroin hydrogels [108]. Bacterial nanocellulose was added, yielding composite hydrogels for 3D printing. Photo-crosslinking using tris(bipyridine)ruthenium(II) chloride as crosslinker generated scaffolds, which differed

in their characteristics depending on the morphology of the nanocellulose used either as a solution, fibers or whiskers. Structural information was related to β-sheet content and silk fibroin nanocellulose interaction, which is defined by inter-domain distance in the silk fibroin determined using small-angle neutron scattering techniques. Scanning electron microscopy images (SEM) showed that cellulose as an additive influenced pore sizes, yielding especially dense structures in the presence of whiskers. Best rheological, tensile and compression behavior was found in the presence of fiber fillers. Printability evaluated by strand-width after printing was similarly high for fiber and whisker additives. However, as a small drawback, these two morphologies were not reported to be cell friendly whilst causing cytotoxicity upon oxidative stress or inflammatory response. Culturing L929 lung fibroblasts on hydrogels, all blends showed proliferation and high viability tested after 1, 3 and 5 days.

In another approach, tyrosinase-crosslinked silk fibroin/gelatine hydrogel blends were cell loaded and 3D printed to gain functional constructs [122]. Silk was functionalized with gelatine and calcium chloride for sustained release of calcium ions from the scaffold, similar to the extracellular release of calcium by osteoclasts during bone modeling (Figure 5). Several aims were followed in this study: First, the material was optimized for 3D printing concerning long-term stability for cell culture, and, therefore, rheological properties were adjusted. It was found that at low shear rates, blend hydrogels showed shear thickening behavior, presumably related to crystallization or entanglement between both components. At higher shear rates, a sudden transition to shear thinning behavior was assumed as a result of compound release. Further, the addition of calcium chloride increased the viscosity in such hydrogel blends up to 100-fold due to faster gelation times and ionic interactions with silk fibroin. Second, the osteogenic profile of the constructs was investigated upon release of calcium ions. Ion release was found for 3 weeks, but the release was not complete at that time point.

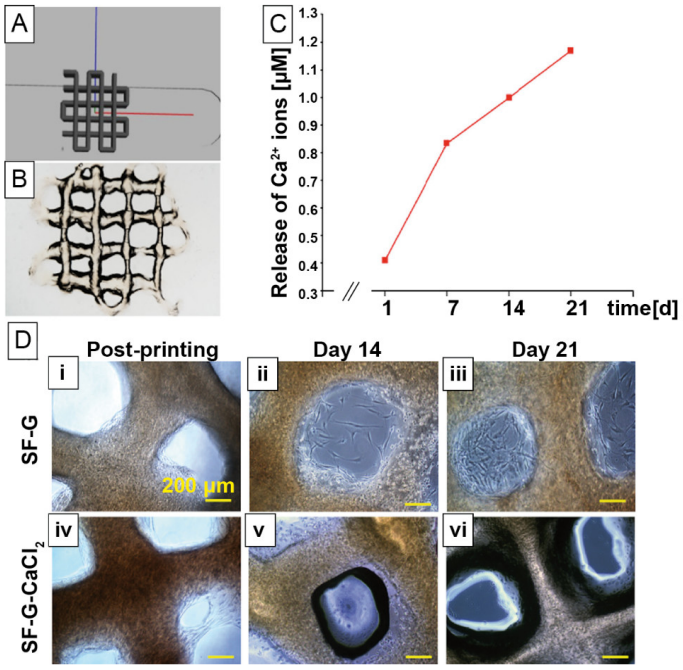

Figure 5. (**A**) CAD sketch of a *Bombyx mori* silk fibroin/gelatine construct and (**B**) its 3D dispense plotted result. (**C**) Release profile of calcium ions from *Bombyx mori* silk fibroin/gelatine hydrogels, loaded with calcium chloride. (**D**) Light microscopy images of unloaded (**i–iii**) and loaded (**iv–vi**)

silk/gelatine hydrogels with human mesenchymal stem cells post-printing (**i**,**iv**), after 14 (**ii**,**v**) and 21 days (**iii**,**vi**). Unloaded scaffolds showed cells present in both the construct and in pores, whereas, in the loaded construct, cells remained in the strands. SF = silk fibroin, G = gelatine. Reprinted and adopted with permission from Sharma, A. et al., Investigating the Role of Sustained Calcium Release in Silk-Gelatin-Based Three-Dimensional Bioprinted Constructs for Enhancing the Osteogenic Differentiation of Human Bone Marrow Derived Mesenchymal Stromal Cells. *ACS Biomater. Sci. Eng.* **2019**, *5*(3), 1518–1533. Copyright (2019) American Chemical Society.

Third, the signaling pathway, which regulates osteogenic differentiation in human bone marrow derived progenitor cells, was analyzed with regard to the influence of calcium ions. The gene expression profile of parts of the canonical Wnt pathway with specific expression of β-catenin, BMP2 and BMP4 was investigated, and their concentration was highest on day 21. It can be therefore assumed that BMP plays an important role in osteogenic differentiation of human bone marrow derived progenitor cells.

The recombinant spider silk protein eADF4(C16), which is based on the consensus sequence of one component of the *A. diadematus* dragline silk [123], was found to form hydrogels in a controlled manner forming a physical fibrillar network [124,125], enabling its processing via 3D printing for tissue engineering [126]. Further, this recombinant spider silk protein could be modified to comprise the well-known RGD cell-binding motif [126]. Modifications of the processing technique, such as creating blends [127,128], incorporation of silica particles [129] or the release of biologicals from hydrogels [130], were investigated and successfully yielded stable gels.

Silk fibroin hybrid materials with two different silk morphologies were studied, for example, by Ding et al. [131] β-sheet rich silk fibroin nanofibers were encapsulated in an amorphous silk matrix. Upon electric field exposition of 50 V for 30 min, nanofibers traveled through the amorphous matrix and aligned. Scaffolds were crosslinked using horseradish peroxidase and yielded anisotropic scaffolds with up to 120 kPa in stiffness. In in vitro studies, bone marrow mesenchymal stem cells showed osteogenic behavior, and an ectopic in vivo bone model was used to investigate osteogenic properties in rate femurs. Between week 8 to 12, newly formed bone was found in the case of the stiffest hydrogel samples, whereas the aligned fiber structure also led to alignment in the new tissue. The fabrication method with two silk fibroin morphologies and electric field alignment was then used by the same group to fabricate gradient hydrogels [132]. In further detail, horseradish crosslinking times and resulting gradient mechanical properties were investigated. The distribution of β-sheet rich silk fibroin nanofibers in the scaffolds also led not only to changed material stiffness but also to gradient pore structures resembling that of native tissue. In cell culture studies, the construct's properties translated into tissue-specific differentiation of bone marrow mesenchymal stem cells, whereas the soft part induced chondro-related genes, gradually triggering bone formation towards the stiffer end of the scaffold, which was also confirmed in in vivo studies.

In another double silk approach, Liu et al. [133] used silk fibroin solutions, which were first autoclaved to induce nanoparticle formation (in the range of 50–300 nm), then embedded in silk fibroin solutions, followed by freeze-drying to form 3D sponges. To stabilize structures in an aqueous environment, low-molecular weight PEG solutions were used to induce β-sheet formation. Nanoparticles could be extracted, yielding cavities and pores in the scaffold. This generated pore structure introduced permeability and flexibility compared to silk sponges without particle loading. After methanol annealing, better cell adhesion, distribution and growth on scaffolds were observed. In general, particle-loaded constructs, especially with bioceramics, have the potential to support bone tissue engineering due to their pre-mineralization, which is discussed in detail in the section below.

5.1.2. Microcarriers for Bone Tissue Engineering

Microcarriers based on silk fibroin and gelatine were fabricated in a top-down approach for bone tissue engineering as injectable units or building blocks for scaffolds (Figure 6) [134]. With a microfluidic asymmetric flow-focusing device, carriers of about 100–350 µm were produced depending on the flow rate ratio of the aqueous and separation oil phase also containing methanol. The material blend was tested as 2D films and 3D microcarriers in rat mesenchymal stem cell culture. With increasing proportion of gelation from 25 to 50 to 75 wt.%, increasing cell adhesion was found. This trend was similarly confirmed for the carrier's mechanical properties, as both higher blend situations were in the range of 183 kPa and 139 kPa, respectively, values which are also described for the osteoid region, where pre-osteoblast differentiation takes place.

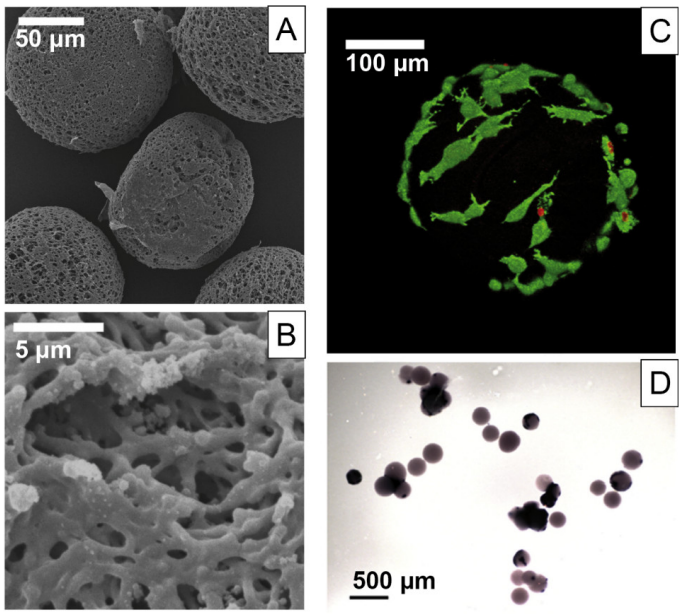

Figure 6. (**A**) Scanning electron microscopy images of blend hydrogel microcarriers using *Bombyx mori* silk fibroin and porcine gelatine at a 3:1 ratio and asymmetric flow focusing in a microfluidic preparation method and (**B**) surface micro-topography at higher magnification. (**C**) Rat mesenchymal stem cells on hydrogel microcarriers visualized using confocal microscopy after 96 h in live/dead staining (dead cells in red, living cells in green) and (**D**) after 28 days. Purple coloring indicates alkaline phosphatase activity of differentiated osteoblasts. Reprinted and adopted with permission Luetchford, K. A.; Chaudhuri, J. B.; De Bank, P. A., Silk fibroin/gelatin microcarriers as scaffolds for bone tissue engineering. *Mater. Sci. Eng. C* **2020**, *106*, 110116. Copyright (2020) Elsevier.

5.1.3. Biomineralized Scaffolds Using Specific Mineralization Tags

As a template for biomineralization, natural spider silk fibers were collected from adult females of *Cupiennius salei*. Biomineralization of dragline silk fibers took place upon subsequent incubation in calcium hydroxide containing solution, followed by incubation in diluted phosphoric acid. The procedure was also used the other way round as reversed biomineralization, and further, both solutions were incubated on the fibers simultaneously (Figure 7). Biomimetic hybrid materials were yielded with controlled hydroxyapatite deposition, forming a homogenous coating on the fibers. The best mineralization results were obtained upon initial incubation in calcium-containing solutions, as silk fibers were assumed to interact with the cations and induce higher

mineralization with less calcium-deficient hydroxyapatite. Mechanical characterization of the mineralized fibers showed similar strength, toughness and Young's Modulus in comparison to the natural supercontracted fibers [110].

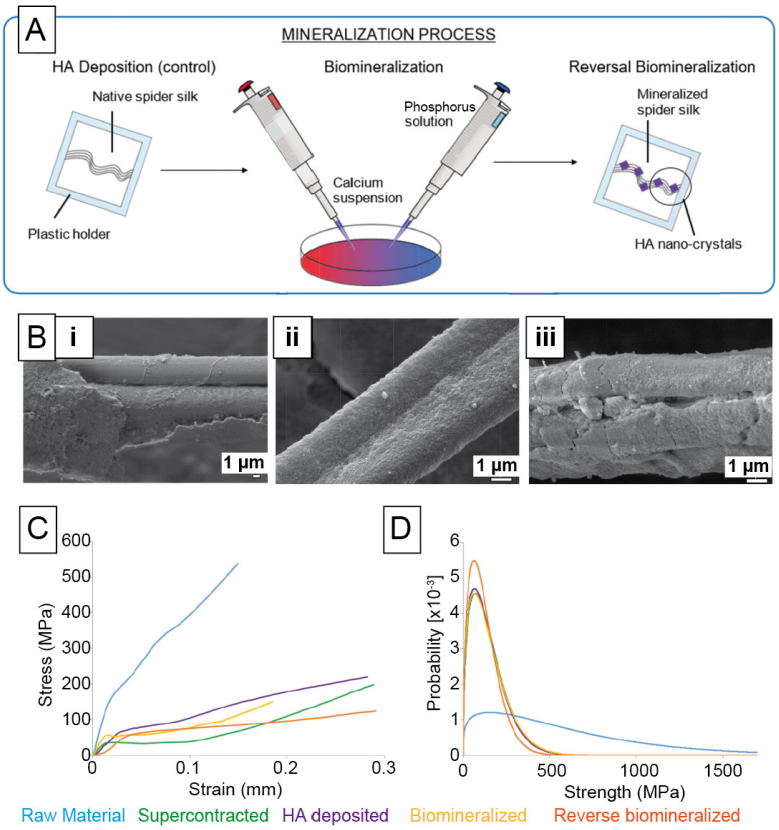

Figure 7. (**A**) Schematic illustration of the mineralization process of natural *Cupiennius salei* spider silk fibers. (**B**) Scanning electron microscopy images of mineralized silk fibers after (**i**) 1 day of biomineralization and day 3 (**ii**) and 7 (**iii**) of reversal biomineralization. Mechanical characterization of fibers in (**C**) stress-strain plots and (**D**) Weibull probability distribution as parameter for homogenous fracture behavior. HA = hydroxyapatite. Reprinted and adopted with permission from Dellaquila, A. et al., Optimized production of a high-performance hybrid biomaterial: biomineralized spider silk for bone tissue engineering. *J. Appl. Polym. Sci.* **2020**, *137*(22), 48739. Copyright (2020) John Wiley and Sons.

Smaller fibers in the sub-micron range were produced using electrospinning of *B. mori* silk fibroin out of formic acid and HFIP mixtures into an ethanol bath for instant crosslinking by β-sheet formation. Freeze-drying of the multilayer yielded a 3D fibrous scaffold, and biomineralization was induced upon incubation in two-fold Simulated Body Fluid for up to 28 days. Imaging of the interconnected pores and the increase in fiber diameter allowed an estimation of the ongoing mineral deposition. The effect of the mineralized layers was investigated in vitro using bone marrow mesenchymal stem cells (BMSC) and in vivo in rat cranial defect models. More migrated cells next to the newly formed bone and capillaries confirmed bone regeneration ability of these biomimetic scaffolds [135]. Strong, ductile and lightweight materials were gained upon self-assembly of silk fibroin nanofibers from an aqueous solution. In further processing steps, biomineralization was initiated out of calcium chloride and sodium dihydrogen

phosphate solutions before chitin nanofibers were introduced in a hierarchical assembly. Mechanical characterization of the scaffolds revealed a very lightweight material like aerogels but with high compressive strength of up to more than 400 MPa [136].

Further, biomineralization was directed through silk components. Therefore, silk extracted sericin was added to dense collagen hydrogels. Due to the sericin's negative charge resulting from amino acid residues such as aspartic and glutamic acid, hydroxyapatite formation could be induced. This acellular mineralization process in Simulated Body Fluid yielded minerals after 3 days, with an ongoing process until 14 days resulting in 90 wt.% mineral phase. SEM, energy-dispersive X-ray spectroscopy and X-ray diffraction studies showed distinct spherulite particles. Mesenchymal stem cells were seeded on mineralized sericin-containing collagen hydrogels, and an osteogenic upregulation was observed in metabolic activity [137].

However, biotechnology allows for tailoring recombinant peptides and proteins, and silk proteins were combined with explicit peptide tags on the DNA level to trigger mineralization processes. Engineered sequences from *N. clavipes* dragline silk MaSp1 were C- or/and N-terminally hybridized with the hydroxyapatite binding peptide VTKHLNQISQSY, which was identified via phage display. Films processed out of these proteins were immersed in calcium chloride and sodium phosphate solutions. Mineral formation, as well as human mesenchymal stem cell differentiation, were especially enhanced in the case of double functionalized constructs [60]. The same silk consensus sequence was functionalized with a bone sialoprotein motif to introduce non-collagenous moieties. In this case, silk films were mineralized in female mice in vivo. At first, a mild inflammatory response could be observed using flow cytometry and also histology, but after 6 weeks, inflammation markers decreased. Finally, no capsule formation was observed [71].

In another approach, recombinant spider silk fusion proteins were engineered with different mineralization and collagen-binding motifs from non-collagenous proteins in bone. Proteins with N- and C-terminal peptide tags were compared concerning their mineralization ability in Simulated Body Fluid and their interaction with MC3T3 E1 mouse pre-osteoblasts. The variants showed mineralization tendency to a different extent but confirmed the formation of calcium and phosphate-containing species. Studying cell adhesion on materials of the protein variants separately, no significant favor of one variant over the other could be observed. However, when processing two materials into a gradient, cell adhesion towards the collagen-binding motif was clearly favored over the mineralization variant and could be maintained for 21 days. Therefore, these materials are also suitable candidates for applications at the tendon-bone-interface [72].

5.1.4. Biomineralization of Scaffolds Using Pre-Mineralization

In one example, 1–10 wt.% alumina nanoparticles were added to 4 wt.% silk solutions, and this emulsion was then lyophilized [111]. Additional mineralization was achieved upon incubation of the scaffolds in Simulated Body Fluid [61] for 28 days, forming an apatite layer in all constructs. The cell attachment of rabbit adipose-derived stem cells was not significantly changed with varying alumina content as this material was already reported to be bioinert, leading to the assumption that mechanical and structural cues were affected by the particles. Osteogenic upregulation in an initial stage was found starting at day 7 using alkaline phosphatase activity and Alizarin red staining of the cultures.

Another lyophilized scaffold comprised silk fibroin titanium dioxide and fluoridated titanium dioxide nanoparticles [138]. Particles acted as bioceramic reinforcement for compressive load. As the compressive modulus is often related to particle content, calculations and experimental data were collected. Both approaches were in good agreement with each other and showed open honeycomb structures in the constructs with a compressive modulus of up to 1.297 ± 0.175 MPa in the presence of 20 wt.% TiO_2.

Magnesium oxide nanoparticle-containing scaffolds at 15/20/25 wt.% were fabricated upon electrospinning of silk fibroin and PCL at a 4:1 *w/w* ratio [139]. Increasing amounts of nanoparticles lead to higher fiber diameters, respectively 651 nm/1055 nm/1251 nm, with visibly entrapped particles. At higher amounts of inorganic fillers, water contact angles decreased (below 30°), turning the materials more hydrophilic and favorable for cells. In comparison, no significant differences between particle-loaded fiber meshes were found concerning cytotoxicity for MC3T3 E1 mouse pre-osteoblast cells. The cumulative release of Mg^{2+} from the meshes reached a plateau after about 15 days. The ion release is often related to osteogenic differentiation and was confirmed with extracts from fiber mats after 21 days using both Alizarin Red and alkaline phosphatase staining. In vivo studies in rat calvarial defects revealed a significant enhancement of bone regeneration using nanofibrous membranes loaded with magnesium oxide particles in the twelfth week post-surgery.

As actuation and dynamic cultures are improving bone regeneration, magnetic particles were incorporated inside silk fibroin scaffolds [140]. Scaffolds were soaked in biomineralization solutions, and Ca/P containing species were obtained. Vibrating Sample Magnetometry was used to characterize magnetic properties, especially for further evaluation of proliferation of MC3T3 E1 cells upon stimulation using a magnetic field. In general, cells grew randomly in the absence and clustered in the presence of a magnetic influence.

Graphene oxide has also been added to silk fibroin solutions, followed by lyophilization. In one study, 3D porous silk fibroin/graphene oxide constructs were freeze-dried from solution, and the graphene oxide induced wrinkled surface nanotopographies [141]. Further, its incorporation into scaffolds decreased the diameter of the interconnected pores from 25–60 nm to 10–30 nm. The compressive modulus was independent of the graphene oxide concentration and ranged between 1.5–2 MPa for 3–10 wt.%. Water uptake is a critical factor for cell compatibility and was found to be related to graphene oxide content. Further, water uptake was also crucial for in vitro biomineralization in Simulated Body Fluid. Although mineralization commenced from day 7 to 14, the crystal morphology in the pores changed towards larger and smoother crystals. In a second approach, the synergistic or individual effect of graphene oxide with β-tricalcium phosphate in a lyophilized silk fibroin/soy protein blend scaffold was studied concerning osteoconductivity [112]. Both particle types influenced mechanical properties in the range of 1 MPa. In vitro biomineralization was induced in Simulated Body Fluid for 14 days, and rather small crystals were found covering the whole construct's surface. Both scaffolds with particles exhibited more minerals than protein blend scaffolds on their own, however, nanocrystal deposition was increased in the presence of graphene oxide only. Alkaline phosphatase activity of rat bone marrow mesenchymal stem cells was highest at day 5, whereas RUNX2 expression as osteogenesis-related gene marker increased until day 14. Osteocalcin expression, as a marker of late osteoblastic differentiation, also increased up to day 14, indicating that the material was suitable for long-term bone regeneration processes. A third filler material was achieved using silk fibroin freeze-dried scaffolds and particles made of nanohydroxyapatite and graphene oxide [113]. Scaffolds with both particle types showed oriented pore structure, similar to lamellae or channels, which was not observed in the other studies. Channel structures were fabricated using a directional temperature field freezing technology, where only one side of the scaffold was exposed to a cold surface and gradually frozen until the fixed structure was freeze-dried. Both oriented and unoriented double-filled materials were fabricated (Figure 8). Mechanical properties were in the kPa range and, therefore, lower than in the two aforementioned studies. Regarding biomineralization, enzyme-directed mineralization was analyzed using alkaline phosphatase activity from bone marrow mesenchymal stem cells. Interestingly, the highest cell viability, and proliferation was found in the case of oriented double-loaded constructs, whereas unoriented constructs showed even lower signals than silk fibroin scaffolds with nanohydroxyapatite only. Besides osteogenic

differentiation, the ability was studied to provide structures for vascularization. Human umbilical vein endothelial cells (HUVECs) seeded on the 3D scaffolds migrated preferentially into the aligned channel-like structures as they might sense orientation similar to blood vessels.

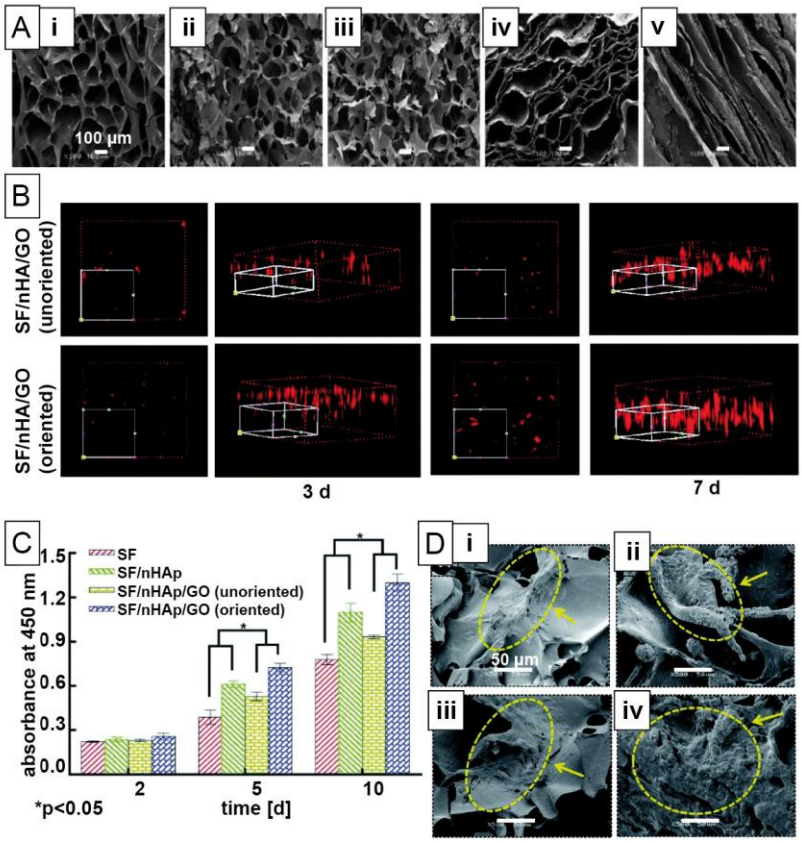

Figure 8. (**A**) Scanning electron microscopy images of lyophilized foam-like scaffolds with different orientation made of plain *Bombyx mori* silk fibroin (**i**), foams loaded with hydroxyapatite nanoparticles (**ii**), foams loaded with hydroxyapatite and graphene oxide nanoparticles in unoriented structures (**iii**), with directional structuring in cross-section (**iv**) and in longitudinal ones (**v**). (**B**) 3D Confocal microscopy images of HUVEC cells in unoriented and oriented foams loaded with hydroxyapatite nanoparticles and graphene oxide nanoparticles after 3 and 7 days. Scale bar not indicated. (**C**) Cell viability assay for bone mesenchymal stem cells after 2, 5 and 10 days. (**D**) Scanning electron microscopy images of bone mesenchymal stem cells in lyophilized foam-like scaffolds after 10 days for (**i**) silk fibroin, (**ii**) foams loaded with hydroxyapatite nanoparticles loaded with hydroxyapatite and graphene oxide nanoparticles without (**iii**) and with (**iv**) directional structuring. SF = silk fibroin, nHA = hydroxyapatite nanoparticles, GO = graphene oxide. Reprinted and adopted with permission from Wang, L.; et al., Preparation and biological properties of silk fibroin/nano-hydroxyapatite/graphene oxide scaffolds with an oriented channel-like structure. *RSC Advances* **2020**, *10*(17), 10118-10128. Copyright (2020) The Royal Society of Chemistry.

For all studies, no phase separation between fillers and silk was observed, indicating good compatibility between the material types. Only at very high filler content, particle aggregates were observed.

5.2. Teeth and Mandible Tissue Engineering

State of the art for the regeneration of hard tissue in the mouth is placing implants at the defect location, which need to integrate well at the implantation site. However, it is important to remain the tooth socket for implants, which is rather relating to bone regeneration than teeth themselves. First approaches into this field of application were realized using *B. mori* silk fibroin based scaffolds, prepared via freeze-drying and studied to preserve the jaw ridge [142]: An appropriate rate of material resorption was found for silk fibroin scaffolds with pore sizes around 200 nm and nano-hydroxyapatite reinforcements, which were additionally mineralized in vitro. These scaffolds showed osteogenic differentiation in pre-osteoblast MC3T3 E1 cells after 21 days. Further, the interaction with human bone marrow stromal cells showed good biocompatibility [142].

Another study focused on culturing stem cells from human exfoliated deciduous teeth on silkworm sponges prepared from cocoon cuts. Cell proliferation could be confirmed, but scaffolds for endodontic repair, which can simulate dynamic dental pulp repair, are still at the beginning to commence the field [115].

5.3. Tissue Engineering of Bone Neighbouring Hard Tissues

Osteochondral defects can be caused by trauma, tumor resection or osteoporosis and can lead to bone loss, osteoarthritis and even full tissue dysfunction when not treated. Scaffolds used for osteochondral defects often show hierarchical arrangement of chondral and bone bilayers. One example of scaffolds are blends of bacterial cellulose with silk fibroin in an interpenetrating hydrogel to create artificial cartilage [143]. Bacterial cellulose spongy cubes were prepared, squeezed to dry, and silk solutions were soaked in. MC3T3 cell cultures showed the best proliferation on plain silk fibroin, followed by the blended scaffold and finally the cellulose sponge.

Ribeiro et al. [114] crosslinked silk fibroin with horseradish peroxidase as subchrondro-layer and a tricalcium phosphate bone-like layer in a bilayered structure. The scaffolds showed homogenous porosity with macro- and micropore sizes (500 and 10 μm) for the regions with denser structures when mineralized with tricalcium phosphate. Human osteoblasts from femoral bone tissue and human articular chondrocytes were seeded on the scaffolds, and tissue distinct expression patterns were found on the bilayered regions in case of the flexible subchondral and supportive bone region.

In a trilayered scaffold, a cartilage layer, a calcified transition layer and a bony layer were generated upon the addition of nanohydroxyapatite and paraffin-spheres [144]. First, hydroxyapatite in silk solution with paraffin was cast and frozen. Then, on top of the construct, the silk solution was applied and exposed to a cold cylinder using a temperature gradient-guided thermal-induced phase separation technique. After leaching of the paraffin spheres, a lamellar structure in the chondral part, round cavities in the bony part, and an intermediate layer were formed (Figure 9). Adipose-derived stromal cells were seeded on the scaffolds, and both sides had cell favorable structures with lamellae, round pores or cavities. Upon induction of differentiation using respective chondral and bone factors, glycosaminoglycans and collagen type II were found in oriented structures indicating chondrogenic differentiation. In the same manner, bone-related matrix content was found, such as calcium and collagen type I in bone pore structures.

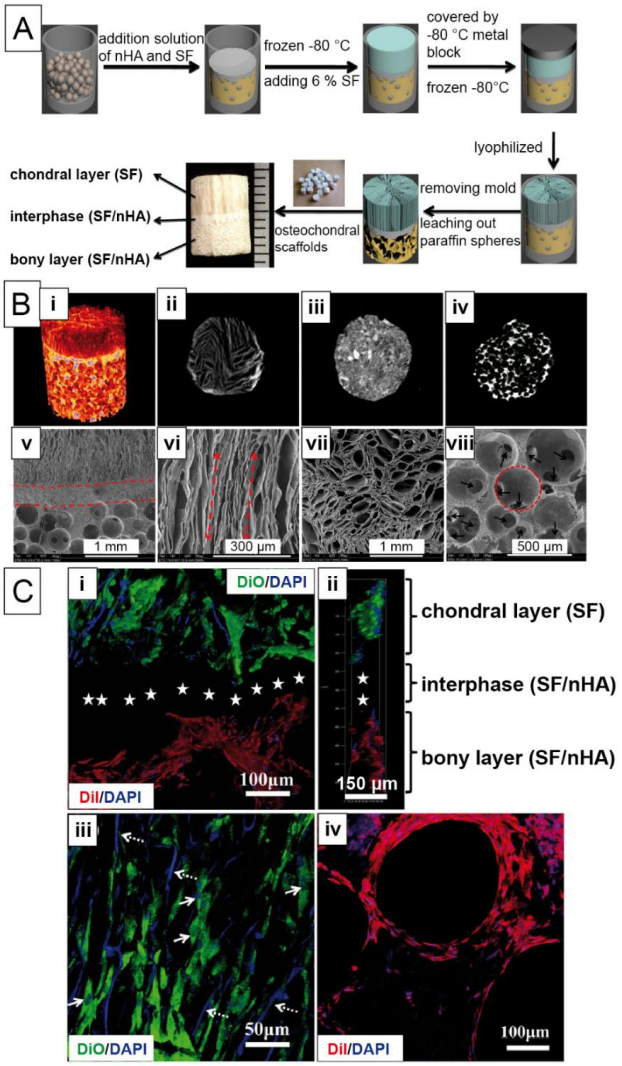

Figure 9. (**A**) Illustration of a trilayer (e.g., chondral, interphase and bony layer) scaffold preparation process using *Bombyx mori* silk. (**B**) Micro-CT (µCT) and scanning electron microscopy (SEM) images of the individual layers in the scaffold: (**i**) µCT of the full construct, (**ii**) µCT of the chondral layer (top), (**iii**) interphase, (**iv**) bony layer (bottom), and (**v**) SEM of a longitudinal section of the full construct (red lines indicate interphases), (**vi**) of a longitudinal section of the chondral layer (red arrows indicate orientation), (**vii**) of cross-sections of the chondral layer, (**viii**) of cross-sections of the bony layer (red circle indicate a macropore and black arrows connections between pores). (**C**) Confocal microscopy images of trilayer scaffolds of rabbit adipose-derived stroma cells after 3 d in (**i**) longitudinal section and (**ii**) profile view with magnifications of (**iii**) the chondral layer and (**iv**) the bony layer. Blue staining indicates cell nuclei (DAPI), green and red staining cell membranes (DiO, DiI) confirming the interphase as an isolation layer. White solid arrows indicate stained cell nuclei, white dashed arrows indicate unspecific staining, white stars indicate the cell-free intermediate layer. SF = silk fibroin, nHA = hydroxyapatite nanoparticles. Reprinted and adopted with permission from Ding, X.; et al. Integrated Trilayered Silk Fibroin Scaffold for Osteochondral Differentiation of Adipose-Derived Stem Cells. *ACS Appl. Mater. Interfaces* **2014**, *6*(19), 16696–16705. Copyright (2014) American Chemical Society.

Having confirmed to be able to control differentiation towards the desired tissues based on structural cues, these scaffolds were then applied for rabbit bone repair in the knee, with and without pre-cell seeding [145]. Defect evaluation took place 4, 8 and 12 weeks after implantation. At all points of evaluation, surface roughness and integrity, bone smoothness and genetic upregulation were higher when cells were already present in the scaffold. However, neither bone strength nor quality was affected thereby.

Neighboring tissues towards bone such as tendon or cartilage can also exhibit mineralized regions in a gradual manner. This gradual change in composition and mechanical properties hinders crack propagation and allows a uniform transmission of loads. Qian et al. [109] fabricated structures from collagen type I and silk fibroin with increasingly aligned structures, generated using unidirectional freezing for application at the tendon-to-bone interface. Aligned collagen structures with knitted silk fibers exhibited the highest order. Implantation into rats showed that rather unoriented structures were favored for bone repair, whereas aligned structures triggered tendon regeneration. Therefore, the optimum structural combination still has to be found, as complex processes are interacting at the joint between two tissues.

Bradner et al. [146] fabricated microfibers out of silk fibroin hydrogels with additional functionalization using bovine serumalbumin and a bio-silica precursor peptide. Biomineralization is required at the tendon-to-bone insertion to transmit loads on mineralized fibrils. Silk at 5 wt.% and BSA at 0.2 wt.% resembled the natural ratio of collagen-to-elastin in the tendon. Fibers were extruded and enzymatically crosslinked using horseradish peroxidase, followed by thermal post-treatment. Fibers were braided or twisted by hand. Fiber toughness was increased up to 125.4 ± 3.50 Jm^{-2} upon the addition of BSA. An explanation for this behavior could be the presence of additional sacrificial bonds, which break before the structure collapses. Pre-and post-strained silk-BSA samples showed a hierarchy-enabling microstructural rearrangement.

6. Outlook

Major biomaterials in the current global orthopedic market are collagen, hydroxyapatite, calcium phosphates, calcium sulfate and hyaluronic acids [147]. While there are no silk-based products in the field of hard tissue engineering available up until now, recent strategies have shown the eligibility of such materials for hard tissue engineering. Tailor-made solutions for patients will enable personalized medicine, and therein individual requirements for defect solutions can be met. However, top-down strategies rely on construct fabrication, and various complex prerequisites need to be approached concerning the choice of material. Biodegradability is thereby of high importance with respect to non-toxic metabolites and material break down along with tissue regeneration. Hence, biomaterials such as silk-based ones are increasingly in the focus of interest for tissue engineering approaches as they are biocompatible, non-toxic and do not evoke a strong immune reaction by the recipient. Additionally, recombinant silks can be genetically fine-tuned and produced biotechnologically in a large scale. The processing of raw silk proteins into a wide range of morphologies such as particles, fibers, foams and hydrogels allows the coverage of scaffold complexity on various hierarchical levels. In hard tissue engineering, construct design relies to a high degree on the fulfillment of biological and mechanical prerequisites, some of which are based on proper mineralization. Biomimetic mineralization of silk scaffolds can be conveyed upon the introduction of binding sites to accumulate ions from the surrounding media. To our knowledge, currently no clinical studies containing silk-based materials are under way for hard tissue engineering. The available tools to modify and process these materials, as well as the presented promising research results, however, are key further developments. For example, silk screws (*B. mori*) applied as orthopedic fixtures, already successful in animal testing, show high potential for clinical trials [148,149]. Especially with the rise of additive manufacturing techniques and the need, as well as the possibility, for

personalized scaffolds within hard tissue engineering, silk-based materials might soon take the next step towards an application.

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References

1. Palmer, L.C.; Newcomb, C.J.; Kaltz, S.R.; Spoerke, E.D.; Stupp, S.I. Biomimetic Systems for Hydroxyapatite Mineralization Inspired by Bone and Enamel. *Chem. Rev.* **2008**, *108*, 4754–4783, doi:10.1021/cr8004422.
2. Beniash, E. Biominerals—hierarchical nanocomposites: The example of bone. *WIRES Nanomed. Nanobi.* **2011**, *3*, 47–69, doi:10.1002/wnan.105.
3. Gower, L.B. Biomimetic Model Systems for Investigating the Amorphous Precursor Pathway and Its Role in Biomineralization. *Chem. Rev.* **2008**, *108*, 4551–4627, doi:10.1021/cr800443h.
4. Weiner, S.; Addadi, L. Crystallization Pathways in Biomineralization. In *Annual Review of Materials Research*; Clarke, D.R., Fratzl, P., Eds; Annual Reviews: Palo Alto, CA, USA, 2011; Volume 41, pp. 21–40.
5. George, A.; Veis, A. Phosphorylated Proteins and Control over Apatite Nucleation, Crystal Growth, and Inhibition. *Chem. Rev.* **2008**, *108*, 4670–4693, doi:10.1021/cr0782729.
6. Anderson, H.C. Matrix vesicles and calcification. *Curr. Rheumatol. Rep.* **2003**, *5*, 222–226, doi:10.1007/s11926-003-0071-z.
7. Zhang, T.; Lin, S.; Shao, X.; Zhang, Q.; Xue, C.; Zhang, S.; Lin, Y.; Zhu, B.; Cai, X. Effect of matrix stiffness on osteoblast functionalization. *Cell Prolif.* **2017**, *50*, e12338, doi:10.1111/cpr.12338.
8. Zioupos, P.; Kirchner, H.O.; Peterlik, H. Ageing bone fractures: The case of a ductile to brittle transition that shifts with age. *Bone* **2020**, *131*, 115176, doi:10.1016/j.bone.2019.115176.
9. Schmid, G.L.; Kühnast, B.; Heise, M.; Deutsch, T.; Frese, T. Ultrasonography in assessing suspected bone fractures: A cross-sectional survey amongst German general practitioners. *BMC Fam. Prac.* **2020**, *21*, 1–7, doi:10.1186/s12875-020-1078-5.
10. Li, J.; Qin, L.; Yang, K.; Ma, Z.; Wang, Y.; Cheng, L.; Zhao, D.W. Materials evolution of bone plates for internal fixation of bone fractures: A review. *J. Mater. Sci. Technol.* **2020**, *36*, 190–208, doi:10.1016/j.jmst.2019.07.024.
11. Langer, R.; Vacanti, J. Tissue engineering. *Science* **1993**, *260*, 920–926.
12. Hoffman, T.; Khademhosseini, A.; Langer, R. Chasing the Paradigm: Clinical Translation of 25 Years of Tissue Engineering. *Tissue Eng. Part A* **2019**, *25*, 679–687, doi:10.1089/ten.tea.2019.0032.
13. Mao, A.S.; Mooney, D.J. Regenerative medicine: Current therapies and future directions. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 14452–14459, doi:10.1073/pnas.1508520112.
14. Ramos, T.; Moroni, L. Tissue Engineering and Regenerative Medicine 2019: The Role of Biofabrication—A Year in Review. *Tissue Eng. Part C: Methods* **2019**, *26*, 91–106, doi:10.1089/ten.tec.2019.0344.
15. Nichol, J.W.; Khademhosseini, A. Modular tissue engineering: Engineering biological tissues from the bottom up. *Soft Matter.* **2009**, *5*, 1312–1319, doi:10.1039/b814285h.
16. Chen, P.; Zhou, Z.; Liu, W.; Zhao, Y.; Huang, T.; Li, X.; Duan, J.; Fang, J. Preparation and Characterization of Poly(L-lactide-co-glycolide-co-ε-caprolactone) Scaffolds by Thermally Induced Phase Separation. *J. Macromol. Sci. Part B* **2020**, *59*, 427–439, doi:10.1080/00222348.2020.1735136.
17. Januariyasa, I.K.; Yusuf, Y. Porous carbonated hydroxyapatite-based scaffold using simple gas foaming method. *J. Asian Ceram. Soc.* **2020**, *8*, 634–641, doi:10.1080/21870764.2020.1770938.
18. Judawisastra, H.; Nugraha, F.R.; Wibowo, U.A. Porous Architecture Evaluation of Silk Fibroin Scaffold from Direct Dissolution Salt Leaching Method. *Macromol. Symp.* **2020**, *391*, 1900187, doi:10.1002/masy.201900187.
19. Khoramgah, M.S.; Ranjbari, J.; Abbaszadeh, H.-A.; Mirakabad, F.S.T.; Hatami, S.; Hosseinzadeh, S.; Ghanbarian, H. Freeze-dried multiscale porous nanofibrous three dimensional scaffolds for bone regenerations. *BioImpacts* **2020**, *10*, 73–85, doi:10.34172/bi.2020.10.
20. Madrid, A.P.M.; Vrech, S.M.; Sanchez, M.A.; Rodriguez, A.P. Advances in additive manufacturing for bone tissue engineering scaffolds. *Mater. Sci. Eng. C* **2019**, *100*, 631–644, doi:10.1016/j.msec.2019.03.037.
21. Gaspar, V.M.; Lavrador, P.; Borges, J.; Oliveira, M.B.; Mano, J.F. Advanced Bottom-Up Engineering of Living Architectures. *Adv. Mater.* **2020**, *32*, e1903975, doi:10.1002/adma.201903975.
22. Elbert, D.L. Bottom-up tissue engineering. *Curr. Opin. Biotechnol.* **2011**, *22*, 674–680, doi:10.1016/j.copbio.2011.04.001.
23. Silva, A.S.; Santos, L.F.; Mendes, M.C.; Mano, J.F. Multi-layer pre-vascularized magnetic cell sheets for bone regeneration. *Biomaterials* **2020**, *231*, 119664, doi:10.1016/j.biomaterials.2019.119664.
24. Chuah, Y.J.; Tan, J.R.; Wu, Y.; Lim, C.S.; Hee, H.T.; Kang, Y.; Wang, D.-A. Scaffold-Free tissue engineering with aligned bone marrow stromal cell sheets to recapitulate the microstructural and biochemical composition of annulus fibrosus. *Acta Biomater.* **2020**, *107*, 129–137, doi:10.1016/j.actbio.2020.02.031.

25. Ahmad, T.; Byun, H.; Lee, J.; Perikamana, S.K.M.; Shin, Y.M.; Kim, E.M.; Shin, H. Stem cell spheroids incorporating fibers coated with adenosine and polydopamine as a modular building blocks for bone tissue engineering. *Biomaterials* **2020**, *230*, 119652, doi:10.1016/j.biomaterials.2019.119652.
26. Kronemberger, G.S.; Matsui, R.A.M.; Miranda, G.D.A.S.D.C.E.; Granjeiro, J.M.; Baptista, L.S. Cartilage and bone tissue engineering using adipose stromal/stem cells spheroids as building blocks. *World J. Stem Cells* **2020**, *12*, 110–122, doi:10.4252/wjsc.v12.i2.110.
27. Toda, S.; Frankel, N.W.; Lim, W.A. Engineering cell–cell communication networks: Programming multicellular behaviors. *Curr. Opin. Chem. Biol.* **2019**, *52*, 31–38, doi:10.1016/j.cbpa.2019.04.020.
28. Amaral, A.J.; Pasparakis, G. Cell membrane engineering with synthetic materials: Applications in cell spheroids, cellular glues and microtissue formation. *Acta Biomater.* **2019**, *90*, 21–36, doi:10.1016/j.actbio.2019.04.013.
29. Qu, M.; Jiang, X.; Zhou, X.; Wang, C.; Wu, Q.; Ren, L.; Zhu, J.; Zhu, S.; Tebon, P.; Sun, W.; et al. Stimuli-Responsive Delivery of Growth Factors for Tissue Engineering. *Adv. Health Mater.* **2020**, *9*, e1901714, doi:10.1002/adhm.201901714.
30. Tang, J.D.; Mura, C.; Lampe, K.J. Stimuli-Responsive, Pentapeptide, Nanofiber Hydrogel for Tissue Engineering. *J. Am. Chem. Soc.* **2019**, *141*, 4886–4899, doi:10.1021/jacs.8b13363.
31. Fathi-Achacheloui, M.; Keskin, D.; Bat, E.; Vrana, N.E.; Tezcaner, A. Dual growth factor delivery using PLGA nanoparticles in silk fibroin/PEGDMA hydrogels for articular cartilage tissue engineering. *J. Biomed. Mater. Res. Part B: Appl. Biomater.* **2019**, *108*, 2041–2062, doi:10.1002/jbm.b.34544.
32. Groll, J.; Boland, T.; Blunk, T.; Burdick, J.A.; Kim, Y.K.; Dalton, P.D.; Derby, B.; Forgacs, G.; Li, Q.; Mironov, V.A.; et al. Biofabrication: Reappraising the definition of an evolving field. *Biofabrication* **2016**, *8*, 013001, doi:10.1088/1758-5090/8/1/013001.
33. Moroni, L.; Boland, T.; Burdick, J.A.; De Maria, C.; Derby, B.; Forgacs, G.; Groll, J.; Li, Q.; Malda, J.; Mironov, V.A.; et al. Biofabrication: A Guide to Technology and Terminology. *Trends Biotechnol.* **2018**, *36*, 384–402, doi:10.1016/j.tibtech.2017.10.015.
34. Divakar, D.D.; Jastaniyah, N.T.; Altamimi, H.G.; Alnakhli, Y.O.; Alkheraif, A.A.; Haleem, S. Enhanced antimicrobial activity of naturally derived bioactive molecule chitosan conjugated silver nanoparticle against dental implant pathogens. *Int. J. Biol. Macromol.* **2018**, *108*, 790–797, doi:10.1016/j.ijbiomac.2017.10.166.
35. Nguyen, M.A.; Camci-Unal, G. Unconventional Tissue Engineering Materials in Disguise. *Trends Biotechnol.* **2020**, *38*, 178–190, doi:10.1016/j.tibtech.2019.07.014.
36. Levato, R.; Jungst, T.; Scheuring, R.G.; Blunk, T.; Groll, J.; Malda, J. From Shape to Function: The Next Step in Bioprinting. *Adv. Mater.* **2020**, *32*, e1906423, doi:10.1002/adma.201906423.
37. Ghassemi, T.; Shahroodi, A.; Ebrahimzadeh, M.H.; Mousavian, A.; Movaffagh, J.; Moradi, A. Current Concepts in Scaffolding for Bone Tissue Engineering. *Arch. Bone Jt. Surg.* **2018**, *6*, 90–99.
38. Raquel Maia, F.; Correlo, V.M.; Oliveira, J.M.; Reis, R.L. Chapter 32—Natural Origin Materials for Bone Tissue Engineering: Properties, Processing, and Performance. In *Principles of Regenerative Medicine*, 3rd ed.; Atala, A., Lanza, R., Mikos, A.G., Nerem, R., Eds.; Academic Press: Boston, MA, USA, 2019; pp. 535–558.
39. Wang, W.; Yeung, K.W. Bone grafts and biomaterials substitutes for bone defect repair: A review. *Bioact. Mater.* **2017**, *2*, 224–247, doi:10.1016/j.bioactmat.2017.05.007.
40. Souza, A.T.P.; Lopes, H.B.; Freitas, G.P.; Ferraz, E.P.; Oliveira, F.S.; Almeida, A.L.G.; Weffort, D.; Beloti, M.M.; Rosa, A.L. Role of embryonic origin on osteogenic potential and bone repair capacity of rat calvarial osteoblasts. *J. Bone Miner. Metab.* **2020**, *38*, 481–490, doi:10.1007/s00774-020-01090-5.
41. Vasquez-Sancho, F.; Abdollahi, A.; Damjanovic, D.; Catalan, G. Flexoelectricity in Bones. *Adv. Mater.* **2018**, *30*, 1705316, doi:10.1002/adma.201705316.
42. Núñez-Toldrà, R.; Vasquez-Sancho, F.; Barroca, N.; Catalan, G. Investigation of The Cellular Response to Bone Fractures: Evidence for Flexoelectricity. *Sci. Rep.* **2020**, *10*, 1–10, doi:10.1038/s41598-019-57121-3.
43. Lin, C.-C.; Chang, W.H.-S.; Cheng, T.-M.; Chiu, L.-H.; Wang, Y.-H.; Lin, C.-A.J.; Ho, Y.-S.; Zuo, C.S.; Wang, Y.-M.; Lai, W.-F.T. Two new, near-infrared, fluorescent probes as potential tools for imaging bone repair. *Sci. Rep.* **2020**, *10*, 1–10, doi:10.1038/s41598-020-59522-1.
44. Saha, S.; Pramanik, K.; Biswas, A. Silk fibroin coated TiO2 nanotubes for improved osteogenic property of Ti6Al4V bone implants. *Mater. Sci. Eng. C* **2019**, *105*, 109982, doi:10.1016/j.msec.2019.109982.
45. Qin, T.; Li, X.; Long, H.; Bin, S.; Xu, Y. Bioactive Tetracalcium Phosphate Scaffolds Fabricated by Selective Laser Sintering for Bone Regeneration Applications. *Materials* **2020**, *13*, 2268, doi:10.3390/ma13102268.
46. Zhou, J.; Gao, C.; Feng, P.; Xiao, T.; Shuai, C.; Peng, S. Calcium sulfate bone scaffolds with controllable porous structure by selective laser sintering. *J. Porous Mater.* **2015**, *22*, 1171–1178, doi:10.1007/s10934-015-9993-x.
47. Shuai, C.; Gao, C.; Nie, Y.; Hu, H.; Zhou, Y.; Peng, S. Structure and properties of nano-hydroxypatite scaffolds for bone tissue engineering with a selective laser sintering system. *Nanotechnology* **2011**, *22*, 285703, doi:10.1088/0957-4484/22/28/285703.
48. Duan, B.; Wang, M.; Zhou, W.Y.; Cheung, W.L.; Li, Z.Y.; Lu, W.W. Three-dimensional nanocomposite scaffolds fabricated via selective laser sintering for bone tissue engineering. *Acta Biomater.* **2010**, *6*, 4495–4505, doi:10.1016/j.actbio.2010.06.024.
49. Simpson, R.L.; Wiria, F.E.; Amis, A.A.; Chua, C.K.; Leong, K.F.; Hansen, U.N.; Chandrasekaran, M.; Lee, M.W. Development of a 95/5 poly(L-lactide-co-glycolide)/hydroxylapatite and β-tricalcium phosphate scaffold as bone replacement material via selective laser sintering. *J. Biomed. Mater. Res. B* **2008**, *84B*, 17-25.
50. Cox, S.C.; Thornby, J.A.; Gibbons, G.J.; Williams, M.A.; Mallick, K.K. 3D printing of porous hydroxyapatite scaffolds intended for use in bone tissue engineering applications. *Mater. Sci. Eng. C* **2015**, *47*, 237–247, doi:10.1016/j.msec.2014.11.024.

51. Ahn, M.-K.; Moon, Y.-W.; Maeng, W.-Y.; Koh, Y.-H.; Kim, H.-E. Design and Production of Continuously Gradient Macro/Microporous Calcium Phosphate (CaP) Scaffolds Using Ceramic/Camphene-Based 3D Extrusion. *Materials* **2017**, *10*, 719, doi:10.3390/ma10070719.
52. Chow, L.C.; Takagi, S. A natural bone cement—A laboratory novelty led to the development of revolutionary new biomaterials. *J. Res. Natl. Inst. Stand. Technol.* **2001**, *106*, 1029–1033, doi:10.6028/jres.106.053.
53. Kretlow, J.D.; Mikos, A.G. Review: Mineralization of Synthetic Polymer Scaffolds for Bone Tissue Engineering. *Tissue Eng.* **2007**, *13*, 927–938, doi:10.1089/ten.2006.0394.
54. Rho, J.; Kuhn-Spearing, L.; Zioupos, P. Mechanical properties and the hierarchical structure of bone. *Med Eng. Phys.* **1998**, *20*, 92–102, doi:10.1016/s1350-4533(98)00007-1.
55. Currey, J.; Butler, G. The mechanical properties of bone tissue in children. *JBJS* **1975**, *57*, 810–814, doi:10.2106/00004623-197557060-00015.
56. Vogel, H.G. Influence of Maturation and Aging on Mechanical and Biochemical Parameters of Rat Bone. *Gerontology* **1979**, *25*, 16–23, doi:10.1159/000212316.
57. Dunlop, J.W.C.; Fratzl, P. Biological Composites. In *Annual Review of Materials Research*; Clarke, D.R., Ruhle, M.; Zok, F., Eds.; Annual Reviews: Palo Alto, CA, USA, 2010; Volume 40, pp. 1–24.
58. Hardy, J.G.; Torres-Rendon, J.G.; Leal-Egaña, A.; Walther, A.; Schlaad, H.; Cölfen, H.; Scheibel, T. Biomineralization of Engineered Spider Silk Protein-Based Composite Materials for Bone Tissue Engineering. *Materials* **2016**, *9*, 560, doi:10.3390/ma9070560.
59. Lee, J.Y.; Choo, J.E.; Choi, Y.S.; Park, J.B.; Min, D.S.; Lee, S.J.; Rhyu, H.K.; Jo, I.H.; Chung, C.P.; Park, Y.J. Assembly of collagen-binding peptide with collagen as a bioactive scaffold for osteogenesis in vitro and in vivo. *Biomaterials* **2007**, *28*, 4257–4267, doi:10.1016/j.biomaterials.2007.05.040.
60. Dinjaski, N.; Plowright, R.; Zhou, S.; Belton, D.J.; Perry, C.C.; Kaplan, D.L. Osteoinductive recombinant silk fusion proteins for bone regeneration. *Acta Biomater.* **2017**, *49*, 127–139, doi:10.1016/j.actbio.2016.12.002.
61. Kokubo, T.; Takadama, H. How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* **2006**, *27*, 2907–2915, doi:10.1016/j.biomaterials.2006.01.017.
62. Giachelli, C.M.; Steitz, S. Osteopontin: A versatile regulator of inflammation and biomineralization. *Matrix Biol.* **2000**, *19*, 615–622, doi:10.1016/s0945-053x(00)00108-6.
63. Jin, R.; Wang, Y.; Liu, Y.; Ding, C.; Xie, J.; Li, J. Biomineralization and osteogenic differentiation modulated by substrate stiffness. *Eur. Polym. J.* **2020**, *122*, 109395, doi:10.1016/j.eurpolymj.2019.109395.
64. Midha, S.; Kumar, S.; Sharma, A.; Kaur, K.; Shi, X.; Naruphontjirakul, P.; Jones, J.R.; Ghosh, S. Silk fibroin-bioactive glass based advanced biomaterials: Towards patient-specific bone grafts. *Biomed. Mater.* **2018**, *13*, 055012, doi:10.1088/1748-605x/aad2a9.
65. Hench, L.L. The story of Bioglass®. *J. Mater. Sci. Mater. Med.* **2006**, *17*, 967–978.
66. Mertgen, A.-S.; Trossmann, V.T.; Guex, A.G.; Maniura-Weber, K.; Scheibel, T.; Rottmar, M. Multifunctional Biomaterials: Combining Material Modification Strategies for Engineering of Cell-Contacting Surfaces. *ACS Appl. Mater. Interfaces* **2020**, *12*, 21342–21367, doi:10.1021/acsami.0c01893.
67. Le Saux, G.; Magenau, A.; Böcking, T.; Gaus, K.; Gooding, J.J. The Relative Importance of Topography and RGD Ligand Density for Endothelial Cell Adhesion. *PLoS ONE* **2011**, *6*, e21869, doi:10.1371/journal.pone.0021869.
68. Wohlrab, S.; Müller, S.; Schmidt, A.; Neubauer, S.; Kessler, H.; Leal-Egaña, A.; Scheibel, T. Cell adhesion and proliferation on RGD-modified recombinant spider silk proteins. *Biomaterials* **2012**, *33*, 6650–6659, doi:10.1016/j.biomaterials.2012.05.069.
69. Bessa, P.C.; Balmayor, E.R.; Azevedo, H.S.; Nürnberger, S.; Casal, M.; Van Griensven, M.; Reis, R.L.; Redl, H. Silk fibroin microparticles as carriers for delivery of human recombinant BMPs. Physical characterization and drug release. *J. Tissue Eng. Regen. Med.* **2010**, *4*, 349–355, doi:10.1002/term.245.
70. Bessa, P.C.; Casal, M.; Reis, R.L. Bone morphogenetic proteins in tissue engineering: The road from laboratory to clinic, part II (BMP delivery). *J. Tissue Eng. Regen. Med.* **2008**, *2*, 81–96, doi:10.1002/term.74.
71. Gomes, S.; Gallego-Llamas, J.; Leonor, I.B.; Mano, J.F.; Reis, R.L.; Kaplan, D.L. In Vivo Biological Responses to Silk Proteins Functionalized with Bone Sialoprotein. *Macromol. Biosci.* **2013**, *13*, 444–454, doi:10.1002/mabi.201200372.
72. Neubauer, V.J.; Scheibel, T. Spider Silk Fusion Proteins for Controlled Collagen Binding and Biomineralization. *ACS Biomater. Sci. Eng.* **2020**, *6*, 5599–5608, doi:10.1021/acsbiomaterials.0c00818.
73. Yousaf, S.S.; Houacine, C.; Khan, I.; Ahmed, W.; Jackson, M.J. Chapter 11—Importance of biomaterials in biomedical engineering. In *Advances in Medical and Surgical Engineering*; Ahmed, W., Phoenix, D.A., Jackson, M.J., Charalambous, C.P., Eds.; Academic Press: Cambridge, MA, USA, 2020; pp. 149–176.
74. Craig, C.L. Evolution of Arthopods Silks. *Annu. Rev. Entomol.* **1997**, *42*, 231–267.
75. Andersson, M.; Johansson, J.; Rising, A. Silk Spinning in Silkworms and Spiders. *Int. J. Mol. Sci.* **2016**, *17*, 1290, doi:10.3390/ijms17081290.
76. Hardy, J.G.; Scheibel, T.R. Production and processing of spider silk proteins. *J. Polym. Sci. Part A: Polym. Chem.* **2009**, *47*, 3957–3963, doi:10.1002/pola.23484.
77. Eisoldt, L.; Smith, A.; Scheibel, T. Decoding the secrets of spider silk. *Mater. Today* **2011**, *14*, 80–86, doi:10.1016/s1369-7021(11)70057-8.
78. Salehi, S.; Koeck, K.; Scheibel, T. Spider Silk for Tissue Engineering Applications. *Molecules* **2020**, *25*, 737, doi:10.3390/molecules25030737.

79. Pollini, M.; Paladini, F. Bioinspired Materials for Wound Healing Application: The Potential of Silk Fibroin. *Materials* **2020**, *13*, 3361, doi:10.3390/ma13153361.
80. Melke, J.J.; Midha, S.; Ghosh, S.; Ito, K.; Hofmann, S. Silk fibroin as biomaterial for bone tissue engineering. *Acta Biomater.* **2016**, *31*, 1–16, doi:10.1016/j.actbio.2015.09.005.
81. Umuhoza, D.; Yang, F.; Long, D.; Hao, Z.; Dai, J.; Zhao, A. Strategies for Tuning the Biodegradation of Silk Fibroin-Based Materials for Tissue Engineering Applications. *ACS Biomater. Sci. Eng.* **2020**, *6*, 1290–1310, doi:10.1021/acsbiomaterials.9b01781.
82. Nagano, A.; Tanioka, Y.; Sakurai, N.; Sezutsu, H.; Kuboyama, N.; Kiba, H.; Tanimoto, Y.; Ma, S.; Asakura, T. Regeneration of the femoral epicondyle on calcium-binding silk scaffolds developed using transgenic silk fibroin produced by transgenic silkworm. *Acta Biomater.* **2011**, *7*, 1192–1201, doi:10.1016/j.actbio.2010.10.032.
83. Saotome, T.; Hayashi, H.; Tanaka, R.; Kinugasa, A.; Uesugi, S.; Tatematsu, K.-I.; Sezutsu, H.; Kuwabara, N.; Asakura, T. Introduction of VEGF or RGD sequences improves revascularization properties of Bombyx mori silk fibroin produced by transgenic silkworm. *J. Mater. Chem. B* **2015**, *3*, 7109–7116, doi:10.1039/c5tb00939a.
84. Gomes, S.C.; Leonor, I.B.; Mano, J.F.; Reis, R.; Kaplan, D.L. Natural and genetically engineered proteins for tissue engineering. *Prog. Polym. Sci.* **2012**, *37*, 1–17, doi:10.1016/j.progpolymsci.2011.07.003.
85. Huang, J.; Wong, C.; George, A.; Kaplan, D.L. The effect of genetically engineered spider silk-dentin matrix protein 1 chimeric protein on hydroxyapatite nucleation. *Biomaterials* **2007**, *28*, 2358–2367, doi:10.1016/j.biomaterials.2006.11.021.
86. Mo, C.; Wu, P.; Chen, X.; Zheng, G. The effect of water on the conformation transition of Bombyx mori silk fibroin. *Vib. Spectrosc.* **2009**, *51*, 105–109, doi:10.1016/j.vibspec.2008.11.004.
87. Arai, T.; Freddi, G.; Innocenti, R.; Tsukada, M. Biodegradation ofBombyx mori silk fibroin fibers and films. *J. Appl. Polym. Sci.* **2003**, *91*, 2383–2390, doi:10.1002/app.13393.
88. Tucker, C.L.; Jones, J.A.; Bringhurst, H.N.; Copeland, C.G.; Addison, J.B.; Weber, W.S.; Mou, Q.; Yarger, J.L.; Lewis, R.V. Mechanical and Physical Properties of Recombinant Spider Silk Films Using Organic and Aqueous Solvents. *Biomacromolecules* **2014**, *15*, 3158–3170, doi:10.1021/bm5007823.
89. Spieß, K.; Wohlrab, S.; Scheibel, T. Structural characterization and functionalization of engineered spider silk films. *Soft Matter* **2010**, *6*, 4168–4174, doi:10.1039/b927267d.
90. Ohgo, K.; Zhao, C.; Kobayashi, M.; Asakura, T. Preparation of non-woven nanofibers of Bombyx mori silk, Samia cynthia ricini silk and recombinant hybrid silk with electrospinning method. *Polymer* **2003**, *44*, 841–846, doi:10.1016/s0032-3861(02)00819-4.
91. Sukigara, S.; Gandhi, M.; Ayutsede, J.; Micklus, M.; Ko, F. Regeneration of Bombyx mori silk by electrospinning—part 1: Processing parameters and geometric properties. *Polymer* **2003**, *44*, 5721–5727, doi:10.1016/s0032-3861(03)00532-9.
92. Keirouz, A.; Fortunato, G.; Zhang, M.; Callanan, A.; Radacsi, N. Nozzle-free electrospinning of Polyvinylpyrrolidone/Poly(glycerol sebacate) fibrous scaffolds for skin tissue engineering applications. *Med. Eng. Phys.* **2019**, *71*, 56–67, doi:10.1016/j.medengphy.2019.06.009.
93. DeSimone, E.; Aigner, T.B.; Humenik, M.; Lang, G.; Scheibel, T. Aqueous electrospinning of recombinant spider silk proteins. *Mater. Sci. Eng. C* **2020**, *106*, 110145, doi:10.1016/j.msec.2019.110145.
94. Keirouz, A.; Zakharova, M.; Kwon, J.; Robert, C.; Koutsos, V.; Callanan, A.; Chen, X.; Fortunato, G.; Radacsi, N. High-throughput production of silk fibroin-based electrospun fibers as biomaterial for skin tissue engineering applications. *Mater. Sci. Eng. C* **2020**, *112*, 110939, doi:10.1016/j.msec.2020.110939.
95. Lammel, A.; Schwab, M.; Slotta, U.; Winter, G.; Scheibel, T. Processing Conditions for the Formation of Spider Silk Microspheres. *ChemSusChem* **2008**, *1*, 413–416, doi:10.1002/cssc.200800030.
96. Jastrzebska, K.; Felcyn, E.; Kozak, M.; Szybowicz, M.; Buchwald, T.; Pietralik, Z.; Jesionowski, T.; Mackiewicz, A.; Dams-Kozlowska, H. The method of purifying bioengineered spider silk determines the silk sphere properties. *Sci. Rep.* **2016**, *6*, 28106, doi:10.1038/srep28106.
97. Rajkhowa, R.; Hu, X.; Tsuzuki, T.; Kaplan, D.L.; Wang, X. Structure and Biodegradation Mechanism of Milled Bombyx mori Silk Particles. *Biomacromolecules* **2012**, *13*, 2503–2512, doi:10.1021/bm300736m.
98. Cao, Z.; Chen, X.; Yao, J.; Huang, L.; Shao, Z. The preparation of regenerated silk fibroin microspheres. *Soft Matter.* **2007**, *3*, 910–915, doi:10.1039/b703139d.
99. Herold, H.M.; Döbl, A.; Wohlrab, S.; Humenik, M.; Scheibel, T. Designed Spider Silk-Based Drug Carrier for Redox- or pH-Triggered Drug Release. *Biomacromolecules* **2020**, *21*, 4904–4912, doi:10.1021/acs.biomac.0c01138.
100. Maniglio, D.; Bonani, W.; Migliaresi, C.; Motta, A. Silk fibroin porous scaffolds by N2O foaming. *J. Biomater. Sci. Polym. Ed.* **2018**, *29*, 491–506, doi:10.1080/09205063.2018.1423811.
101. Lv, Q.; Feng, Q. Preparation of 3-D regenerated fibroin scaffolds with freeze drying method and freeze drying/foaming technique. *J. Mater. Sci. Mater. Electron.* **2006**, *17*, 1349–1356, doi:10.1007/s10856-006-0610-z.
102. Schacht, K.; Vogt, J.; Scheibel, T. Foams Made of Engineered Recombinant Spider Silk Proteins as 3D Scaffolds for Cell Growth. *ACS Biomater. Sci. Eng.* **2016**, *2*, 517–525, doi:10.1021/acsbiomaterials.5b00483.
103. Hoffman, A.S. Hydrogels for biomedical applications. *Adv. Drug Deliv. Rev.* **2012**, *64*, 18–23, doi:10.1016/j.addr.2012.09.010.
104. Schacht, K.; Jungst, T.; Schweinlin, M.; Ewald, A.; Groll, J.; Scheibel, T. Biofabrication of Cell-Loaded 3D Spider Silk Constructs. *Angew. Chem. Int. Ed.* **2015**, *54*, 2816–2820, doi:10.1002/anie.201409846.
105. Chameettachal, S.; Midha, S.; Ghosh, S. Regulation of Chondrogenesis and Hypertrophy in Silk Fibroin-Gelatin-Based 3D Bioprinted Constructs. *ACS Biomater. Sci. Eng.* **2016**, *2*, 1450–1463, doi:10.1021/acsbiomaterials.6b00152.

106. Wang, Y.; Zheng, Z.; Cheng, Q.; Kaplan, D.L.; Li, G.; Wang, X. Ductility and Porosity of Silk Fibroin Films by Blending with Glycerol/Polyethylene Glycol and Adjusting the Drying Temperature. *ACS Biomater. Sci. Eng.* **2019**, *6*, 1176–1185, doi:10.1021/acsbiomaterials.9b01567.
107. Sartika, D.; Hsin, W.C.; Wang, D.-H.; Cherng, J.-H.; Chang, S.-J.; Fan, G.-Y.; Wang, Y.-W.; Lee, C.-H.; Hong, P.-D.; Wang, C. Human Adipose-Derived Mesenchymal Stem Cells-Incorporated Silk Fibroin as a Potential Bio-Scaffold in Guiding Bone Regeneration. *Polymers* **2020**, *12*, 853, doi:10.3390/polym12040853.
108. Dorishetty, P.; Balu, R.; Athukoralalage, S.S.; Greaves, T.L.; Mata, J.; De Campo, L.; Saha, N.; Zannettino, A.C.W.; Dutta, N.K.; Choudhury, N.R. Tunable Biomimetic Hydrogels from Silk Fibroin and Nanocellulose. *ACS Sustain. Chem. Eng.* **2020**, *8*, 2375–2389, doi:10.1021/acssuschemeng.9b05317.
109. Qian, S.; Wang, Z.; Zheng, Z.; Ran, J.; Zhu, J.; Chen, W. A Collagen and Silk Scaffold for Improved Healing of the Tendon and Bone Interface in a Rabbit Model. *Med Sci. Monit.* **2019**, *25*, 269–278, doi:10.12659/msm.912038.
110. Dellaquila, A.; Greco, G.; Campodoni, E.; Mazzocchi, M.; Mazzolai, B.; Tampieri, A.; Pugno, N.M.; Sandri, M. Optimized production of a high-performance hybrid biomaterial: Biomineralized spider silk for bone tissue engineering. *J. Appl. Polym. Sci.* **2019**, *137*, 48739, doi:10.1002/app.48739.
111. Zafar, B.; Mottaghitalab, F.; Shahosseini, Z.; Negahdari, B.; Farokhi, M. Silk fibroin/alumina nanoparticle scaffold using for osteogenic differentiation of rabbit adipose-derived stem cells. *Materials* **2020**, *9*, 100518, doi:10.1016/j.mtla.2019.100518.
112. Liu, F.; Liu, C.; Zheng, B.; He, J.; Liu, J.; Chen, C.; Lee, I.-S.; Wang, X.; Liu, Y. Synergistic Effects on Incorporation of β-Tricalcium Phosphate and Graphene Oxide Nanoparticles to Silk Fibroin/Soy Protein Isolate Scaffolds for Bone Tissue Engineering. *Polymer* **2020**, *12*, 69, doi:10.3390/polym12010069.
113. Wang, L.; Fang, M.; Xia, Y.; Hou, J.; Nan, X.; Zhao, B.; Wang, X. Preparation and biological properties of silk fibroin/nano-hydroxyapatite/graphene oxide scaffolds with an oriented channel-like structure. *RSC Adv.* **2020**, *10*, 10118–10128, doi:10.1039/c9ra09710d.
114. Ribeiro, V.P.; Pina, S.; Costa, J.B.; Cengiz, I.F.; García-Fernández, L.; Fernández-Gutiérrez, M. d. M.; Paiva, O.C.; Oliveira, A.L.; San-Román, J.; Oliveira, J.M.; et al. Enzymatically Cross-Linked Silk Fibroin-Based Hierarchical Scaffolds for Osteochondral Regeneration. *ACS Appl. Mater. Interfaces* **2019**, *11*, 3781–3799.
115. Collado-González, M.; Pecci-Lloret, M.P.; García-Bernal, D.; Aznar-Cervantes, S.D.; Oñate-Sánchez, R.E.; Moraleda, J.M.; Cenis, J.L.; Rodríguez-Lozano, F.J. Biological effects of silk fibroin 3D scaffolds on stem cells from human exfoliated deciduous teeth (SHEDs). *Odontology* **2018**, *106*, 125–134, doi:10.1007/s10266-017-0310-9.
116. Wang, Q.-S.; Han, G.-C.; Yan, S.-Q.; You, R.-C.; Zhang, Q. Facile Preparation of Silk Fibroin Scaffold Via Direct Solvent Exchange. *J. Fiber Bioeng. Inform.* **2020**, *13*, 13–21.
117. Siavashani, A.Z.; Mohammadi, J.; Rottmar, M.; Senturk, B.; Nourmohammadi, J.; Sadeghi, B.; Huber, L.; Maniura-Weber, K. Silk fibroin/sericin 3D sponges: The effect of sericin on structural and biological properties of fibroin. *Int. J. Biol. Macromol.* **2020**, *153*, 317–326, doi:10.1016/j.ijbiomac.2020.02.316.
118. Xingtong, L.; Zhentao, W.; Tingting, D.; Hongbo, G.; Juan, G. Hydrogels from silk fibroin and multiarmed hydrolyzed elastin peptide. *Surf. Innov.* **2020**, *8*, 216–223.
119. Wu, N.; Yu, H.; Sun, M.; Li, Z.; Zhao, F.; Ao, Y.; Chen, H. Investigation on the Structure and Mechanical Properties of Highly Tunable Elastomeric Silk Fibroin Hydrogels Cross-Linked by γ-Ray Radiation. *ACS Appl. Biomater.* **2019**, *3*, 721–734, doi:10.1021/acsabm.9b01062.
120. Laomeephol, C.; Guedes, M.; Ferreira, H.; Reis, R.L.; Kanokpanont, S.; Laomeephol, C.; Neves, N.M. Phospholipid-induced silk fibroin hydrogels and their potential as cell carriers for tissue regeneration. *J. Tissue Eng. Regen. Med.* **2020**, *14*, 160–172, doi:10.1002/term.2982.
121. Nguyen, T.T.; Ratanavaraporn, J.; Yodmuang, S. Alginate-silk fibroin Bioink: A printable hydrogel for tissue engineering. In Proceedings of the 2019 12th Biomedical Engineering International Conference (BMEiCON), Ubon Ratchathani, Thailand, 19–22 November 2019; pp. 1–4.
122. Sharma, A.; Desando, G.; Petretta, M.; Chawla, S.; Bartolotti, I.; Manferdini, C.; Paolella, F.; Gabusi, E.; Trucco, D.; Ghosh, S.; et al. Investigating the Role of Sustained Calcium Release in Silk-Gelatin-Based Three-Dimensional Bioprinted Constructs for Enhancing the Osteogenic Differentiation of Human Bone Marrow Derived Mesenchymal Stromal Cells. *ACS Biomater. Sci. Eng.* **2019**, *5*, 1518–1533, doi:10.1021/acsbiomaterials.8b01631.
123. Huemmerich, D.; Helsen, C.W.; Quedzuweit, S.; Oschmann, J.; Rudolph, A.R.; Scheibel, T. Primary Structure Elements of Spider Dragline Silks and Their Contribution to Protein Solubility†. *Biochemistry* **2004**, *43*, 13604–13612, doi:10.1021/bi048983q.
124. Schacht, K.; Scheibel, T. Controlled Hydrogel Formation of a Recombinant Spider Silk Protein. *Biomacromolecules* **2011**, *12*, 2488–2495, doi:10.1021/bm200154k.
125. DeSimone, E.; Schacht, K.; Scheibel, T. Cations influence the cross-linking of hydrogels made of recombinant, polyanionic spider silk proteins. *Mater. Lett.* **2016**, *183*, 101–104, doi:10.1016/j.matlet.2016.07.044.
126. DeSimone, E.; Schacht, K.; Jungst, T.; Groll, J.; Scheibel, T. Biofabrication of 3D constructs: Fabrication technologies and spider silk proteins as bioinks. *Pure Appl. Chem.* **2015**, *87*, 737–749, doi:10.1515/pac-2015-0106.
127. DeSimone, E.; Schacht, K.; Pellert, A.; Scheibel, T. Recombinant spider silk-based bioinks. *Biofabrication* **2017**, *9*, 044104, doi:10.1088/1758-5090/aa90db.
128. Thamm, C.; DeSimone, E.; Scheibel, T. Characterization of Hydrogels Made of a Novel Spider Silk Protein eMaSp1s and Evaluation for 3D Printing. *Macromol. Biosci.* **2017**, *17*, doi:10.1002/mabi.201700141.

129. Kumari, S.; Bargel, H.; Scheibel, T. Recombinant Spider Silk-Silica Hybrid Scaffolds with Drug-Releasing Properties for Tissue Engineering Applications. *Macromol. Rapid Commun.* **2019**, *41*, e1900426, doi:10.1002/marc.201900426.
130. Kumari, S.; Bargel, H.; Anby, M.U.; LaFargue, D.; Scheibel, T.; LaFargue, D. Recombinant Spider Silk Hydrogels for Sustained Release of Biologicals. *ACS Biomater. Sci. Eng.* **2018**, *4*, 1750–1759, doi:10.1021/acsbiomaterials.8b00382.
131. Ding, Z.; Lu, G.; Cheng, W.; Xu, G.; Zuo, B.; Lu, Q.; Kaplan, D.L. Tough Anisotropic Silk Nanofiber Hydrogels with Osteoinductive Capacity. *ACS Biomater. Sci. Eng.* **2020**, *6*, 2357–2367, doi:10.1021/acsbiomaterials.0c00143.
132. Xu, G.; Ding, Z.; Lu, Q.; Zhang, X.; Zhou, X.; Xiao, L.; Lv, G.; Kaplan, D.L. Electric field-driven building blocks for introducing multiple gradients to hydrogels. *Protein Cell* **2020**, *11*, 267–285, doi:10.1007/s13238-020-00692-z.
133. Liu, J.; Chen, H.; Wang, Y.; Li, G.; Zheng, Z.; Kaplan, D.L.; Wang, X. Flexible Water-Absorbing Silk-Fibroin Biomaterial Sponges with Unique Pore Structure for Tissue Engineering. *ACS Biomater. Sci. Eng.* **2020**, *6*, 1641–1649, doi:10.1021/acsbiomaterials.9b01721.
134. Luetchford, K.A.; Chaudhuri, J.B.; De Bank, P.A. Silk fibroin/gelatin microcarriers as scaffolds for bone tissue engineering. *Mater. Sci. Eng. C* **2020**, *106*, 110116, doi:10.1016/j.msec.2019.110116.
135. Li, Z.; Chu, D.; Gao, Y.; Jin, L.; Zhang, X.; Cui, W.; Li, J. Biomimicry, biomineralization, and bioregeneration of bone using advanced three-dimensional fibrous hydroxyapatite scaffold. *Mater. Today Adv.* **2019**, *3*, 100014, doi:10.1016/j.mtadv.2019.100014.
136. Zhang, W.; Zheng, K.; Ren, J.; Fan, Y.; Ling, S. Strong, ductile and lightweight bionanocomposites constructed by bioinspired hierarchical assembly. *Compos. Commun.* **2020**, *17*, 97–103, doi:10.1016/j.coco.2019.11.017.
137. Griffanti, G.; Jiang, W.; Nazhat, S.N. Bioinspired mineralization of a functionalized injectable dense collagen hydrogel through silk sericin incorporation. *Biomater. Sci.* **2019**, *7*, 1064–1077, doi:10.1039/c8bm01060a.
138. Johari, N.; Hosseini, H.R.M.; Samadikuchaksaraei, A. Mechanical modeling of silk fibroin/TiO_2 and silk fibroin/fluoridated TiO2 nanocomposite scaffolds for bone tissue engineering. *Iran. Polym. J.* **2020**, *29*, 219–224, doi:10.1007/s13726-020-00789-6.
139. Xing, X.; Cheng, G.; Yin, C.; Cheng, X.; Cheng, Y.; Ni, Y.; Zhou, X.; Deng, H.; Li, Z. Magnesium-containing silk fibroin/polycaprolactone electrospun nanofibrous scaffolds for accelerating bone regeneration. *Arab. J. Chem.* **2020**, *13*, 5526–5538, doi:10.1016/j.arabjc.2020.03.031.
140. Tanasa, E.; Zaharia, C.; Hudita, A.; Radu, I.-C.; Costache, M.; Galateanu, B. Impact of the magnetic field on 3T3-E1 preosteoblasts inside SMART silk fibroin-based scaffolds decorated with magnetic nanoparticles. *Mater. Sci. Eng. C* **2020**, *110*, 110714, doi:10.1016/j.msec.2020.110714.
141. Narimani, M.; Teimouri, A.; Shahbazarab, Z. Synthesis, characterization and biocompatible properties of novel silk fibroin/graphene oxide nanocomposite scaffolds for bone tissue engineering application. *Polym. Bull.* **2019**, *76*, 725–745, doi:10.1007/s00289-018-2390-2.
142. Nie, L.; Zhang, H.; Ren, A.; Li, Y.; Fu, G.; Cannon, R.D.; Ji, P.; Wu, X.-H.; Yang, S. Nano-hydroxyapatite mineralized silk fibroin porous scaffold for tooth extraction site preservation. *Dent. Mater.* **2019**, *35*, 1397–1407, doi:10.1016/j.dental.2019.07.024.
143. Wang, K.; Ma, Q.; Zhang, Y.; Han, G.-T.; Qu, C.-X.; Wang, S. Preparation of bacterial cellulose/silk fibroin double-network hydrogel with high mechanical strength and biocompatibility for artificial cartilage. *Cellulose* **2020**, *27*, 1845–1852, doi:10.1007/s10570-019-02869-0.
144. Ding, X.; Zhu, M.; Xu, B.; Zhang, J.; Zhao, Y.; Ji, S.; Wang, L.; Wang, L.; Li, X.; Kong, D.; et al. Integrated Trilayered Silk Fibroin Scaffold for Osteochondral Differentiation of Adipose-Derived Stem Cells. *ACS Appl. Mater. Interfaces* **2014**, *6*, 16696–16705, doi:10.1021/am5036708.
145. Zhao, Y.; Ding, X.; Dong, Y.; Sun, X.; Wang, L.; Ma, X.; Zhu, M.; Xu, B.; Yang, Q. Role of the Calcified Cartilage Layer of an Integrated Trilayered Silk Fibroin Scaffold Used to Regenerate Osteochondral Defects in Rabbit Knees. *ACS Biomater. Sci. Eng.* **2019**, *6*, 1208–1216, doi:10.1021/acsbiomaterials.9b01661.
146. Bradner, S.A.; McGill, M.; Golding, A.; Grudt, R.; Kaplan, D.L. Silk Hydrogel Microfibers for Biomimetic Fibrous Material Design. *Macromol. Mater. Eng.* **2019**, *304*, 1900045, doi:10.1002/mame.201900045.
147. The Business Research Company. *Orthopedic Biomaterials Market Global Report 2020-30: Covid 19 Growth and Challenges*; The Business Research Company: London, UK, 2020. Available online: https://www.thebusinessresearchcompany.com/report/orthopedic-biomaterials-market-global-report-2020-30-covid-19-growth-and-change (accessed on 31 January 2021).
148. Perrone, G.S.; Leisk, G.G.; Lo, T.J.; Moreau, J.E.; Haas, D.S.; Papenburg, B.J.; Golden, E.B.; Partlow, B.P.; Fox, S.E.; Ibrahim, A.M.S.; et al. The use of silk-based devices for fracture fixation. *Nat. Commun.* **2014**, *5*, 3385, doi:10.1038/ncomms4385.
149. Li, C.; Guo, C.; Fitzpatrick, V.; Ibrahim, A.; Zwierstra, M.J.; Hanna, P.; Lechtig, A.; Nazarian, A.; Lin, S.J.; Kaplan, D. Design of biodegradable, implantable devices towards clinical translation. *Nat. Rev. Mater.* **2019**, *5*, 61–81, doi:10.1038/s41578-019-0150-z.

7.3 Teilarbeit III

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Spider Silk Fusion Proteins for Controlled Collagen Binding and Biomineralization

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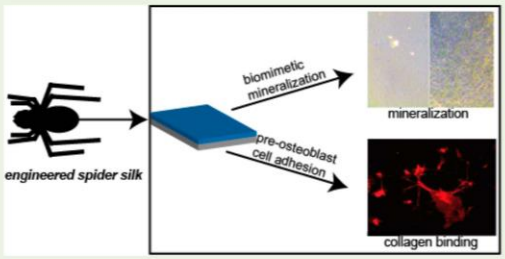
ABSTRACT: The development of biomaterials for the interface between tendon and bone is important for realizing functional tendon replacements. Toward the development of new materials for such applications, engineered recombinant spider silk proteins were modified with peptide tag sequences derived from noncollagenous proteins in bone, so-called SIBLING proteins, such as osteopontin and sialoprotein, which are known to interact with collagen and to initiate mineralization. Materials made of these spider silk−SIBLING hybrids were analyzed concerning mineralization and interaction with cells. They showed enhanced calcium phosphate formation upon incubation in mineralization agents. In gradient films, MC3T3-E1 mouse preosteoblasts adhered preferentially along the gradient toward the variant with a collagen binding motif.



KEYWORDS: *recombinant spider silk, calcium phosphate, biomineralization, osteopontin, sialoprotein, gradient materials*

1. INTRODUCTION

The tendon-to-bone interface is one example where a high control over mineralization processes of (collagen-based) biopolymeric materials is required, because in a steep gradient toward the bone, hydroxyapatite mineralization, collagen fibril disorientation, and Young's modulus increase significantly.[1,2] Initial nucleation of biogenic minerals and complex formation with collagen fibrils is mediated by noncollagenous proteins in bone, which exhibit the ability of mineralization together with collagen binding to build a composite material with high strength.[3] This tendon interface region is referred to as enthesis, which hinders crack propagation while uniformly transmitting high loads when muscles are contracted and pull on the tendon.[1,2,4] Once ruptured, tendons heal badly because they are populated with only few cells or vasculature which limits the ability to recover the tissue, and implants need to fit the mechanical requirements of the native material.[1] In nature, gradual integration of tendon into bone is controlled by noncollagenous proteins, such as osteopontin and sialoprotein, which were found to influence gradient mineralization and collagen binding at the enthesis.[5]

Biomineralization, in general, is a process of mineral formation by cellular metabolism yielding, for example, support structures in vertebrates or protective outer shells in mussels or diatoms.[6,7] Besides the high percentage of inorganic material of mainly carbonated hydroxyapatite platelets (dahllite, 60−70% w/w) in mineralized human tissues,[3] proteins serve as flexible fillers but also provide anchoring points for tissue-specific cells because of the exposure of recognition motifs.[8] Although collagen type I fibrils are the main organic component with up to 90% w/w in human bone, they are not found to initiate mineralization.[3] Noncollagenous proteins, which comprise the remaining 10% w/w,[3] like the small integrin binding ligand N-linked glycoprotein (SIBLING) family, exhibit on the one hand peptide motifs which can bind to collagen fibrils while others trigger mineralization in bone by exposing highly, mainly negatively charged peptide sequences, rich in glutamic and aspartic acid.[3] Besides complex glycosylation patterns,[3] the charged sequence motifs were found to specifically attract ions from the surrounding media such as blood or intestinal fluid, initiating mineralization and yielding a composite matrix with collagen.[9] During the process of new bone formation, after the recruitment of preosteoblasts, SIBLING proteins were found to be produced in highly concerted patterns switching mineralization "on and off" to prevent putative pathogenic overmineralization.[10,11] Upon ongoing mineralization and densifying the surrounding matrix, osteoblasts lose the easy access to nutrients and oxygen and differentiate into osteocytes only passively supplied by diffusion.[6]

Besides charge effects, the orientation of the SIBLING proteins and their conformation, immobilized or in solution, have been shown to influence crystal nucleation.[3] The exposition of acidic motifs was found to play a crucial role

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therein, and their stereochemistry can alter the facing direction of the crystal on the protein template.[12] To date, several short mineralization motifs[13] derived from native proteins have been examined in regard to their hydroxyapatite binding ability[14] or mineralization in calcium and phosphate containing solutions.[15] Further, peptide sequences were identified via phage display providing hydroxyapatite binding affinity and performing mineralization ability.[16−18]

In some previous set-ups, silk derivatives were connected to mineralization peptides.[19−21] An amphipathic protein comprising the main sequence of *Bombyx mori* silk fibroin with an 8-fold N-terminal repeat of glutamic acid was recombinantly produced or alternatively via solid phase synthesis to study calcium binding affinities.[21] Further, recombinant spider silk proteins were designed based on the *Nephila clavipes* repetitive dragline silk sequence and fused with hydroxyapatite binding domains identified using phage display, at the C- and N-terminus of the protein for *in vitro* bone formation.[19] Further, a peptide motif from bone sialoprotein was fused with a hexamer of the engineered *Nephila clavipes* sequence to investigate the *in vivo* biocompatibility for bone regeneration applications in mice.[20] In other approaches, the influence of noncollagenous protein analogues on collagen fibril and mineral interaction was studied: Collagen mineralization was allowed at simultaneous collagen fibril assembly and calcium phosphate precipitation from solution, yielding higher interaction between the formed apatite-phase and collagen fibrils in the presence of polyaspartate as noncollagenous analogue.[22] Further, synthetic interaction supporters and mineralization templates as substitutes for noncollagenous proteins were found in brushlike polymers rich in carboxyl moieties, which can similarly trigger intrafibrillar collagen calcium phosphate mineralization.[23]

In one of our previous studies, composite films from synthetic polymers and an engineered spider silk protein were investigated regarding their biocompatibility and application potential in bone tissue engineering upon calcium phosphate biomineralization.[24] Herein, we report the design of fusions of engineered spider silk proteins with biomimetic mineralization and collagen binding peptide sequences from native osteopontin and sialoprotein SIBLING proteins. Films made thereof were tested concerning mineralization and cell binding.

2. MATERIALS AND METHODS

2.1. Molecular Cloning and Protein Preparation. The engineered spider silk protein eADF4(C16) is based on the consensus gene sequence of the MaSp 2 dragline silk fibroin 4 of the European garden spider *Araneus diadematus*. The consensus C-module (GSSAAAAAAAASGPGGYGPENQGPSGPGGYGPGGP) is repeated 16 times and N-terminally fused with a T7-tag for detection purposes. After codon optimization of the desired amino acid tag sequences, two single-stranded oligo-nucleotides were annealed in a PCR step. DNA cloning cassettes with sticky ends were designed for genetic modification of the eADF4(C16) gene using a seamless cloning strategy as reported previously.[25] The oligo sequences were for the osteo tag 5′ AGG AGG ATC CAT GGG CGG GCT GCG TTC AAA GTC GAA AAA GTT TCG TCG CCC TGA TAT TCA ATA TCC AGA CGC CAC CGA TGA GGA CAT CAC AAG TCA TAT GGG GTA ATG AAA GCT TAC TG 3′; for the sialoN tag 5′ AGG AGG ATC CAT GGG CAT GGA TTC ATC GGA AGA GAA CGG GAA TGG CGA CTC AAG TGA GGA AGA AGA AGA AGA AGA AGA AAC ATC TGG GTA ATG AAA GCT TAC TG 3′; and for the sialoC tag 5′ AGG AGG ATC CAT GGG CGA GGA TGA GTC AGA CGA GGA AGA AGA AGA AGA AGA AGA AGA AGA AGG GTA ATG AAA GCT TAC TG 3′. The DNA sequences were confirmed by sequencing (eurofins genomics, Germany). The DNA sequences encoded the following amino acids: osteo-tag GLRSKSKKFRRPDIQYPDATDEDITSHM, sialoN-tag DSSEENGNGDSSEEEEEEEETS, and sialoC-tag EDESDEEEEEEEEE. Recombinant protein production using *E. coli* BL21Gold and purification were carried out according to Huemmerich *et al.*[25]

2.2. Film Preparation and Biomineralization Methods. The proteins eADF4(C16-osteo) (M_W = 51 030 g·mol^{-1}), eADF4(SN-C16) (M_W = 50 230 g·mol^{-1}), eADF4(SN-C16-osteo) (M_W = 53 618 g·mol^{-1}), eADF4(C16-SC) (M_W = 49 622 g·mol^{-1}), and eADF4-(C16) (AMSilk, Germany, M_W = 47 698 g·mol^{-1}) were dissolved in 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP) at a concentration of 10 mg/mL and cast in unmodified 48-wellplates (Thermo Fisher Scientific, Germany, Nunc). Air-dried films had a weight of 0.7 mg/cm^2. Films referred to as "post-treated" were incubated in 100% v/v MeOH for 1 h and air-dried for 24 h. To induce biomineralization, the films were incubated in 500 μL of simulated body fluid (SBF), which was prepared according to Kokubo *et al.*,[26] for 4 days at 37 °C, and SBF was stored in plastic containers up to 2 weeks after preparation at 4 °C for further use according to the published protocol. Alternatively, films were mineralized in 500 μL of Dulbecco's Modified Eagle Media (Biochrom, Germany) with supplemented 10% v/v fetal calve serum (Biochrom, Germany), 1% v/v GlutaMax (Invitrogen, United States), and 0.1% v/v gentamycin sulfate (Sigma-Aldrich, Germany) at 5% CO_2, 95% relative humidity, and 37 °C in a cell culture incubator for 4 days (HERAcell 150i, Thermo Scientific, Germany). Mineralized films were washed 3 times with Milli-Q water and air-dried for 24 h before the respective measurements.

2.3. Microscopy. Light microscopy images to monitor mineralization progress and cell cultivation were recorded in media using a Leica DM IL LED microscope (Leica, Germany) and were processed using LAS 4.8 software (Leica, Germany). Fluorescently stained cell samples were studied using a Leica DMI3000B fluorescence microscope (Leica, Germany) and processed using LAS AF 2.7 software (Leica, Germany) and ImageJ (NIH, United States). The film surfaces and chemical composition of the minerals were studied after carbon sputtering (20 nm) using SEM-imaging with a ZEISS Sigma 300 VP and Sigma 500 chamber equipped with an EDS detector (EDAX Pegasus and Octane Super Detector, 60 mm^2 chip, Zeiss, Germany) with an acceleration voltage of 7.5 kV to excite the Kα shell.

2.4. ATR-FTIR. Attenuated total reflectance-Fourier transformation infrared spectroscopy (ATR-FTIR) was used to confirm the protein integrity as well as the identity of the formed minerals before and after the biomineralization process. As a control, hydroxyapatite particles (Sigma-Aldrich, Germany) were used. Spectra (n = 3) were recorded 24 h after film preparation using a Bruker Tensor 27 (Ettlingen, Germany) with a germanium crystal and a resolution of 2 cm^{-1}, using 100 scans and an atmospheric compensation algorithm in OPUS 8.0 software (Bruker, Ettlingen, Germany) to correct water vapor and carbon dioxide fluctuations during the measurement.

2.5. Contact Angle Measurements. Wetting behavior of film surfaces was studied by water contact angle measurements using the sessile drop method and Surftens (OEG, Germany) by dropping 2 μL water on the surface and recording the water drop with the equipped camera after settling for 10 s.

2.6. X-ray Diffraction. X-ray diffraction (XRD) diffractograms of films were recorded using Bragg−Brentano-geometry on an Empyrean diffractometer (Malvern PANalytical B.V., Netherlands) and Cu Kα radiation from 20 to 80° 2θ. Diffractograms were used for peak assignment in X'Pert HighScore Plus (Version 3.0, Malvern PANalytical B.V., Netherlands) to identify mineral species. Mineralized spider silk films could unfortunately not be removed from polystyrene substrates for analysis. However, for three out of five samples, diffractograms could be recorded with mineral signals. This might be due to little mineral formed on the spider silk films or due to the measurement mode in the a/b direction along film orientation.

2.7. Cell Culture Experiments: Cultivation, Quantification, and Staining. As-cast and mineralized film surfaces were UV-treated

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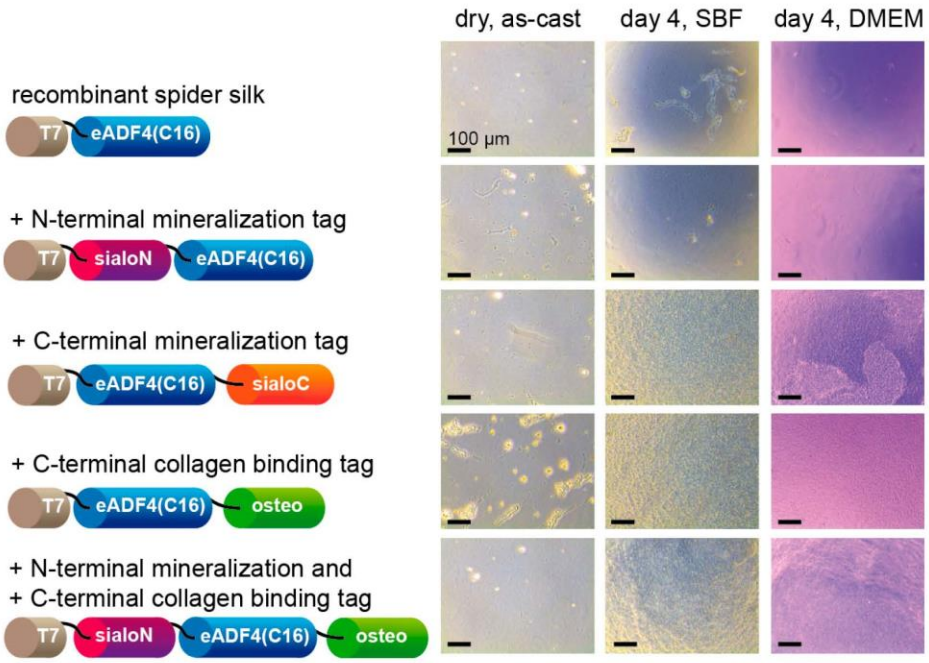

Figure 1. Illustration of recombinant spider silk variants with peptide tags ("sialoN", N-terminal mineralization tag from sialoprotein; "sialoC", C-terminal mineralization tag from sialoprotein; "osteo", C-terminal collagen binding tag from osteopontin) and light microscopy images of dry as-cast recombinant spider silk films, after incubation in simulated body fluid (SBF) for 4 days at 37 °C, or after incubation in Dulbecco's Modified Eagle Media (DMEM) with supplements for 4 days at 37 °C/5% CO_2/95% relative humidity; scale bars: 100 μm.

for 30 min to sterilize them prior to use in cell culture and washed with 1× phosphate buffered saline (PBS). MC3T3 subclone E1 mouse preosteoblasts (ATCC, CLL-163.2) were subsequently cultured in Alpha Modified Eagle Media (Sigma-Aldrich, Germany) with supplemented 10% v/v fetal calve serum (Biochrom, Germany), 1% v/v GlutaMax (Invitrogen, United States) and 0.1% v/v gentamycin sulfate (Sigma-Aldrich, Germany) at 5% CO_2, 95% relative humidity and 37 °C in a cell culture incubator (HERAcell 150i, Thermo Scientific, Germany). The adhesion test on film surfaces was carried out at passage 18 of cultivation, confirming cell viability with trypan blue (Sigma-Aldrich, U.K.) using an automatic cell counter (TC20, Bio-Rad Laboratories Ltd., U.K.). As controls, unmodified (Thermo Fisher Scientific, Germany, Nunc) and tissue-culture-treated 48-wellplates (Thermo Fisher Scientific, Germany, Nunclon) were used. On each test surface (n = 3), 37 500 preosteoblasts/cm^2 were seeded for 4 h. To quantify the adherent cells, 10% v/v CellTiter-Blue reagent (Promega, Germany) in media was incubated on washed samples for 2.5 h. The reaction of resazurin to resofurin was detected using 100 μL of sample and a plate reader (Mithras LB 940, Berthold Technologies, Germany) at 590 nm. Significance was calculated using ANOVA statistic in Origin (Northampton, Massachusetts, United States) in a Tukey test with $p < 0.05$.

Preosteoblast cell culture tests were carried out at passage 22 with a total of 50 000 cells (5 000 cells/cm^2) on the gradient films. The culture was maintained in the described growth media for 10 days, then in differentiation media supplemented with additional 10.2 mM ascorbate-2-phosphate and 10 mM β-glycerophosphate from day 11 to day 21. Controls for cell differentiation were obtained with a lower initial cell density of 1 000 cells/cm^2 on tissue-culture-treated 6-wellplates (Thermo Fisher Scientific, Germany, Nunclon) to prevent cell death at high confluence.

For staining and imaging, cells were fixed in 3.7% para-formaldehyde for 30 min at ambient conditions. To increase permeability of the cell membrane, samples were incubated at ambient conditions in 0.1% v/v Triton X-100 (Carl Roth, Germany) in 1× PBS. Fluorescence staining of actin filaments was achieved upon incubation in 100 nM Phalloidin-rhodamine (Sigma-Aldrich, Germany) for 60 min in the dark.

Alizarin red staining (Carl Roth, Germany) of calcium phosphate species on cell culture samples was carried out on fixed samples with 2% w/v Alizarin Red powder in MQ-water for 30 s. Stained samples were washed thoroughly.

2.8. Gradient Film Production. Gradient films were prepared by defining flow rates in a sawtooth profile from 0.01 to 1 μL/s for eADF4(C16-osteo) and 1 to 0.01 μL/s for eADF4(SN-C16) with a step time of 2 s and a total time of 5 min per run. eADF4(C16-osteo) and eADF4(SN-C16) were dissolved in 1,1,1,3,3,3-hexafluoro-2-isopropanol at a concentration of 10 mg/mL. After applying the protein solutions in 1 mL of gastight glass syringes (Hamilton, Reno, NV, United States), the syringes were fixed in a neMESYS syringe pump (Cetoni, Germany) and allowed to extrude the respective volumes according to the gradient flow rates. Both protein solutions were combined in an in-house built conical polyethylene tip mixing unit before being applied on a Petri dish surface of 2 cm × 5 cm (Thermo Fisher Scientific, Germany) delimited by a PDMS mold. The conical mixing unit was an in-house built adapter from a polyethylene tip where the two endings of the tubings were combined in a small chamber and allowed for the mixing of both gradually applied solutions before releasing the mixed solution dropwise onto the substrate. Additional walls were applied in the Petri dish upon casting and cutting PDMS into defined shapes to yield a reproducible film size of 2 cm × 5 cm and comparable film thickness of 1 mg/cm^2. This mold was also used during cell culture experiments to guarantee cells were located on the film and not on the surrounding dish surface. The step motor of a Harvard syringe pump (Model 33, Harvard Apparatus, United States) was used with a defined speed of 0.8 mL/min to control the movement speed of the substrate, whereas the solution application tip was stationary. The function of the second syringe pump was to use the device's step motor to allow a controlled

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movement of the substrate fixed on top, while the application tip of the conical mixer was stationary. This was the requirement for steady and homogeneous application of the solutions onto the substrate during gradient formation without manual manipulation. Therefore, the second syringe pump was not further depicted and is only referred to as "movable substrate" in Figure 6. The resulting film had an area of 10 cm^2 with 1 mg/cm^2 protein in total and was air-dried for 24 h before use.

3. RESULTS AND DISCUSSION

Our previously established cloning system[25] allows the fusion of spider silk genes with N- and C-terminal[27] tags. Here, sequences encoding peptide motifs well-known from non-collagenous SIBLING proteins, which can bind to collagen fibrils and further mediate their mineralization toward a composite bone material, were fused to that of the previously established engineered spider silk protein eADF4(C16).[15,28] The GLRSKSKKFRRPDIQYPDATDEDITSHM sequence of the osteo-tag is the collagen binding domain (CBD) of human osteopontin (amino acids 167−194[29]) and has a slightly positive net charge while displaying acidic and basic amino acids on its surface. Further, two highly acidic motifs with repetitive glutamic acid residues, generating a strong negative net charge, sialoN-tag DSSEENGNGDSSEEEEEEEETS and sialoC-tag EDESDEEEEEEEEE peptides, were found to be present N- and C-terminally in bone sialoprotein (amino acids 65−86 and 146−160[30−32]) and were fused with eADF4(C16). The osteo tag[15] and sialo tag sequences[8] were previously identified to nucleate hydroxyapatite formation.

3.1. Mineralization of Silk Fusion Films Is Independent of the Mineralization Agent. In a first experimental setup, the mineralization behavior of films made of the five different spider silk variants (Figure 1) was tested. Films were cast out of HFIP (0.7 mg/cm^2) and allowed to air-dry until complete solvent evaporation. The films were first incubated in simulated body fluid (SBF), an established model solution for the evaluation of putative bone formation because of its ion composition and concentration close to that of human blood plasma.[33] SBF was prepared as described by Kokubo *et al.*,[26] and the metastable solution was stored as advised in a plastic container at 4 °C to prevent premature mineral formation on the container walls.[11] Incubation of silk films in SBF at 37 °C showed an increase in surface roughness and opaqueness for distinct variants (Figure 1).

Similar results were observed when films were incubated in cell culture supplemented Dulbecco's Modified Eagle Media (DMEM) (Figure 1), as the ion concentration and composition was similar to that of SBF. When immersed in media, the films were incubated at elevated relative humidity of 95%, 5% CO_2 and 37 °C to evaluate the mineralization behavior at cell culture conditions and to maintain a stable pH for mineralization purposes. The spider silk variants comprising the C-terminal positively charged tag (eADF4(C16-osteo) and eADF4(SN-C16-osteo)) as well as the variant with the C-terminal negatively charged tag (eADF4(C16-SC)) showed a higher apparent mineralization tendency compared to that of the unmodified eADF4(C16) or the variant comprising the N-terminal negatively charged tag (eADF4(SN-C16)). A C-terminal position of the tag, regardless of its charge, seemed to be preferred regarding mineralization, which might be a result of an improved tag availability on the film surface. Interestingly, all four new variants were stable without post-treatment under the chosen conditions in mineralization solutions (see below). Post-treatment of films in MeOH for 1 h did passivate the surface and the visible mineralization declined (Figure S1).

3.2. Characterization of the Mineralized Film Surface. To identify the formed minerals on the films, ATR-FTIR spectra were recorded of washed and dried films. The spectrum of eADF4(C16) (Figure 2A−C, left panel, black) was subtracted from the spectra of the variants, displaying differences in peaks heights. For better identification, hydroxyapatite particles were used as an internal reference (Figure 2B,C, left panel, red). For as-cast films, no significant differences in protein spectra were visible (Figure 2A, right panel). After incubation in SBF, protein films remained highly intact, as seen by preserved amide I and II bands (1790−1520 cm^{-1}). However, slight shifts were identified for the variants, indicative of β-sheet formation.[34] The eADF4(C16) films showed lower amide peaks (Figure 2B, right panel). This indicated a dissolution of the eADF4(C16) film, which is in accordance with previous studies showing its water solubility when not post-treated.[35] Further, distinct vibrations of hydroxyapatite-like phosphate could be detected for all films

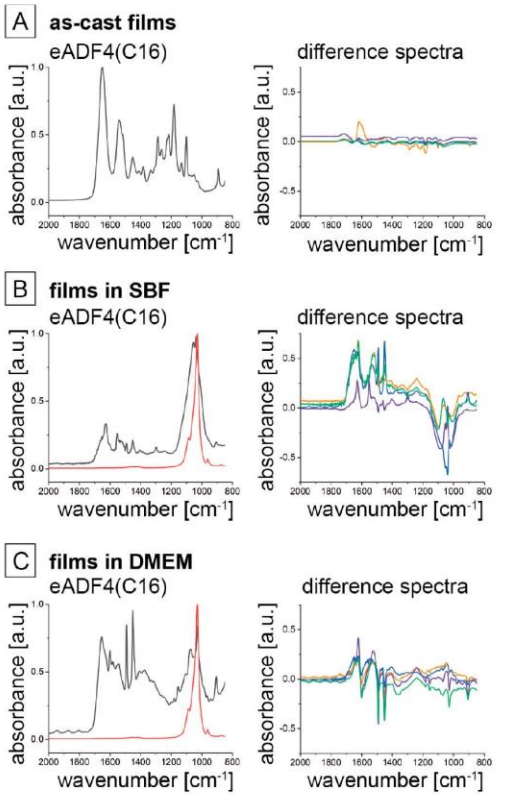

Figure 2. Mean ATR-FTIR spectra of as-cast films (A) of spider silk variants, after incubation in simulated body fluid (SBF) for 4 days (B), after incubation in Dulbecco's Modified Eagle Media (DMEM) with supplements for 4 days (C) and hydroxyapatite particles as control (B and C, red). The spectrum of eADF4(C16) (A−C, left panels, black) was subtracted from the variants' spectra to show variations in peak intensities (right panel, difference spectra): eADF4(C16-osteo) orange, eADF4(C16-SC) violet, eADF4(SN-C16) blue, eADF4(SN-C16-osteo) green (A−C).

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Table 1. Water Contact Angle Measurements for Post-Treated Films and for Mineralized Films (Washed and Dried) in Simulated Body Fluid (SBF) and in Dublecco's Modified Eagle Media (DMEM) after 4 Days[a]

recombinant spider silk variant	eADF4(C16)	eADF4(SN-C16)	eADF4(C16-SC)	eADF4(C16-osteo)	eADF4(SN-C16-osteo)
	[deg] ± STD	[deg] ± STD	[deg] ± STD	[deg] ± STD	[deg] ± STD
tag	no tag	N-terminal mineralization	C-terminal mineralization	C-terminal collagen binding	N-terminal mineralization and C-terminal collagen binding
in SBF	52 ± 8	8 ± 2	11 ± 3	16 ± 3	48 ± 5
in DMEM	46 ± 13	32 ± 5	42 ± 2	51 ± 5	46 ± 11
post-treated	34 ± 5	32 ± 4	28 ± 5	35 ± 5	39 ± 1
post-treated, in SBF	43 ± 5	32 ± 2	30 ± 1	34 ± 3	31 ± 7
post-treated, in DMEM	54 ± 4	46 ± 6	46 ± 5	50 ± 6	43 ± 6

[a]STD, standard deviation.

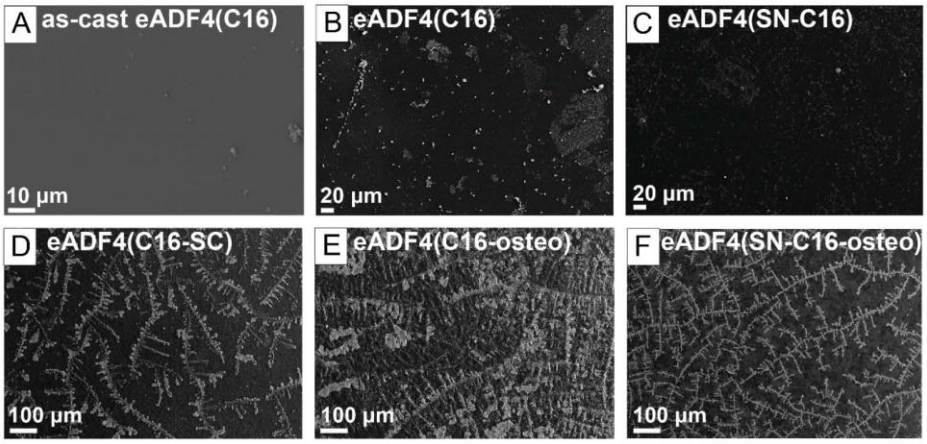

Figure 3. SEM imaging of (A) as-cast eADF4(C16) film surface without further incubation and (B–F) film surfaces of spider silk variants after incubation in simulated body fluid for 4 days.

at wavenumbers 1089, 1032, and 962 cm^{-1},[36] which were not present in as-cast, dried films. When comparing all films incubated in DMEM after 4 days, noisier spectra were achieved (Figure 2C, right panel). This result likely indicated a simultaneous precipitation of minerals and proteins from the complex supplemented cell culture media.

eADF4(C16) spectra showed decreased amide bands compared to that of the variants. Upon post-treatment of the films (Figure S2) mineralization was largely diminished and could be seen only on the eADF4(SN-C16-osteo) film surface. Post-treatment might influence not only the secondary structure of the underlying spider silk proteins but also lead to a different mineralization behavior on the films surfaces.

As-cast films were stable during the mineralization process at equilibrium conditions because of simultaneously occurring post-treatment but dissolved in pure water as expected. Accordingly, data for water contact angle measurements of as-cast films could not be depicted. After treatment with SBF, films showed a hydrophilic behavior with low water contact angles when minerals were deposited.[37] This behavior was different upon incubation in DMEM for both as-cast and post-treated films, as adsorption of proteins from the cell culture media on the film surfaces influenced the contact angles[38] (Table 1). Although the surface charge of the films might have influenced the water contact angle, a shielding effect has to be taken into account based on deposited media components on the film surfaces. Post-treatment of films yielded water contact angles of 28°–39°, which was in good agreement with previously recorded results of post-treated recombinant spider silk films on polystyrene surfaces.[34] The observed slight increase in water contact angles after treatment in SFB and more pronounced increase after DMEM incubation could be an indication for starting mineral deposition but also for protein adsorption, covering the surface.[39]

Noncollagenous proteins in bone are known to carry acidic residues, mainly glutamic and aspartic acid. These proteins are assumed to show disordered structures in solution but form intermolecular assemblies during absorption on a surface. SIBLING proteins were identified to form Ca^{2+} salt bridges at the protein–mineral interface.[3] Reducing the electrostatic repulsion of the side chains initiated β-sheet formation.[3] Similar processes were previously observed for other acidic synthetic proteins.[3] When immobilized on a surface, acidic side chains tend to arrange periodically and thereby generate mineralization patterns by templating crystal nucleation and growth.[3] Although eADF4(C16) is carrying a negative net charge, mineralization patterns were not observed. Upon functionalization with highly charged C-terminal mineralization tags, periodic patterns of crystal formation could be seen using SEM imaging, similar to the reported ones in the literature (Figure 3). The availability of C-terminal tags on the films' surface arranged the crystals in patterns, because charge density attracted ions from the mineralization agents. Variants with negatively charged C-terminal tags created highly similar patterns, while eADF4(C16-osteo) films with a positively charged tag showed a slightly different pattern (Figure 3).

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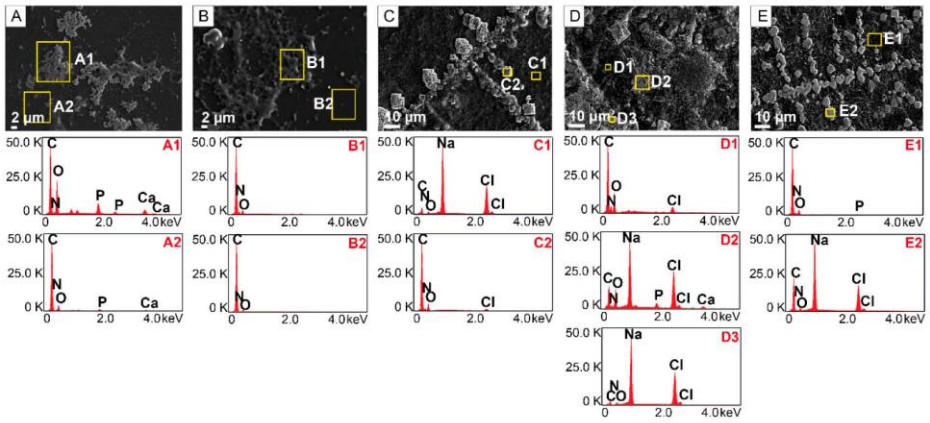

Figure 4. SEM-EDX imaging of mineralized film surfaces and element analysis of (A) eADF4(C16), (B) eADF4(SN-C16), (C) eADF4(C16-SC), (D) eADF4(C16-osteo), and (E) eADF4(SN-C16-osteo) surfaces after 4 days in simulated body fluid.

SEM-EDX analysis revealed Ca and P within the crystals, next to precipitated NaCl from SBF (Figure 4). However, as 4 days of mineralization did not lead to a full mineral coverage of the films, mineralized patterns and nonmineralized areas were still visible. The weak Ca/P-signal obtained on film surfaces was in contrast to phosphate vibrations using ATR-FTIR, which might result from a larger film area covered by the ATR-FTIR spectra.

Diffractograms for mineral peaks assignment using XRD could not be recorded for all mineralized spider silk films because of preparation issues, but for eADF4(C16-osteo), eADF4(SN-C16), and eADF4(SN-C16-osteo), diffractograms were achieved (Figure S3). Predominantly calcium pyrophosphate ($Ca_2O_7P_2$) and calcium oxide (CaO) species were indicated. The formation of hydroxyapatite was not confirmed. Calcium phosphate and calcium carbonate was previously found in biomineralized composite films from synthetic polymers and eADF4(C16) using two set-ups to trigger specific mineral deposition. Calcite crystals were previously localized in spots on eADF4(C16) films, whereas calcium phosphate covered the entire film surface.[24] In contrast, here, more complex mineralization agents were used and more possible mineral phases could be expected to deposit from solution.

3.3. Performance of Spider Silk Fusion Films in Cell Adhesion. In a second experimental setup, we tested films of all spider silk-fusion variants regarding cell adhesion. On the one hand, films were used as-cast after drying, and on the other hand, followed by incubation in SBF for 4 days, which is termed "mineralized" films (Figure 5). Upon incubation of cells in their respective media on as-cast film surfaces, similar mineralization and protein adsorption effects from the media can be expected as described above for mineralization experiments in parallel to cell adhesion. Films were rendered stable and were compared concerning cell adhesion upon incubation with MC3T3 subclone E1 mouse preosteoblasts. eADF4(C16) films, tissue-culture-treated and nontreated plates served as control. As it is recommended to cultivate low cell numbers for the preosteoblast cell line to prevent cell death by confluence, 37 500 cells/cm^2 were seeded on the film surfaces. After 4 h, Cell Titer Blue Assay was performed to quantify the metabolism of the adherent cells. The quantification was normalized to tissue-culture-treated plates as 100%. For as-cast mineralized surfaces, the same trend for all variants could be observed (Figure 5B). In general, calcium phosphate deposition on surfaces was found to enhance osteoblast adhesion, proliferation, and differentiation.[19,24,40−43] The incubation in SBF for 4 days did not seem to influence cell adhesion properties, as by then only low amounts of mineral species were deposited in this early phase of mineralization.[40] However, ongoing mineralization can be expected to enhance cell adhesion on mineralized spider silk surfaces. All film samples showed less cell adhesion than the negative control (nontreated plate) and significant differences to the positive control (tissue-culture-treated plate). This behavior has already been shown for eADF4(C16) surfaces with low cell adhesion.[27,44]

Further, a typical MC3T3 morphology was only found on tissue-culture-treated plates (Figure 5A). For the other film samples and nontreated plates, fewer cells were observed in accordance with the quantified adhesion as well as fewer focal adhesions.

3.4. Generation and Evaluation of Silk-Fusion Gradient Films. Since MC3T3 cells were previously found to form mineralized matrices on their own upon incubation in differentiation media in long-term cell culture studies,[45,46] self-mineralization behavior and gradual cell adhesion were finally studied at those conditions. Biomimetic mineralization in conjunction with controllable cell adhesion are important design criteria for a functional osteoconductive material, for example, at the tendon-to-bone interface. The natural enthesis is marked by a gradual increase in collagen fibril disorientation and increase in the amount of hydroxyapatite minerals and preosteoblast population.[2,4,47]

Therefore, we produced gradient spider silk films with one end made of eADF4(C16-osteo) and the other of eADF4(SN-C16). The gradient films were realized by using a syringe pump system, which allowed us to continuously change the flow rates of both spider silk fusion proteins (Figure 6A).[48] Films were not post-treated or pre-mineralized to evaluate mineralization induced by MC3T3 preosteoblast cells. Preosteoblast cell adhesion showed no significant differences for films of the variants after 4 h (Figure 5). However, slightly different mineralization patterns were detected (Figure 3). Upon providing the two chosen tags in the gradient, it was

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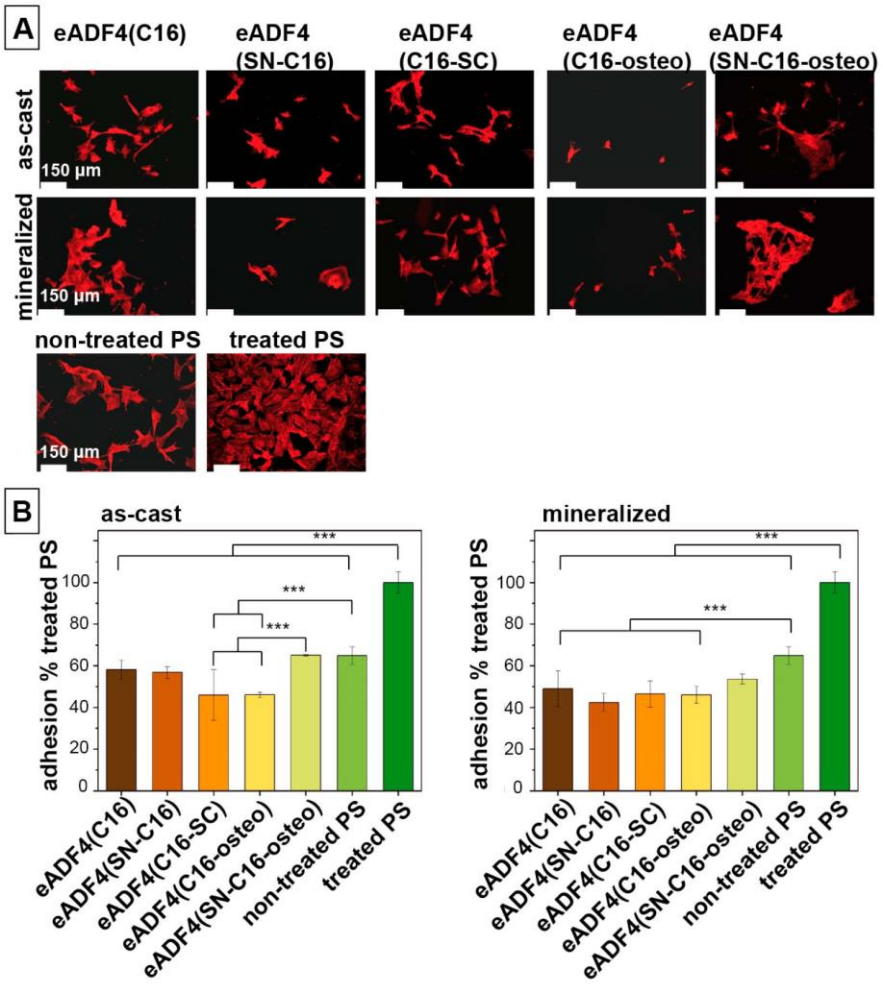

Figure 5. (A) Fluorescence microscopy images of MC3T3 mouse preosteoblast cells incubated on as-cast and mineralized films of eADF4(C16), eADF4(SN-C16), eADF4(C16-SC), eADF4(C16-osteo), and eADF4(SN-C16-osteo) after 4 h of cultivation; tissue-culture-treated and nontreated culture polystyrene (PS) plates served as control; Phalloidin/rhodamine staining of F-actin (red); scale bars 150 μm. (B) Normalized cell adhesion of MC3T3 mouse preosteoblasts on as-cast films and films from spider silk variants after incubation in simulated body fluid for 4 days using cell titer blue assay. Adhesion to tissue-culture-treated polystyrene (PS) plates was set to 100%.

assumed that gradient effects might occur rather in long-term incubations.

On as-cast film surfaces, a total of 50 000 (5 000 cells/cm^2) MC3T3 preosteoblasts were seeded and cultured for 10 days, followed by 11 days of cultivation in differentiation media, supplemented with 10.2 mM ascorbate-2-phosphate and 10 mM β-glycerophosphate. This cell density was chosen to allow cell proliferation gradually toward the side of the preferred fusion protein. Interestingly, throughout the entire cultivation period, a cell number gradient toward the mineralization variant eADF4(C16-osteo) was maintained. The DAPI-staining of the cell nuclei confirmed a persistent gradual cell adhesion after 21 days (Figure 6B).

In former studies, MC3T3 mouse preosteoblasts were found to form a mineralized matrix in the presence of ascorbic acid and inorganic phosphate components.[45,46] Control experiments in tissue-culture-treated 6-wellplates confirmed the MC3T3 preosteoblasts' ability to differentiate and deposit a mineralized matrix when highly confluent (day 10) in differentiation media for 11 days (Figure S4B). However, a specific Alizarin Red staining of the gradient film on day 21 did not show any significant organic crystal formation upon cell deposition on the construct, which might be a result of too low cell densities, although initial cell densities were higher than for the control. Further, upon addition of ascorbic acid to the differentiation media, crystal deposition from the media is unlikely for apatite species because of the acidic pH. Still, there is a clear effect of the gradient on preosteoblast adhesion and proliferation (Figure S4A). The deposition of a collagen containing extracellular matrix and macromolecular crowding of the media with synthetic or natural polymers previously led to the creation of functional microenvironments for mesenchymal stem cells.[49] Similarly, collagen deposited on the gradient film by preosteoblasts might interact with the collagen binding peptide tag and support long-term cell adhesion toward this side of the gradient in a microenvironment.

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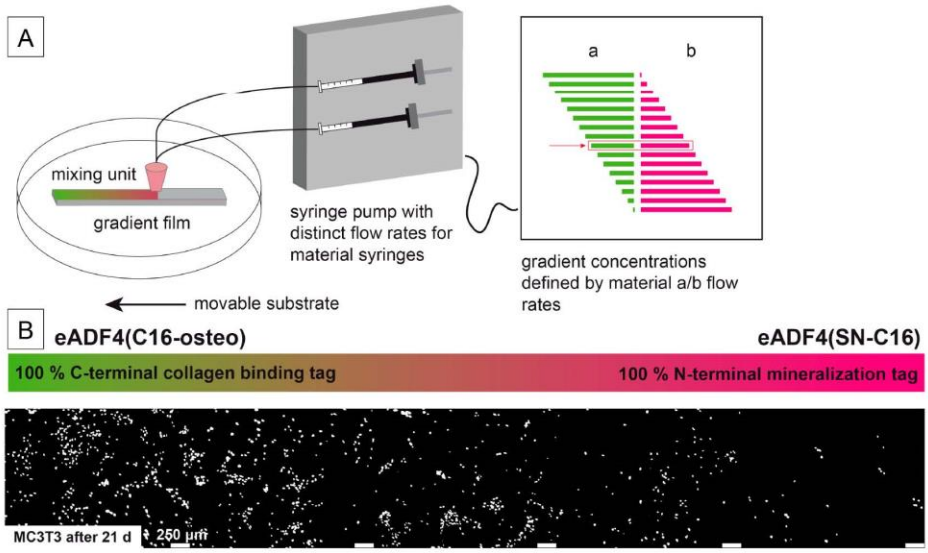

Figure 6. (A) Schematic illustration of gradient film preparation using a syringe pump system. (B) Fluorescence microscopy images of five equally distanced locations on a 100% eADF4(C16-osteo) to 100% eADF4(SN-C16) gradient film after 21 days of MC3T3 cell culture. DAPI-stained cell nuclei are depicted in white; scale bars: 250 μm.

4. CONCLUSION

Osteoconductive spider silk fusion proteins were designed with different mineralization and collagen binding tags inspired by peptide motifs from SIBLING proteins. The variants were found to distinctly induce mineralization in two different media. The film surfaces displayed Ca/P mineral species and pattern formation after mineralization together with changes in surface wetting behavior. In a time-dependent study, a preosteoblast cell gradient was achieved and maintained for 21 days toward the spider silk variant with the collagen binding tag, indicating a favorable substrate for the cells by possible binding of the secreted collagen. This work provides evidence that such gradient materials could be used to generate materials to support tendon-to-bone connections in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.0c00818.

Light microscopy of mineralization, ATR-FTIR spectra, XRD diffractograms, and light microscopy images of cell adhesion on gradient films (PDF)

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Notes

The authors declare the following competing financial interest(s): T.S. is co-founder and shareholder of AMSilk GmbH.

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■ REFERENCES

(1) Baldino, L.; Cardea, S.; Maffulli, N.; Reverchon, E. Regeneration techniques for bone-to-tendon and muscle-to-tendon interfaces reconstruction. *Br. Med. Bull.* **2016**, *117* (1), 25−37.

(2) Thomopoulos, S.; Williams, G. R.; Gimbel, J. A.; Favata, M.; Soslowsky, L. J. Variation of biomechanical, structural, and compositional properties along the tendon to bone insertion site. *J. Orthop. Res.* **2003**, *21* (3), 413−419.

(3) Beniash, E. Biominerals—hierarchical nanocomposites: the example of bone. *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.* **2011**, *3* (1), 47−69.

(4) Shaw, H. M.; Vázquez, O. T.; McGonagle, D.; Bydder, G.; Santer, R. M.; Benjamin, M. Development of the human Achilles tendon enthesis organ. *J. Anat.* **2008**, *213* (6), 718−724.
(5) Marinovich, R.; Soenjaya, Y.; Wallace, G. Q.; Zuskov, A.; Dunkman, A.; Foster, B. L.; Ao, M.; Bartman, K.; Lam, V.; Rizkalla, A.; Beier, F.; Somerman, M. J.; Holdsworth, D. W.; Soslowsky, L. J.; Lagugne-Labarthet, F.; Goldberg, H. A. The role of bone sialoprotein in the tendon-bone insertion. *Matrix Biol.* **2016**, *52*−*54*, 325−338.
(6) Palmer, L. C.; Newcomb, C. J.; Kaltz, S. R.; Spoerke, E. D.; Stupp, S. I. Biomimetic Systems for Hydroxyapatite Mineralization Inspired By Bone and Enamel. *Chem. Rev.* **2008**, *108* (11), 4754−4783.
(7) Hildebrand, M. Diatoms, Biomineralization Processes, and Genomics. *Chem. Rev.* **2008**, *108* (11), 4855−4874.
(8) Harris, N. L.; Rattray, K. R.; Tye, C. E.; Underhill, T. M.; Somerman, M. J.; D'Errico, J. A.; Chambers, A. F.; Hunter, G. K.; Goldberg, H. A. Functional analysis of bone sialoprotein: identification of the hydroxyapatite-nucleating and cell-binding domains by recombinant peptide expression and site-directed mutagenesis. *Bone* **2000**, *27* (6), 795−802.
(9) Hoang, Q. Q.; Sicheri, F.; Howard, A. J.; Yang, D. S. C. Bone recognition mechanism of porcine osteocalcin from crystal structure. *Nature* **2003**, *425* (6961), 977−980.
(10) Hunter, G. K.; Hauschka, P. V.; Poole, A. R.; Rosenberg, L. C.; Goldberg, H. A. Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. *Biochem. J.* **1996**, *317*, 59−64.
(11) Jahromi, M. T.; Yao, G.; Cerruti, M. The importance of amino acid interactions in the crystallization of hydroxyapatite. *J. R. Soc., Interface* **2013**, *10* (80), 20120906.
(12) Addadi, L.; Weiner, S. Interactions between acidic proteins and crystals: stereochemical requirements in biomineralization. *Proc. Natl. Acad. Sci. U. S. A.* **1985**, *82* (12), 4110−4114.
(13) Chen, C. L.; Rosi, N. L. Peptide-Based Methods for the Preparation of Nanostructured Inorganic Materials. *Angew. Chem., Int. Ed.* **2010**, *49* (11), 1924−1942.
(14) Gilbert, M.; Shaw, W. J.; Long, J. R.; Nelson, K.; Drobny, G. P.; Giachelli, C. M.; Stayton, P. S. Chimeric peptides of statherin and osteopontin that bind hydroxyapatite and mediate cell adhesion. *J. Biol. Chem.* **2000**, *275* (21), 16213−16218.
(15) Lee, J. Y.; Choo, J. E.; Choi, Y. S.; Park, J. B.; Min, D. S.; Lee, S. J.; Rhyu, H. K.; Jo, I. H.; Chung, C. P.; Park, Y. J. Assembly of collagen-binding peptide with collagen as a bioactive scaffold for osteogenesis in vitro and in vivo. *Biomaterials* **2007**, *28* (29), 4257−4267.
(16) Chung, W. J.; Kwon, K. Y.; Song, J.; Lee, S. W. Evolutionary Screening of Collagen-like Peptides That Nucleate Hydroxyapatite Crystals. *Langmuir* **2011**, *27* (12), 7620−7628.
(17) Gungormus, M.; Fong, H.; Kim, I. W.; Evans, J. S.; Tamerler, C.; Sarikaya, M. Regulation of in vitro calcium phosphate mineralization by combinatorially selected hydroxyapatite-binding peptides. *Biomacromolecules* **2008**, *9* (3), 966−973.
(18) Roy, M. D.; Stanley, S. K.; Amis, E. J.; Becker, M. L. Identification of a highly specific hydroxyapatite-binding peptide using phage display. *Adv. Mater.* **2008**, *20* (10), 1830−1836.
(19) Dinjaski, N.; Plowright, R.; Zhou, S.; Belton, D. J.; Perry, C. C.; Kaplan, D. L. Osteoinductive recombinant silk fusion proteins for bone regeneration. *Acta Biomater.* **2017**, *49*, 127−139.
(20) Gomes, S.; Gallego-Llamas, J.; Leonor, I. B.; Mano, J. F.; Reis, R. L.; Kaplan, D. L. In Vivo Biological Responses to Silk Proteins Functionalized with Bone Sialoprotein. *Macromol. Biosci.* **2013**, *13* (4), 444−454.
(21) Nagano, A.; Sato, H.; Tanioka, Y.; Nakazawa, Y.; Knight, D.; Asakura, T. Characterization of a Ca binding-amphipathic silk-like protein and peptide with the sequence (Glu)8(Ala-Gly-Ser-Gly-Ala-Gly)4 with potential for bone repair. *Soft Matter* **2012**, *8* (3), 741−748.
(22) Bradt, J.-H.; Mertig, M.; Teresiak, A.; Pompe, W. Biomimetic Mineralization of Collagen by Combined Fibril Assembly and Calcium Phosphate Formation. *Chem. Mater.* **1999**, *11* (10), 2694−2701.
(23) Yu, L.; Martin, I. J.; Kasi, R. M.; Wei, M. Enhanced Intrafibrillar Mineralization of Collagen Fibrils Induced by Brushlike Polymers. *ACS Appl. Mater. Interfaces* **2018**, *10* (34), 28440−28449.
(24) Hardy, J. G.; Torres-Rendon, J. G.; Leal-Egana, A.; Walther, A.; Schlaad, H.; Coelfen, H.; Scheibel, T. R. Biomineralization of Engineered Spider Silk Protein-Based Composite Materials for Bone Tissue Engineering. *Materials* **2016**, *9* (7), 560.
(25) Huemmerich, D.; Helsen, C. W.; Quedzuweit, S.; Oschmann, J.; Rudolph, R.; Scheibel, T. Primary Structure Elements of Spider Dragline Silks and Their Contribution to Protein Solubility. *Biochemistry* **2004**, *43* (42), 13604−13612.
(26) Kokubo, T.; Takadama, H. How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* **2006**, *27* (15), 2907−2915.
(27) Wohlrab, S.; Müller, S.; Schmidt, A.; Neubauer, S.; Kessler, H.; Leal-Egana, A.; Scheibel, T. Cell adhesion and proliferation on RGD-modified recombinant spider silk proteins. *Biomaterials* **2012**, *33* (28), 6650−6659.
(28) George, A.; Veis, A. Phosphorylated Proteins and Control over Apatite Nucleation, Crystal Growth, and Inhibition. *Chem. Rev.* **2008**, *108* (11), 4670−4693.
(29) Kiefer, M. C.; Bauer, D. M.; Barr, P. J. The cDNA and derived amino acid sequence for human osteopontin. *Nucleic Acids Res.* **1989**, *17* (8), 3306−3306.
(30) Ganss, B.; Kim, R. H.; Sodek, J. Bone Sialoprotein. *Crit. Rev. Oral Biol. Med.* **1999**, *10* (1), 79−98.
(31) Tye, C. E.; Rattray, K. R.; Warner, K. J.; Gordon, J. A. R.; Sodek, J.; Hunter, G. K.; Goldberg, H. A. Delineation of the Hydroxyapatite-nucleating Domains of Bone Sialoprotein. *J. Biol. Chem.* **2003**, *278* (10), 7949−7955.
(32) Wazen, R. M.; Tye, C. E.; Goldberg, H. A.; Hunter, G. K.; Smith, C. E.; Nanci, A. In Vivo Functional Analysis of Polyglutamic Acid Domains in Recombinant Bone Sialoprotein. *J. Histochem. Cytochem.* **2007**, *55* (1), 35−42.
(33) Juhasz, J. A.; Best, S. M.; Auffret, A. D.; Bonfield, W. Biological control of apatite growth in simulated body fluid and human blood serum. *J. Mater. Sci.: Mater. Med.* **2008**, *19* (4), 1823−1829.
(34) Wohlrab, S.; Spieß, K.; Scheibel, T. Varying surface hydrophobicities of coatings made of recombinant spider silk proteins. *J. Mater. Chem.* **2012**, *22* (41), 22050−22054.
(35) Slotta, U.; Tammer, M.; Kremer, F.; Koelsch, P.; Scheibel, T. Structural Analysis of Spider Silk Films. *Supramol. Chem.* **2006**, *18* (5), 465−471.
(36) Rehman, I.; Bonfield, W. Characterization of hydroxyapatite and carbonated apatite by photo acoustic FTIR spectroscopy. *J. Mater. Sci.: Mater. Med.* **1997**, *8* (1), 1−4.
(37) Bico, J.; Thiele, U.; Quéré, D. Wetting of textured surfaces. *Colloids Surf., A* **2002**, *206* (1), 41−46.
(38) Lampin, M.; Warocquier-Clérout, R.; Legris, C.; Degrange, M.; Sigot-Luizard, M. F. Correlation between substratum roughness and wettability, cell adhesion, and cell migration. *J. Biomed. Mater. Res.* **1997**, *36* (1), 99−108.
(39) Weiss, A. C. G.; Herold, H. M.; Lentz, S.; Faria, M.; Besford, Q. A.; Ang, C.-S.; Caruso, F.; Scheibel, T. Surface Modification of Spider Silk Particles to Direct Biomolecular Corona Formation. *ACS Appl. Mater. Interfaces* **2020**, *12* (22), 24635−24643.
(40) Griffanti, G.; Jiang, W.; Nazhat, S. N. Bioinspired mineralization of a functionalized injectable dense collagen hydrogel through silk sericin incorporation. *Biomater. Sci.* **2019**, *7* (3), 1064−1077.
(41) Zafar, B.; Mottaghitalab, F.; Shahosseini, Z.; Negahdari, B.; Farokhi, M. Silk fibroin/alumina nanoparticle scaffold using for osteogenic differentiation of rabbit adipose-derived stem cells. *Materialia* **2020**, *9*, 100518.
(42) Narimani, M.; Teimouri, A.; Shahbazarab, Z. Synthesis, characterization and biocompatible properties of novel silk fibroin/graphene oxide nanocomposite scaffolds for bone tissue engineering application. *Polym. Bull.* **2019**, *76* (2), 725−745.

https://dx.doi.org/10.1021/acsbiomaterials.0c00818
ACS Biomater. Sci. Eng. 2020, 6, 5599−5608

(43) Liu, F.; Liu, C.; Zheng, B.; He, J.; Liu, J.; Chen, C.; Lee, L.-S.; Wang, X.; Liu, Y. Synergistic Effects on Incorporation of β-Tricalcium Phosphate and Graphene Oxide Nanoparticles to Silk Fibroin/Soy Protein Isolate Scaffolds for Bone Tissue Engineering. *Polymers* **2020**, *12* (1), 69.
(44) Schacht, K.; Juengst, T.; Schweinlin, M.; Ewald, A.; Groll, J.; Scheibel, T. Biofabrication of Cell-Loaded 3D Spider Silk Constructs. *Angew. Chem., Int. Ed.* **2015**, *54* (9), 2816−2820.
(45) Franceschi, R. T.; Iyer, B. S.; Cui, Y. Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J. Bone Miner. Res.* **1994**, *9* (6), 843−854.
(46) Wang, D.; Christensen, K.; Chawla, K.; Xiao, G.; Krebsbach, P. H.; Franceschi, R. T. Isolation and Characterization of MC3T3-E1 Preosteoblast Subclones with Distinct In Vitro and In Vivo Differentiation/Mineralization Potential. *J. Bone Miner. Res.* **1999**, *14* (6), 893−903.
(47) Genin, G. M.; Kent, A.; Birman, V.; Wopenka, B.; Pasteris, J. D.; Marquez, P. J.; Thomopoulos, S. Functional Grading of Mineral and Collagen in the Attachment of Tendon to Bone. *Biophys. J.* **2009**, *97* (4), 976−985.
(48) Claussen, K. U.; Lintz, E. S.; Giesa, R.; Schmidt, H. W.; Scheibel, T. Protein Gradient Films of Fibroin and Gelatine. *Macromol. Biosci.* **2013**, *13* (10), 1396−1403.
(49) Prewitz, M. C.; Stißel, A.; Friedrichs, J.; Träber, N.; Vogler, S.; Bornhäuser, M.; Werner, C. Extracellular matrix deposition of bone marrow stroma enhanced by macromolecular crowding. *Biomaterials* **2015**, *73*, 60−69.

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Supporting Information

Spider Silk Fusion Proteins for Controlled Collagen Binding and Biomineralization

Vanessa J. Neubauer[a] *and Thomas Scheibel*[*a,b,c,d,e]

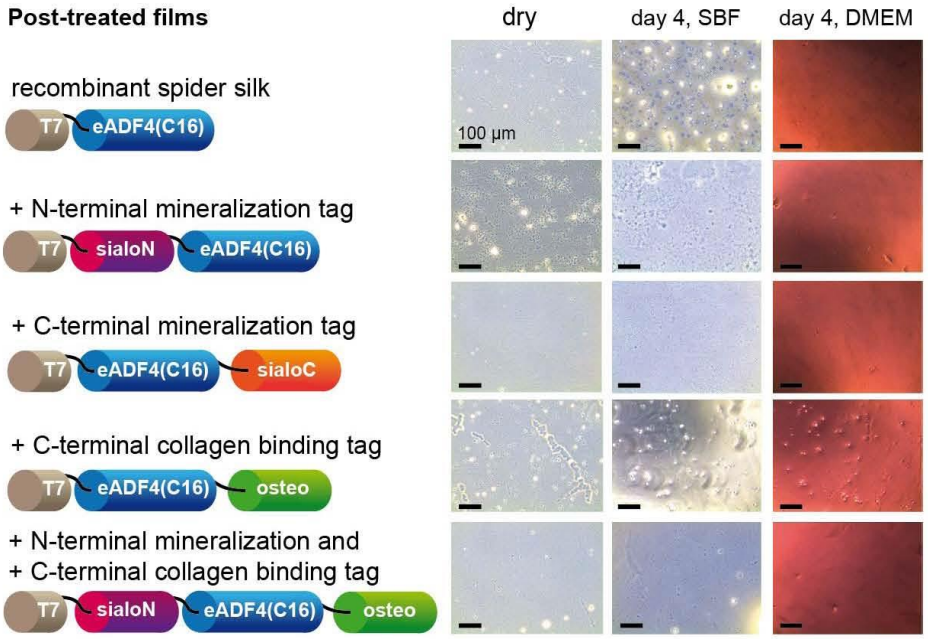
Content on pages S1-S5

S1: Light microscopy of mineralization.

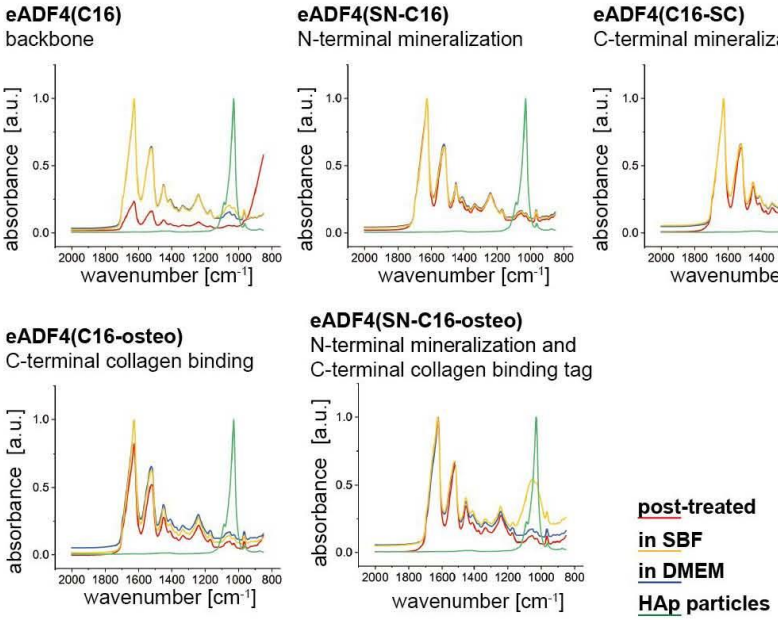
S2: ATR-FTIR.

S3: XRD.

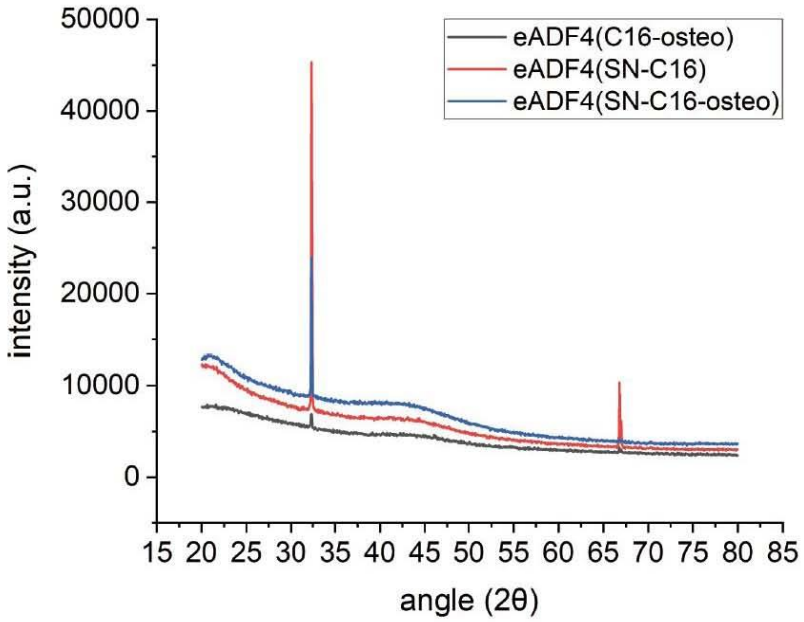
S4: Light microscopy of cell adhesion on gradient films.



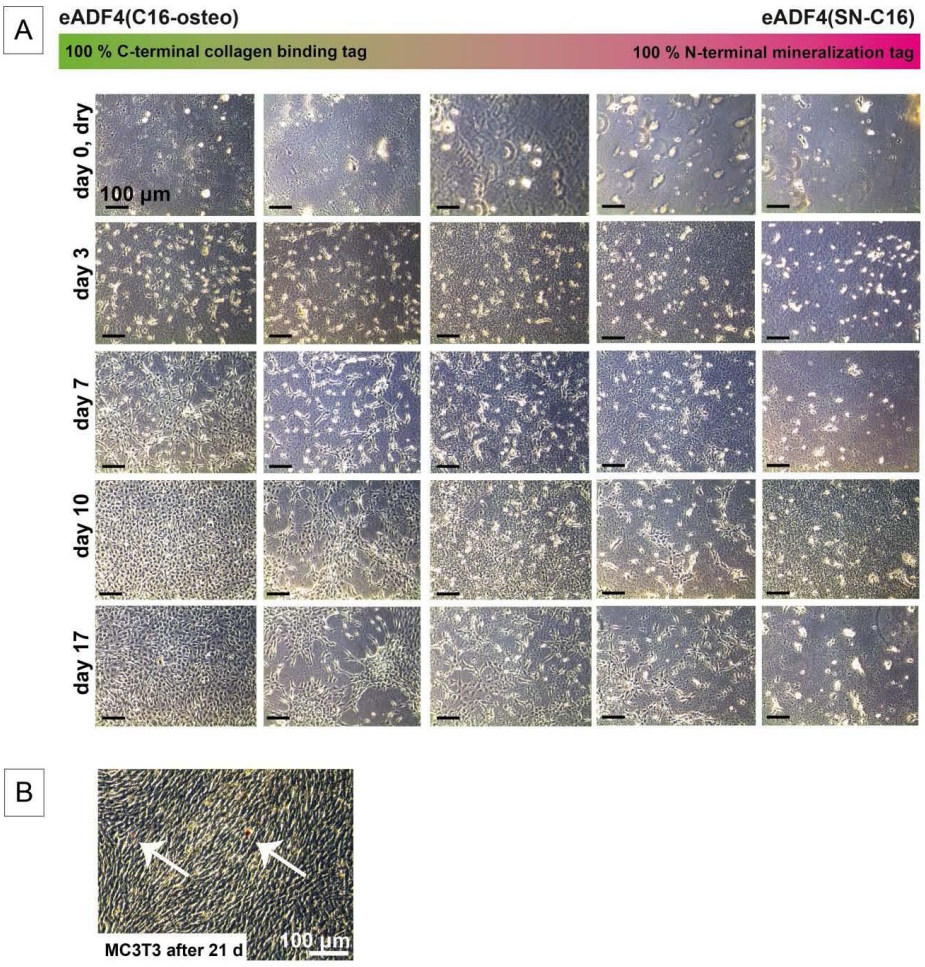
S1: Illustration of recombinant spider silk variants with peptide tags ("sialoN" = N-terminal mineralization tag from sialoprotein; "sialoC" = C-terminal mineralization tag from sialoprotein, "osteo" = C-terminal collagen binding tag from osteopontin) and light microscopy images of dry recombinant spider silk films post-treated in MeOH followed by incubation in Simulated Body Fluid (SBF) for 4 d at 37 °C, or after incubation in Dulbecco's Modified Eagle Media (DMEM) with supplements for 4 d at 37 °C / 5 % CO_2 / 95 % relative humidity; scale bars: 100 µm.



S2: Mean ATR-FTIR spectra of eADF4(C16), eADF4(SN-C16), eADF4(C16-SC), eADF4(C16-osteo) post-treated films (red), followed by incubation in Simulated Body Fluid (SBF, yellow) for 4 d, or followed by incubation in Dulbecco's Modified eagle Media (DMEM) with supplements (blue) for 4 d. Spectra of hydroxyapatite particles are implemented as internal control (green).



S3: XRD diffractograms for mineralized spider silk variants in SBF for 4 d at 37 °C, in dry state after washing.



S4: (A) Light microscopy images of five equally distanced locations of a 100 % eADF4(C16-osteo) to 100 % eADF4(SN-C16) gradient as-cast film in dry state, and after 3, 7, 10 and 17 d of cultivation of MC3T3 osteoblasts on top; scale bars: 100 µm. (B) Light microscopy images of Alizarin red stained MC3T3 cells grown on tissue-culture-treated plates after 21 d of culture as control, arrows indicate the presence of stained minerals.

S5

7.4 Teilarbeit IV

Teilarbeit IV wurde 2021 unter dem Titel *„Flow Simulation and Gradient Printing of Fluorapatite loaded Recombinant Spider Silk Hydrogels*“ vorbereitet.

Neubauer, V. J.; Hüter, F.; Wittmann, J.; Trossmann, V. T.; Kleinschrodt, C.; Alber-Laukant, B.; Rieg, F.; Scheibel, T. (2021) Flow Simulation and Gradient Printing of Fluorapatite loaded Recombinant Spider Silk Hydrogels. *Manuskript in der Vorbereitung*

Flow Simulation and Gradient Printing of Fluorapatite and Cell loaded Recombinant Spider Silk Hydrogels

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Abstract

Hierarchical structures are elemental in almost all tissues of the human body. Therefore, it is essential for tissue engineering approaches to mimic hierarchical structures to gain function of the new tissue. Here, the focus was on the so-called enthesis which is a gradient structure located between tendon and bone, bridging the mechanical properties to allow the perfect force transmission from the muscle to the skeleton. A numerical approach was used to simulate gradient formation by computational flow as prerequisite for real printing of such gradients followed by 3D printing of hydrogels. Composites of spider silk hydrogels with fluorapatite rods were used to generate mineralized gradients in a dispense plotting set-up. Further, BALB/3T3 fibroblasts were encapsulated in spider silk-fluorapatite hydrogels and gradually printed against the pure spider silk hydrogel. Thereby, adjustable gradient features were achieved, useful for tissue engineering at the tendon/bone interface.

Keywords: Gradient 3D Printing, Computational Fluid Dynamics, Spider Silk Hydrogels, Apatite Particles, Single Cartridge Set-up

1. Introduction

The hierarchical organization of tissues is essentially contributing to their function[1] on both a structural/morphological and a functional level. Gradient materials often bridge two materials with different mechanical properties to gain smooth transitions and therefore to prevent material failure at their interface.[2] This concept can be found for example at the tendon-bone-insertion, the so-called enthesis, with material gradients and resulting gradually changing mechanical properties.[3] Previously, biomechanics were investigated to determine threshold parameters for material design[4], and biomimetic gradient materials were generated for tendon-bone-interface reconstruction using a gradual calcium phosphate coating on electrospun nanofiber meshes.[5, 6] Another approach was focussing on the mechanical requirements of heterogeneously composed materials with changes in local stiffness.[7]

A different approach for gradient design is 3D printing, a versatile tool to generate predefined complex hierarchical structures. The simultaneous printing of two or more materials has so far been realized using co-printing approaches from separate cartridges resulting in multilayer constructs[8-11] or concentration patterns[12, 13] or from combined cartridges with a mixing unit[14] to generate scaffolds usable in tissue engineering approaches.[15, 16] The scaffolds have to be biocompatible, biodegradable and should show no toxicity or immune reaction.[17]

To adopt the properties of tissue scaffolds, filler materials can be implemented in soft hydrogel carriers to yield hard tissue engineering materials with increasing mechanical properties, and further providing cell binding motives or chemical cues.[18-20] An interesting type of fillers for hard tissue engineering are fluorapatite particles. Fluoride is known as an essential trace element typically found in enamel and interconnected with dental applications.[21, 22] However, fluoride is also required for bone formation from blood plasma and can induce mineralization processes in collagen or gelatine composites.[23, 24] Fluorapatite (FAp, $Ca_5(PO_4)_3F$) can be produced using precipitation methods[22], or using ultrasonication.[25, 26] FAp has already been used for osteoporosis treatment[21] and bone tissue engineering[27] or as bioactive particles in composite scaffolds[28].

In this study, a combined numerical and experimental approach was used to generate 3D gradient constructs using dispense plotting out of one printer cartridge filled with two different materials. Computational fluid dynamics allowed the prediction of gradient formation using the U-profile of a laminar material flow in a commercial printer cartridge filled with an AB material block system. The findings were transferred to an experimental approach using hydrogels made of the recombinant spider silk protein eADF4(C16). Recombinant spider silk proteins[29] have been previously established concerning hydrogel formation[30], for 3D printing of cell loaded constructs[31] or material blends with gelatine[32]. Dispense plotted gradients were obtained using spider silk-fluorapatite composites, and in addition BALB/3T3 fibroblasts were encapsulated confirming the possibility to generate particle and cell loaded gradient hydrogels in one set-up with controllable properties, which is useful for biofabrication of soft-hard tissue interfaces.

2. Materials and Methods

2.1 Computational Flow Simulation

ANSYS Version15 CFX was used to numerically simulate gradient formation, which follows the Finite Volume Method as discretization technique.[33] Due to symmetry, the printer cartridge was simplified to a quarter model as depicted in Figure SI 1a. Only the fluid domain was modelled within the simulation set-up, where the inner wall of the real cartridge was mimicked by the outer contour of the fluid domain. The piston crown was approximated by a planar wall, as the distance to the outlet was large enough to negelct the influence of the real piston geometry. In addition, extrusion adapters, such as cannulas or cones were not taken into account to simplfy the model. The two phases have been designed as two separate blocks, referred to as AB block system, with a flat contact interface in between. The ratio of the lengths of blocks A and B was 1:2. Both liquids A and B were modelled as homogenous non-Newtonian fluids. The power law according to Ostwald de Waele[34] was used for simulating the shear thinning properties of the recombinant spider silk eADF4(C16) hydrogel:

$$\tau = k\dot{\gamma}^n$$

where $\tau\ [Pa]$ denotes the shear stress and $\dot{\gamma}\ [s^{-1}]$ the shear rate. The flow consistency index was set to $k = 148.89\ Pa * s^n$ with a flow behaviour index of $n = 0.2025$. The material parameters were fitted from measured rheological data within a range of $\dot{\gamma} = 0.1 - 10\ s^{-1}$.

The boundary conditions were defined as depicted in Figure SI 1b. Slip boundary conditions were applied to the outer boundaries of the cartridge, whereas free slip behaviour was defined for the fluid interface of the domains and at symmetry planes of the quarter model.

During the printing process, the piston crown moved towards the outlet activating fluid flow motion. A displacement boundary condition was applied, causing the piston crown wall to move at a speed of $v = 5\ \mathrm{mm/s}$. Since domain B was compressed during the printing process, a mesh motion condition was defined for domain B. The transient flow simulation was performed using a second order backward Euler solution algorithm[35] with a time step size of $\Delta t = 0.1\ \mathrm{s}$.

2.2 3D Dispense Plotting

A regenHU 3D Discovery Gen1 (Switzerland) bioplotter was used for dispense plotting, equipped with cartridges size 3cc and according conical pistons. Luer lock conical needles were adapted to the cartridges with an inner diameter of 14 G, 16 G and 20 G. The different materials were filled in the cartridges manually using block volumes of 0.5-1 mL. Polystyrene Petri dishes were used as substrate (diameter 8 cm, Sarstedt, Germany). A round shaped monolayer construct was designed, and the according G-code was generated using the regenHU BioCAD V1.1 printer software. The applied pressure was set to 0.1 bar for all hydrogels. The printing speed was pre-set to 20 mm/s.

2.3 Recombinant Spider Silk Protein Production, Protein Labelling and Hydrogel Preparation

The amino acid sequence of eADF4(C16) is based on the consensus sequence of the repetitive core domain of the dragline silk fibroin 4 of the European garden spider *Araneus*

diadematus, the so-called C-module sequence (GSSAAAAAAAASGPGG YGPENQGPSGPGGYGPGGP). The C-module is repeated 16 times to yield eADF4(C16). In eADF4(κ16), all glutamic acid residues are replaced by lysine ones. Recombinant spider silk proteins were produced and purified as previously described.[36, 37] Hydrogels formed after protein dialysis and concentration adjustment by water removal using dialysis against PEG (Carl Roth, Germany).[30] Covalent coupling of NHS-fluorescein (Thermo Scientific, Germany) to the amino-terminus of eADF4(C16) was conducted as reported previously[30] with a 10-fold molar excess of dye.

2.4 Fluorescence Analysis and Fluorescence Spectroscopy

Fluorescence images of printed gradient recombinant spider silk hydrogels with and without fluorescent labelling were recorded at CY2 mode (Ex 480/Em 530 nm, exposure 0.05) using an Ettan DIGE imager (GE Healthcare, Sweden).

Fluorescence spectra (n1 = 3 samples per spot on scaffold; n2 = 3 scaffolds) were recorded using a fluorescence spectrometer FP-6300 (JASCO, Germany) and an excitation wavelength of 488 nm at 20 °C. 3 µL of hydrogel samples were taken at equal distances (3 cm) from the printed strands and resuspended in 0.25 mL 10 mM Tris buffer, pH 7.5 before each measurement.

2.5 Fluorapatite Particle Synthesis

14.6 g $CaCl_2$ (Carl Roth, Germany), 14.3 g Na_2HPO_4 (Carl Roth, Germany) and 0.8 g NaF (Carl Roth, Germany) were mixed in dry state, and 40 mL of MilliQ water were added shortly before ultrasonication[38] for 5 min at an energy intake of 18 kJ using a Sonoplus Ultrasonic Homogenizer (Bandelin, Germany) and a KE76 probe. Particles were washed with MilliQ water and air dried at 50 °C over night.

FAp particles were coated in aqueous solution using eADF4(κ16) dissolved in 6 M guanidinium thiocyanate and dialysed against 10 mM Tris buffer, pH 7.5 for 16 h. The coating was performed using 10 mg particles in 1 mg/ml protein solution for 4 h, followed by centrifugation at 13 000 rpm for 10 min and washing in MilliQ water.

2.6 Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy

Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectra (n=3) of particle species were recorded in dry state, using a germanium crystal mounted on a Bruker Tensor 27 (Ettlingen, Germany) at a resolution of 2 cm^{-1} using 100 scans. An atmospheric compensation algorithm was performed using in OPUS 8.0 software to correct water vapour and carbon dioxide fluctuations during the measurement.

2.7 Microscopy

Fluorapatite particles were studied by SEM-imaging after carbon sputtering (20 nm) using a ZEISS Sigma 300 VP and Sigma 500 chamber equipped with an EDS detector (EDAX Pegasus and Octane Super Detector, 60 mm^2 chip, Zeiss, Germany) at an acceleration voltage of 7.5 kV to excite the Kα shell. For SEM imaging of particle-loaded hydrogels, samples were freeze-dried to maintain pore structures before platinum sputtering (2 nm). Images were recorded using a Thermo Scientific (FEI) Apreo VS with a Field Emission Gun at 2 kV and a SE2-detector.

For transmission electron microscopy (TEM) images of fluorapatite particles and hydrogels with FAp particles, samples were immobilized on Pioloform-coated 100-mesh copper grids (Plano GmbH, Germany) and stained with Uranyl acetate. JEM-2100 TEM (JEOL, Japan) was operated at 80 kV, and images were taken using a 4 000 × 4 000 charge-coupled device camera (UltraScan 4000, Gatan, USA) and Gatan Digital Micrograph software (version 1.83.842). Particle size was determined from 10 individual particles using ImageJ software (NHI, USA).

Light microscopy images of cells and hydrogels were recorded in wet state using a Leica DM IL LED microscope (Leica, Germany) and processed using LAS 4.8 software (Leica, Germany).

Confocal laser scanning microscopy was carried out in wet state using a Leica CLSM TCS SP8 (Leica, Germany) and LAS software, and images were processed using ImageJ (NHI, USA).

2.8 Rheology

Rheological data were recorded as triplicate using a Discovery Hybrid Rheometer 3 (TA, USA) with a plate-plate geometry (diameter 25 mm) and a sample volume of 500 µL (n = 3) and a gap size of 500 µm at 25 °C. A wet sponge adapter around the geometry served to prevent the premature drying of the hydrogels. Frequency sweep experiments were recorded at angular frequencies between 0.1-100 rad/s and 100-0.1 rad/s for recovery at 50 % strain. Amplitude sweeps were recorded at 31.4 rad/s and a strain of 0.1-1 000 %.

2.9 Dynamic Light Scattering

Dynamic light scattering (DLS) was measured using a Litesizer^{TM} 500 (Anton Paar, Austria). Diluted particle samples were recorded in 10 mM Tris buffer, pH 7.5 in omega cuvettes at 25 °C. Mean values of zeta potentials were automatically calculated from internal spectra using the Kalliope software and the Smoluchowski model[39].

2.10 Cell culture

Cytotoxicity was analyzed according to DIN EN ISO 10993-5 using BALB/3T3 mouse fibroblasts. BALB/3T3 mouse fibroblasts (ATCC, ACC210) were subsequently cultured in Dulbecco's Modified Eagle Media (Biochrom, Germany) with supplemented 10 % v/v fetal calve serum (Bio&Sell, Germany), 1 % v/v GlutaMax (Invitrogen, USA) and 0.1 % v/v gentamycin sulphate (Sigma-Aldrich, Germany) at 37 °C, 5 % CO_2 and 95 % relative humidity in a cell culture incubator (Thermo Scientific, Germany, HERAcell 150i). Sub-confluent cultures were seeded at 25 000 cells/cm^2 on treated tissue culture plates (TCP, Thermo Fisher Scientific, Germany, Nunclon) 24 h prior to the test (passage 9). Particles, high density polyethylene and organotin-stabilized polyurethane were UV treated for 30 min prior to use (n = 3). For extraction, 10 mg of fluorapatite or eADF4(k16)-coated fluorapatite particles were incubated in 1 mL cell culture media for 24 h at 37 °C. For direct tests, between 10-80 % of the test area were covered with the particles. To quantify cell viability, 10 % v/v CellTiter-Blue reagent (Promega, Germany) were incubated on washed cells for 2.5 h. 100 µL of the sample were analysed concerning resazurin to resofurin transformation at 590 nm using a plate reader (Mithras LB 940, BertholdTechnologies, Germany). Significance was calculated using ANOVA statistic in Origin (Northampton, Massachusetts, USA) in a Tukey test with $p<0.05$.

For gradient printing of cells, 10^6 cells/mL were seeded (at passage 10) with 15 % cell culture media in 3 % w/v spider silk pre-gel solutions complemented with 1 % w/v eADF4(k16)-coated FAp particles and gelled at 37 °C. Cell viability was confirmed using trypan blue (Sigma-Aldrich, UK) and an automatic cell counter (TC20, Bio-Rad). Cells were live-dead stained with calcein acetoxymethylester (calcein AM) and ethidium homodimer I (Invitrogen, Thermo Fisher Scientific, Germany) for 45 min before imaging.

3. Results and Discussion

Dispense plotting depends on parameters such as extrusion velocity, printing speed, strand thickness by nozzle diameter, but also rheological properties of the used materials. There are different types of dispense plotting, and here the focus was on pneumatic extrusion printing of hydrogels. A new set-up was established to process two separate materials out of one printer cartridge. The aim was to generate scaffolds with gradient properties without strand breakup.

Flow Simulation of Gradient Material Generation during 3D Printing

When two materials are present in one printer cartridge, a backmixing effect can be seen both in flow simulations and in printing experiments. This effect was related to the U-profile of a laminar material flow creating a velocity gradient. With the exemplary extrusion velocity of 5 mm/s, a backmixing of two materials could be shown in a cartridge model, creating a gradient profile in the extruded strand. The concentration curve for both fluids at the cartridge outlet is shown in Figure 1 (additional Movie SI 1). When running the simulation with no slip conditions assumed at the cartridge walls, a core-shell effect of both materials was found in addition to mixing as reported earlier[40]. Furthermore, the residual material A at the cartridge walls was extruded after the piston came into contact with the piston crown.

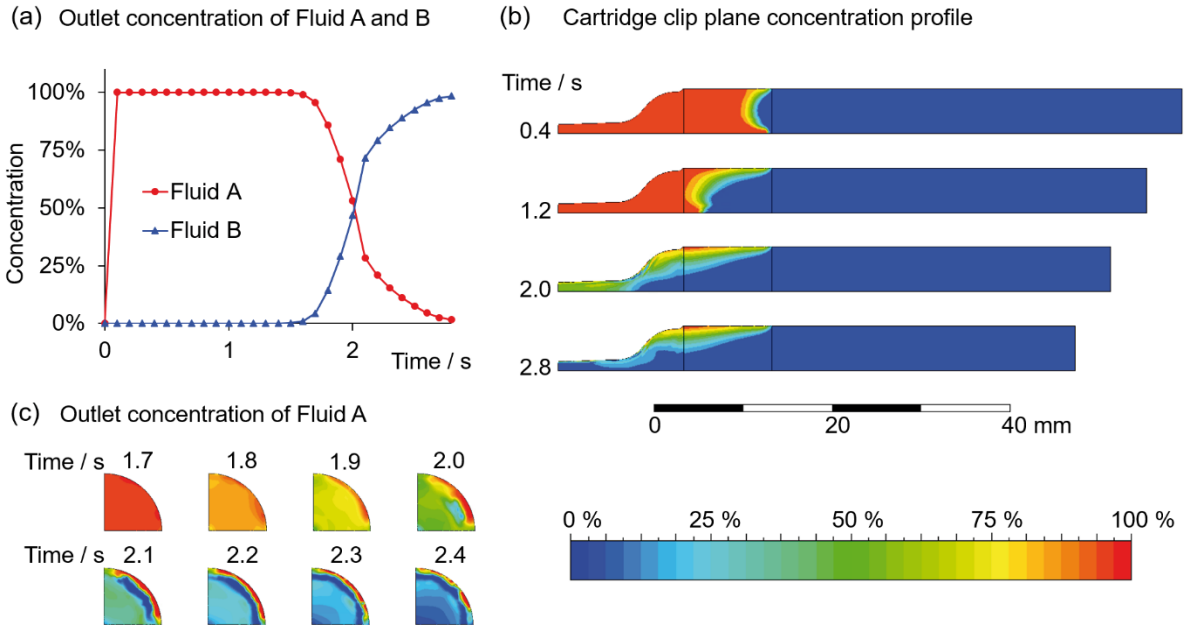

Figure 1: a) Concentration of A and B blocks of an AB block system in a dispense plotting cartridge at the outlet. b) The concentration profile within the cartridge during extrusion shows a backmixing effect. c) Core-shell effect before the final material extrusion.

Experimental Confirmation of Gradient Formation using Dispense Plotting

A commercial 3cc printer cartridge providing a conical piston and a 20 G conical needle was used in a regenHU 3D Discovery printer with a printing speed of 20 mm/s and 0.1 bar. Two different materials, 0.5 mL each, were filled into one cartridge. First, coloured and uncoloured face cream were tested for visualization (Figure SI 2). Then, hydrogels made of 3 % w/v eADF4(C16)[30] were prepared as material block A. In block B, the 3 % w/v eADF4(C16) hydrogel was fluorescently labelled with 10 % w/w of FITC-eADF4(C16). Only a slight decrease in viscosity and stiffness was observed in rheological measurements in case of the eADF4(C16)/FITC-eADF4(C16) blend hydrogel (Figure SI 3), which was in accordance with previous observations.[30] A visual colourization from material B at one side of the printed construct to a transparent hydrogel of material A was obtained after printing (Figure 2a). Fluorescent imaging of the construct showed the gradual increase in fluorescence in accordance to the increase of FITC-eADF4(C16) content (Figure 2b). Small amounts of the hydrogel were retracted from the construct at distinct locations for quantified fluorescence analysis. From unlabelled to fluorescence-labelled hydrogels, an increase in fluorescence intensity was measured using three independent scaffolds (Figure 2c). This profile perfectly fitted the simulated material profile and confirmed the simulation to be in accordance with the experimental set-up. However, after reaching maximum fluorescence, a plateau phase and finally an intensity decrease was recorded. This is indicating the occurrence of an inner filter effect, typically observed at high concentrations of fluorophores.[41]

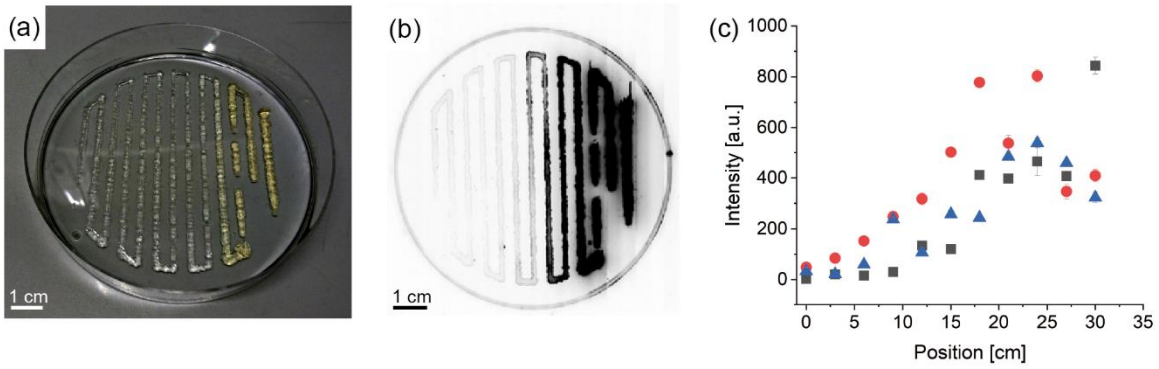

Figure 2: Photographs of 3D printed recombinant spider silk hydrogels (3 % w/v). a) Gradient from non-labelled to fluorescently labelled (yellow) hydrogel in a Petri dish. b) Fluorescence image of the gradient. c) Quantification of fluorescence at distinct locations along the printed strand (red, blue and black symbols are representing measurements for three individual scaffolds).

Spider Silk Fluorapatite Composite Hydrogels and Bioactive Gradient Printing

The tendon-to-bone-interface shows a gradient in mechanical, compositional and structural cues, and, therefore, the applicatability of the presented system was tested in biofabricating such interface. First, to mimic apatite mineralization towards the bone side, fluorapatite particles were gradually integrated.

Fluorapatite (FAp) particles were synthesized using an ultrasonication approach from dry components as previously reported by Willigeroth[38], yielding rod-shaped FAp particles

with 92 ± 27 nm in length and 21 ± 6 nm width as visualized using SEM and TEM (Figure SI 4a+b). The chemical integrity of the particles was identified in comparison to the quantitative occurrence of the elements Ca, F, O and P in FAp stoichiometry using SEM-EDX for element analysis (Figure SI 4c). The variations between calculated and experimentally determined ratios for Ca:F and Ca:P was 2 % and for the Ca:O ratio 21 %. This is a result of a higher content of O than expected, which could be explained by atmospheric or intracrystalline water. Furthermore, ATR-FTIR spectra of FAp particles were compared to commercially available hydroxyapatite particles (Figure SI 4d). Both materials showed similar band assignments as previously reported for both apatite species[42].

The implementation of fluorapatite particles into spider silk solutions before gelation enabled an incorporation into the generated hydrogels as detected using SEM of freeze-dried hydrogels (Figure 3a+b, arrow indicating free particles in a pore) and TEM which indicated intertwined FAp particles with eADF4(C16) fibrils (Figure 3c). Concerning particle content, an increase from 1 % to 3 % w/v yielded a higher initial storage modulus of the hydrogel (Figure 3d). At higher strains, severe differences were visible, as one material started to flow whilst the other was still static. For simultaneous printing of blank and particle-filled hydrogels from one cartridge it was crucial that both materials had flow points in the same order of magnitude. Therefore, a material blend of 3 % w/v eADF4(C16) and 1 % w/v FAp particles was used further on.

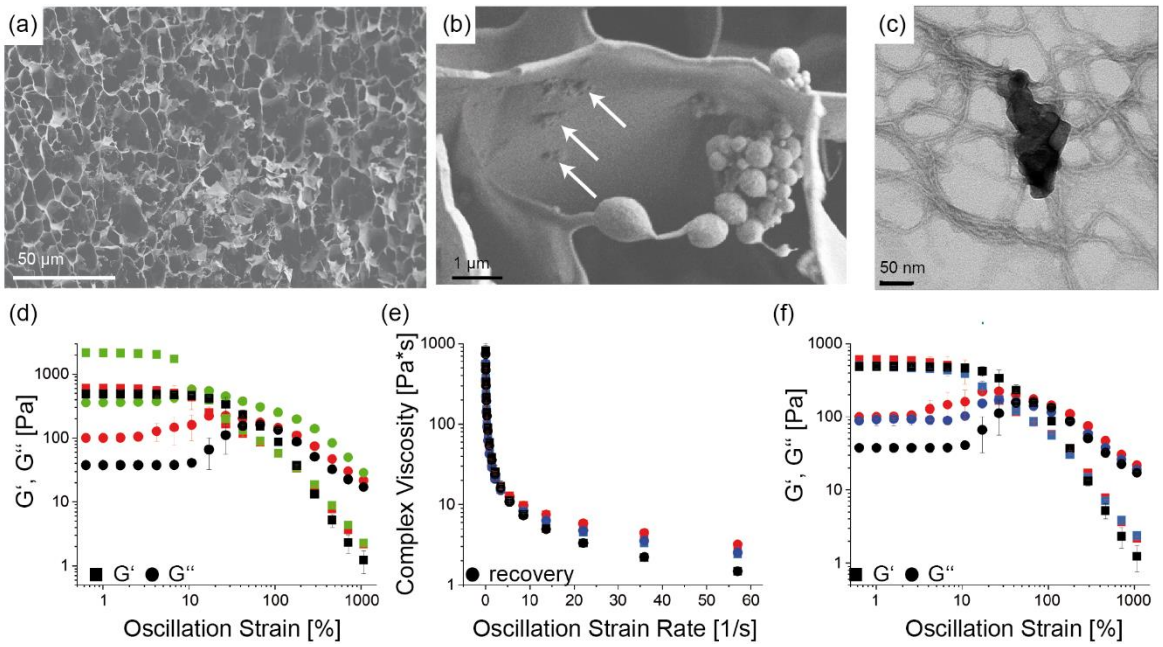

Figure 3: Fluorapatite particles-eADF4(C16) hydrogel composites. a) SEM image of 3 % w/v hydrogels with 1 % w/v FAp. b) SEM image of magnified FAp particles (white arrows indicate particle agglomerates). c) TEM image of eADF4(C16) fibrils, intertwined FAp particles. d) Mean rheological amplitude sweep measurements of 3 % w/v eADF4(C16) (black), 3 % w/v eADF4(C16) + 1 % w/v FAp (red) and 3 % w/v eADF4(C16) + 3 % w/v FAp (green) composite hydrogels. e) Mean rheological frequency sweep measurements of 3 % w/v eADF4(C16) (black), 3 % w/v eADF4(C16) + 1 % w/v FAp (red) and 3 % w/v eADF4(C16) + 1 % w/v kFAp (blue) composite hydrogels. f) Mean rheological amplitude sweep measurements of 3 % w/v eADF4(C16) (black), 3 % w/v eADF4(C16) + 1 % w/v FAp (red) and 3 % w/v eADF4(C16) + 1 % w/v kFAp (blue) composite hydrogels.

One important property of eADF4(C16) hydrogels is their shear thinning behaviour.[30-32] In the presence of FAp particles, the hydrogels also showed shear-thinning behaviour like a non-Newtonian fluid at increasing shear rates and recovery at decreasing shear rates, which is important to gain solid structures after strand deposition upon 3D printing (Figure 3e).

Gradient printing of recombinant spider silk hydrogels with FAp particles (material A) and without (material B) was realized (Figure 4a) with the identical printing parameters as used for single material hydrogels. There was a slight dewetting effect visible between position II and IV (Figure 4a) without influencing the general outcome. Light microscopy images at distinct positions revealed a decreasing particle density in the hydrogel (Figure 4b, I-VI). Small particle aggregates in the lower micrometer range were visible, especially at regions with higher particle concentrations due to particle aggregation.

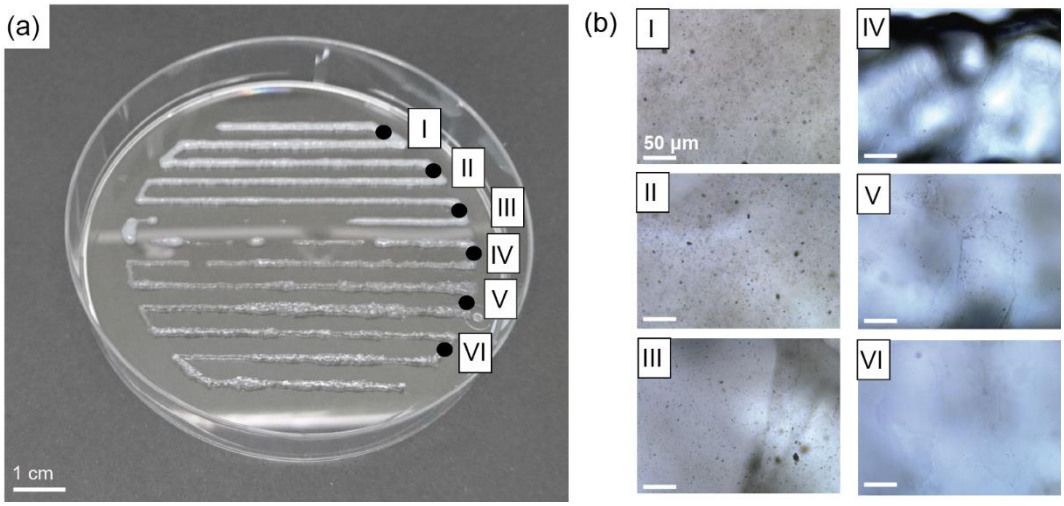

Figure 4: Photograph of a 3D printed gradient of recombinant spider silk hydrogels with and without fluorapatite particles: a) Gradient from 3 % w/v eADF4(C16) + 1 % w/v kFAp (white) to 3 % w/v eADF4(C16) (transparent). b) Light microscopy images at distinct locations of the construct showing decreasing particle concentration (highest at position I and lowest at position VI), scale bar 50 µm.

Biofabrication of Particle and Cell-Loaded Recombinant Spider Silk Hydrogels

As FAp particles showed a negative zeta potential (-22.5 ± 0.9 mV), the interaction of the particles with negatively charged eADF4(C16) in solution could be increased by coating FAp particles with the positively charged spider silk variant eADF4(κ16), referred to as kFAp (zeta potential + 16.5 ± 0.4 mV). The rheological behaviour of the blended hydrogels was not influenced significantly (Figure 3f).

In order to use the composite hydrogels in biofabrication, the incorporated particles were individually tested regarding cell toxicity according to DIN EN ISO 10993-5. An extract test and a direct contact test were carried out. A cytotoxic effect is considered when cell viability is reduced by 30 % referred to high density polyethylene used as positive control. As negative control, organotin-stabilized polyurethane was used. Fluorapatite particles were compared to eADF4(k16)-coated fluorapatite particles in both the extract and contact test. In both cases, the protein coating enhanced the cell viability significantly (Figure SI 5a). Cell toxic effects of apatite particles on cells had already been reported in literature.[43] Here,

cell viablity decreased in the presence of uncoated fluorapatite particles in the direct contact test to 15.7 ± 8.1 % and to 34.8 ± 2.4 % in the extract test. Particles sticked to the cells and could hardly be washed off (Figure SI 5b). In contrast, silk-coated fluorapatite particles showed a much better cell viability of 65.9 ± 4.4 % in the direct contact test and 57.8 ± 10.6 % in the extract test. Besides these enhancing effects on cell viability, a silk-coating could not prevent cytotoxicity to less than 30 % referred to the positive control.

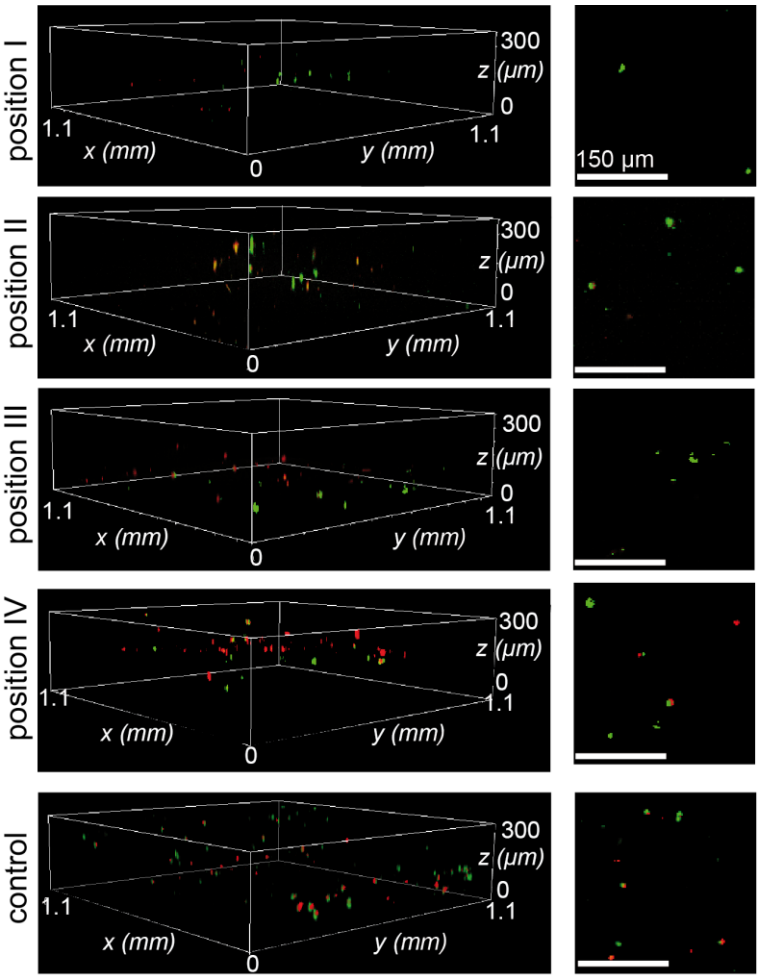

Figure 5: Confocal laser scanning microscopy of BALB/3T3 fibroblasts along a printed gradient from 3 % w/v eADF4(C16) to 3 % w/v eADF4(C16) + 1% w/v kFAp (position I lowest and position III highest concentration) using ethidium homodimer I (red: dead cells)/calcein AM (green: living cells) staining. Not printed cell-loaded 3 % w/v eADF4(C16) + 1 % kFAp w/v hydrogels served as control. Left: side view, right: top view. Scale bars left row 150 µm.

For biofabrication, BALB/3T3 mouse fibroblasts were seeded with 10^6 cells/mL in 3 % w/v eADF4(C16) hydrogels together with 1 % w/v kFAp particles. The AB block system comprised 0.5 mL each of 3 % w/v eADF4(C16) + 1 % w/v kFAp + fibroblasts (material A) and 3 % w/v eADF4(C16) hydrogel (material B). Printing was carried out using a 16 G conical needle. Hydrogels with cells[44] and particles were slightly stiffer as the non-cell-loaded ones. The obtained scaffolds were stained using calcein AM/ethidium homodimer I for live-dead evaluation of the cells. Spider silk hydrogels showed slight red autofluorescence as published previously.[31] The confocal laser scanning microscopy

images at distinct positions on the scaffold confirmed a successful gradient printing of BALB/3T3 fibroblasts (Figure 5). The simultanous processing of apatite particles with a cell-friendly protein coating along with BALB/3T3 cells showed that this printing set-up offers not only the possibility to generate gradients from one printer cartridge but also to incorporate multiple gradient features into one scaffold (here particles and cells), relevant for e.g. biofabrication of tissues at the tendon/bone interface.

4. Conclusions

Computational fluid dynamics combined with dispense plotting enabled to achieve an *in situ* generated gradient construct from one printer cartridge. This set-up could be applied for various material combinations and complexity levels, and mineralization gradients or even mineralization gradients together with cell gradients could be produced. The combination of inorganic fillers and cells simultaneously in a material gradient can yield similar conditions as found at the enthesis, enabling future applications of such set-up in biofabricating tissue for the tendon-to bone interface.

Author Contributions

The concept for this study was initiated by TS and VJN; the manuscript was written by VJN, edited by TS and proofread by all authors; all experimental conduction was done by VJN; simulation was conducted by JW and FH; confocal scanning laser microscopy was done by VTT; CK and B. A.-L. contributed in scientific discussions; TS and FR acquired the funding.

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Declaration of Interest

TS is co-founder and shareholder of AMSilk GmbH.

References

[1] Zylstra U. 1992 *Synthese* **91** (*1*) 111-33.
[2] Claussen KU, Scheibel T, Schmidt HW, Giesa R. 2012 *Macromol Mater Eng* **297** (*10*) 938-57.
[3] Shaw HM, Vázquez OT, McGonagle D, Bydder G, Santer RM, Benjamin M. 2008 *J Anat* **213** (*6*) 718-24.
[4] Genin GM, Kent A, Birman V, Wopenka B, Pasteris JD, Marquez PJ, et al. 2009 *Biophys J* **97** (*4*) 976-85.
[5] Li X, Xie J, Lipner J, Yuan X, Thomopoulos S, Xia Y. 2009 *Nano Lett* **9** (*7*) 2763-8.
[6] Xie JW, Li XR, Lipner J, Manning CN, Schwartz AG, Thomopoulos S, et al. 2010 *Nanoscale* **2** (*6*) 923-6.

[7] Libanori R, Erb RM, Reiser A, Le Ferrand H, Suess MJ, Spolenak R, et al. 2012 *Nat Commun* **3** (*1265*) 1-9.
[8] Gao F, Xu Z, Liang Q, Liu B, Li H, Wu Y, et al. 2018 *Adv Funct Mater* **28** (*13*) 1706644.
[9] Rutz AL, Hyland KE, Jakus AE, Burghardt WR, Shah RN. 2015 *Adv Mater* **27** (*9*) 1607-14.
[10] Kolesky DB, Truby RL, Gladman AS, Busbee TA, Homan KA, Lewis JA. 2014 *Adv Mater* **26** (*19*) 3124-30.
[11] Trachtenberg JE, Placone JK, Smith BT, Fisher JP, Mikos AG. 2017 *J Biomater Sci Polym Ed* **28** (*6*) 532-54.
[12] Khan MS, Fon D, Li X, Tian JF, Forsythe J, Garnier G, et al. 2010 *Colloids Surf B* **75** (*2*) 441-7.
[13] Miller ED, Phillippi JA, Fisher GW, Campbell PG, Walker LM, Weiss LE. 2009 *Comb Chem High Throughput Screen* **12** (*6*) 604-18.
[14] Kokkinis D, Bouville F, Studart AR. 2018 *Adv Mater* **30** (*19*) 1705808.
[15] Mironov V, Prestwich G, Forgacs G. 2007 *J Mater Chem* **17** (*20*) 2054-60.
[16] Mironov V, Visconti RP, Kasyanov V, Forgacs G, Drake CJ, Markwald RR. 2009 *Biomaterials* **30** (*12*) 2164-74.
[17] Mironov V, Trusk T, Kasyanov V, Little S, Swaja R, Markwald R. 2009 *Biofabrication* **1** (*2*) 1-16.
[18] Inzana JA, Olvera D, Fuller SM, Kelly JP, Graeve OA, Schwarz EM, et al. 2014 *Biomaterials* **35** (*13*) 4026-34.
[19] Lin S, T. PS, Daisuke S, Xiuli W, A. LJ, L. KD. 2012 *Adv Healthc Mater* **1** (*6*) 729-35.
[20] Ding Z, Han H, Fan Z, Lu H, Sang Y, Yao Y, et al. 2017 *ACS Appl Mater & Inter* **9** (*20*) 16913-21.
[21] Azami M, Jalilifiroozinezhad S, Mozafari M, Rabiee M. 2011 *Ceram Int* **37** (*6*) 2007-14.
[22] Liu J, Jin TC, Chang S, Czajka-Jakubowska A, Clarkson BH. 2011 *J Biomed Mater Res Part A* **96A** (*3*) 528-34.
[23] Kawska A, Hochrein O, Brickmann J, Kniep R, Zahn D. 2008 *Angew Chem-Int Edit* **47** (*27*) 4982-5.
[24] Tlatlik H, Simon P, Kawska A, Zahn D, Kniep R. 2006 *Angew Chem-Int Edit* **45** (*12*) 1905-10.
[25] Michael FM, Khalid M, Ratnam CT, Chee CY, Rashmi W, Hoque ME. 2016 *Ceram Int* **42** (*5*) 6263-72.
[26] Cao L-Y, Zhang C-B, Huang J-F. 2005 *Ceram Int* **31** (*8*) 1041-4.
[27] Liu J, Wang X, Jin Q, Jin T, Chang S, Zhang Z, et al. 2012 *Biomaterials* **33** (*20*) 5036-46.
[28] Niu Y, Cao L, Wei J, Ma Y, Song S, Weng W, et al. 2014 *J Mat Chem B* **2** 1174.
[29] Leal-Egana A, Scheibel T. 2010 *Biotechnol Appl Biochem* **55** 155-67.
[30] Schacht K, Scheibel T. 2011 *Biomacromolecules* **12** (*7*) 2488-95.
[31] Schacht K, Juengst T, Schweinlin M, Ewald A, Groll J, Scheibel T. 2015 *Angew Chem Int Ed* **54** (*9*) 2816-20.
[32] DeSimone E, Schacht K, Pellert A, Scheibel T. 2017 *Biofabrication* **9** (*4*) 1-14.
[33] Malalasekera W, Versteeg HK. 2007 An Introduction to Computational Fluid Dynamics-The finite volume method. 2 ed. Essex, England: Pearson Education Limited.
[34] Morrison FA. 2001 Understanding Rheology: Oxford University Press.

[35] ANSYS I. ANSYS CFX-Solver Theory Guide. Secondary. 16.2 ed. Canonsburg, USA: ANSYS, Inc.; 2015.
[36] Huemmerich D, Helsen CW, Quedzuweit S, Oschmann J, Rudolph R, Scheibel T. 2004 *Biochemistry* **43** (*42*) 13604-12.
[37] Doblhofer E, Scheibel T. 2015 *J Pharm Sci* **104** (*3*) 988-94.
[38] Willigeroth SF. 2002 Strategien zur Synthese kolloidaler Fluorapatitpartikel und Nanokomposite-Dissertation: Christian-Albrechts-Universität Kiel.
[39] Smoluchowski Mv. 1917 *Z Phys Chem* **92** 129-68.
[40] Hausmann MK, Rühs PA, Siqueira G, Läuger J, Libanori R, Zimmermann T, et al. 2018 *ACS Nano* **12** (*7*) 6926-37.
[41] Kumar Panigrahi S, Kumar Mishra A. 2019 *J Photochem Photobiol C* **41** 100318.
[42] Baddiel CB, Berry EE. 1966 *Spectrochim Acta* **22** (*8*) 1407-16.
[43] Zhao X, Ng S, Heng BC, Guo J, Ma L, Tan TTY, et al. 2013 *Arch Toxicol* **87** (*6*) 1037-52.
[44] DeSimone E, Schacht K, Scheibel T. 2016 *Mater Lett* **183** 101-4.

Supplementary Information

S1: Simulation model.

S2: Gradient printing pre-studies.

S3: Rheology.

S4: Fluorapatite characterization.

S5: DIN EN ISO 10993-5 results for particle species.

Additional Information: Video S6 Flow Simulation.

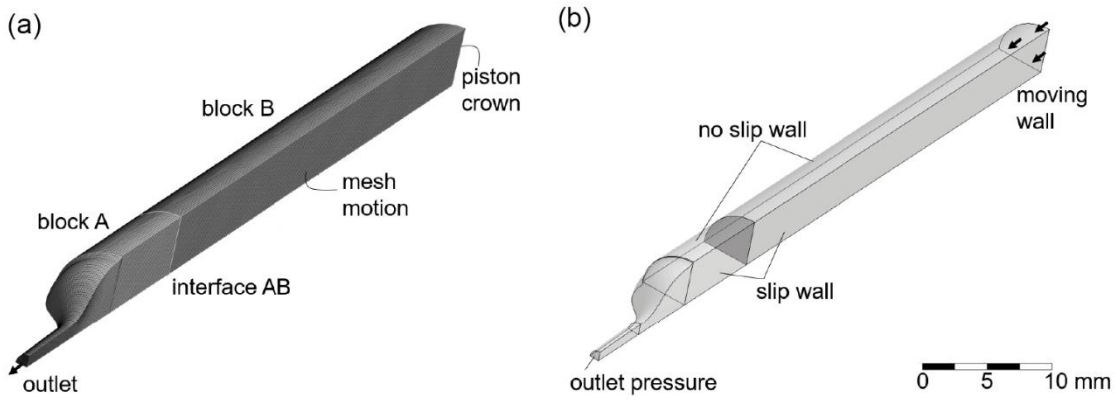


Figure SI 1: Model of the a) block-system and mesh as slip wall and b) boundary conditions.

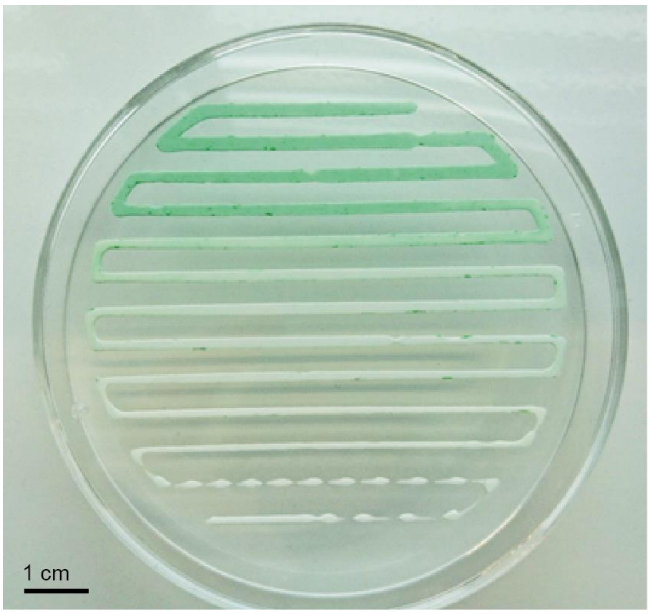


Figure SI 2: Photograph of 3D printing results with coloured water-in-oil emulsion as an exemplary AB block system.

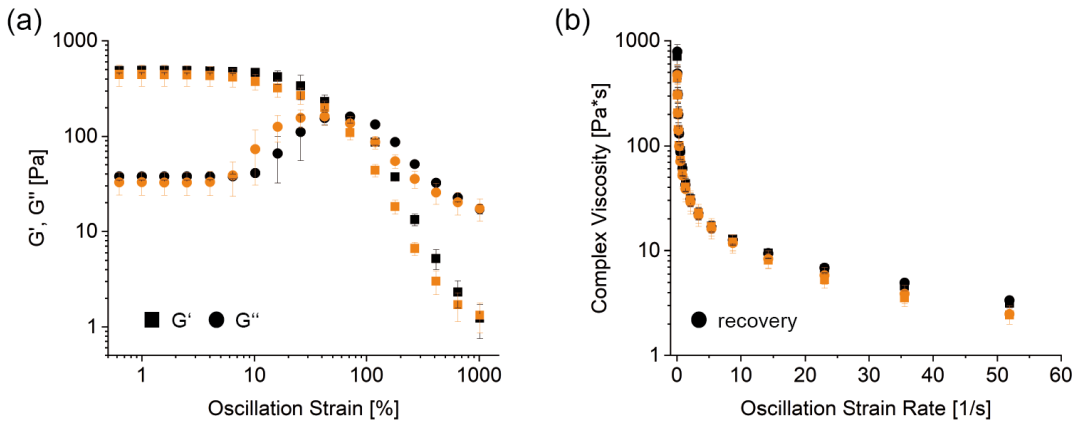
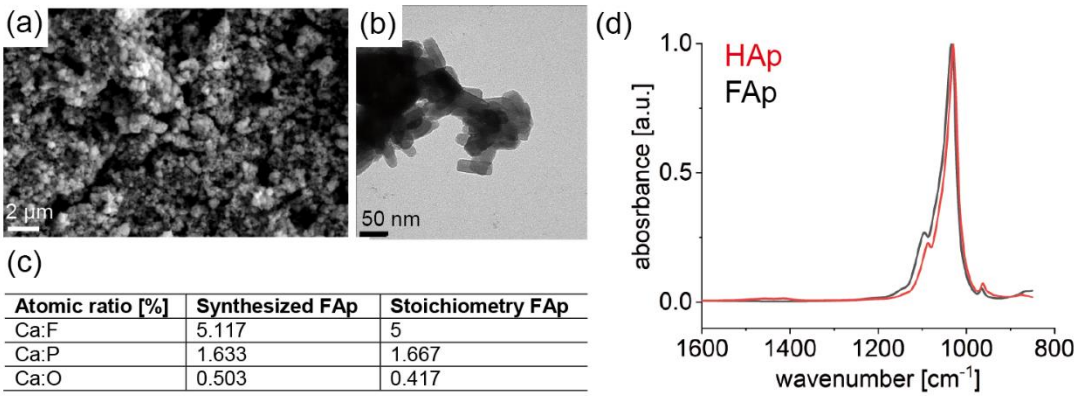


Figure SI 3: Rheological characterization of 3 % w/v eADF4(C16) (black) and eADF4(C16)/FITC-eADF4(C16) (orange) hydrogels: a) Mean amplitude sweep measurements with yield points at the G' and G'' cross-over. b) Mean frequency sweep measurements showing shear-thinning behaviour and recovery.



(c)

Atomic ratio [%]	Synthesized FAp	Stoichiometry FAp
Ca:F	5.117	5
Ca:P	1.633	1.667
Ca:O	0.503	0.417

Figure SI 4: Characterization of fluorapatite particles: a) SEM image of dry FAp particles. b) TEM images of FAp particles. c) SEM-EDX analysis derived atomic ratio of typical elements in fluorapatite compared to its stoichiometry in %. d) Mean ATR-FTIR spectra overlay of synthesized fluorapatite particles with commercially available hydroxyapatite particles as reference.

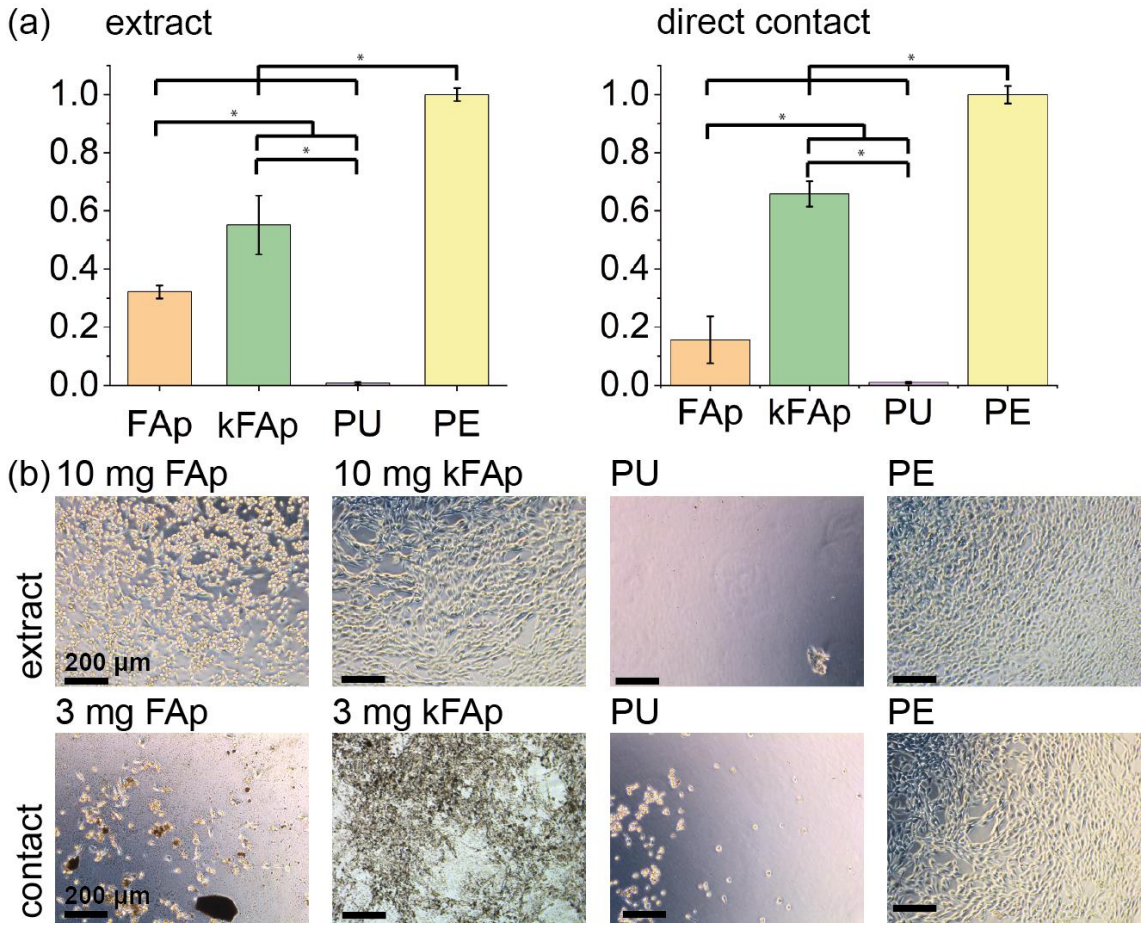


Figure SI 5: a) Quantified viability using Cell Titer Blue assay and b) cell morphology of BALB/3T3 fibroblasts upon contact directly or with an extract of fluorapatite particles or eADF4(κ16) coated fluorapatite particles using a DIN EN ISO 10993-5 test. High density polyethylene served as positive and organotin-stabilized polyurethane as negative control for extract and direct contact test (n=3 for all conditions). Extract and direct contact tests were analsyed using light microscopy, scale bar 200 µm.

7.5 Teilarbeit V

Teilarbeit V erschien am 26.03.2021 unter dem Titel *„Recombinant Spider Silk Gels Derived from Aqueous–Organic Solvents as Depots for Drugs“ in Angewandte Chemie International Edition* und *Angewandte Chemie*.

Neubauer, V. J.; Trossmann, V.T.; Jacobi, S.; Döbl, A.; Scheibel, T. Recombinant Spider Silk Gels Derived from Aqueous–Organic Solvents as Depots for Drugs. *Angewandte Chemie International Edition* **2021**, *60*, 11847-11851. doi.org/10.1002/anie.202103147

Deutsche Version

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Recombinant Spider Silk Gels Derived from Aqueous–Organic Solvents as Depots for Drugs

*Vanessa J. Neubauer, Vanessa T. Trossmann, Sofia Jacobi, Annika Döbl, and Thomas Scheibel**

Abstract: *Hydrogels are widely used in various biomedical applications, as they cannot only serve as materials for biofabrication but also as depots for the administration of drugs. However, the possibilities of formulation of water-insoluble drugs in hydrogels are rather limited. Herein, we assembled recombinant spider silk gels using a new processing route with aqueous–organic co-solvents, and the properties of these gels could be controlled by the choice of the co-solvent. The presence of the organic co-solvent further enabled the incorporation of hydrophobic drugs as exemplarily shown for 6-mercaptopurine. The developed gels showed shear-thinning behaviour and could be easily injected to serve, for example, as drug depots, and they could even be 3D printed to serve as scaffolds for biofabrication. With this new processing route, the formulation of water-insoluble drugs in spider silk-based depots is possible, circumventing common pharmaceutical solubility issues.*

Hydrogels are used for biomedical applications such as tissue engineering, drug delivery, and recently also biofabrication.[1] Besides polysaccharides, such as alginate[2] or chitosan,[3] proteins such as *Bombyx mori* silkworm silk[4] or gelatine[5] have been processed into hydrogels using various methods. Also, hydrogel formation of the recombinant spider silk protein eADF4(C16), which is engineered based on the repetitive core domain of one of the *Araneus diadematus* dragline silk proteins,[6] has been investigated thoroughly.[7] Spider silk hydrogels exhibit shear-thinning behaviour,[7a] which is a crucial requirement for 3D printing and biofabrication[8] or the administration as drug depots.[9] Modifications during processing allowed incorporating water-soluble biologicals in eADF4(C16) hydrogels and their sustained release.[9] However, several pharmaceutical active agents show only poor water solubility or stability and cannot be delivered using hydrogels. Therefore, the aim of this study was to provide drug depots based on gels with the possibility to formulate both water-soluble and water-insoluble drugs.

Ions such as potassium or phosphate effect folding[10] of spider silk proteins,[11] and this kosmotropic ion-triggered structure formation is part of the natural assembly process of spider silk.[12] However, protein folding effects can be also achieved using non-physiological organic co-solvents when they are fully miscible in water.[13] The presence of organic co-solvents with varying polarity can change the solvation conditions, often leading to protein conformational changes.[13] The assumption that especially hydrophobic interactions and hydrogen bonds are driving forces of protein folding is commonly agreed with.[14] Besides intramolecular folding, also intermolecular structure formation can be obtained in aqueous–organic solvents yielding protein assembly and fibrillisation.[15]

Therefore, we investigated a novel gelation route of recombinant spider silk proteins upon fibril assembly in aqueous–organic micro-heterogeneous phases, including the underlying assembly mechanism, and we provide evidence for the use of gels made therewith as drug depots as well as their 3D printability.

Recombinant spider silk proteins have previously been reported to assemble into fibrils and/or particles upon addition of potassium phosphate, and the gained morphology depends on the concentration of the kosmotropic salt.[6,11,16] Here, we investigated assembly in the presence of co-solvents and -solutes such as DMSO and potassium phosphate and obtained characteristic fibrillisation-based sigmoidal turbidity curves[7a] (Figure 1 A). In order to gain more mechanistic insights into this behaviour, three spider silk protein variants differing in their net charge but with otherwise identical amino acid compositions were analysed (see Experimental Section). Potassium phosphate induced fibrillisation of the negatively charged eADF4(C16),[6] which was used as positive control, and its mechanism of fibrillisation has been reported previously.[11] The positively charged eADF4(κ16)[17] showed accelerated protein aggregation and phase separation in the

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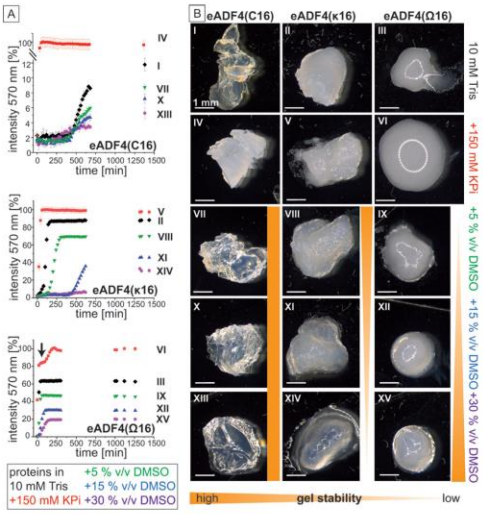


Figure 1. Assembly of three eADF4 variants in presence of different Tris/DMSO volume ratios in comparison to that in presence of 150 mM potassium phosphate (KPi). I–III in 10 mM Tris pH 7.5, IV–VI in presence of 150 mM KPi, VII–IX in presence of 5% (v/v) DMSO, X–XII in presence of 15% (v/v) DMSO, and XIII–XV in presence of 30% (v/v) DMSO. A) Turbidity measurements of eADF4(C16), eADF4(κ16), and eADF4(Ω16) as indicated during fibril formation. Fibril assembly is normalised to KPi samples. The arrow indicates particle formation in case of eADF4(Ω16). B) Stereomicroscopic images of gels at conditions as indicated; scale bars 1 mm. Image VI shows reflections of the light source appearing as a ring. Qualitative gel stabilities are indicated by stability bars.

presence of potassium phosphate, and the uncharged eADF4(Ω16)[18] showed particle formation, indicating a shift towards lower critical potassium phosphate concentrations for particle formation for this variant. The impact of DMSO was analysed in aqueous–organic binary mixtures[19] with different volumes. Like in potassium phosphate, in DMSO the uncharged variant showed the fastest nucleation and fibril growth based on its low electrostatic repulsion, which was least controllable. The positively charged variant showed a fast turbidity increase assuming an aggregation-driven process, yielding gelly morphologies, but no stable gels. The negative variant exhibited the longest lag-phase and yielded well-controllable gels. As this protein is based on the naturally occurring spider silk consensus sequence, the best-controlled assembly behaviour was expected. It has to be mentioned that the addition of DMSO yielded decreased turbidity for all variants and additionally slowed down fibrillisation for eADF4(Ω16) and extended the lag-phase for eADF4(κ16).

Interestingly, gel stability was dependent on both the net charge of the eADF4 variant, as already mentioned above, and the organic additive (Figure 1B), indicating charge-dependent intra- and intermolecular structure formation, as the variants differed in only one amino acid per module in the repetitive sequence. Hydrogen bonds between DMSO and glutamic acid residues within eADF4(C16) were likely the reason for higher gel stability but were independent of the DMSO concentration. In contrast, DMSO seemed to stabilise eADF4(Ω16) gels with increasing concentration, based on hydrogen bonds with glutamine residues. The stability of eADF4(κ16) gelly morphologies decreased with increasing concentration of DMSO, which forms fewer hydrogen bonds with lysine residues.

To investigate the influence of solvent polarity, 3% (w/v) eADF4(C16) solutions, as the best controllable ones, were used in water, and in blends with less polar DMF and with least polar DMSO. Additionally, to gradually increase the concentration of DMSO, 3% (w/v) eADF4(C16) solutions were dialysed against this solvent. The organo-dialysis step allowed a fast solvent exchange and simultaneously lead to highly transparent gels. The effect on protein structure formation was analysed using ATR-FTIR spectroscopy. Secondary structures in gels were derived from the peaks of the amide I and II bands at 1720–1490 cm^{-1} (Figure 2A) and were quantified using Fourier self-deconvolution.

The highest β-sheet content (39 ± 1%) was found in case of Tris-hydrogels as well as gels made in DMSO blends. Tris/DMSO-gels from organo-dialysis showed a lower β-sheet content of 28 ± 5%, which might be caused by faster gelation.

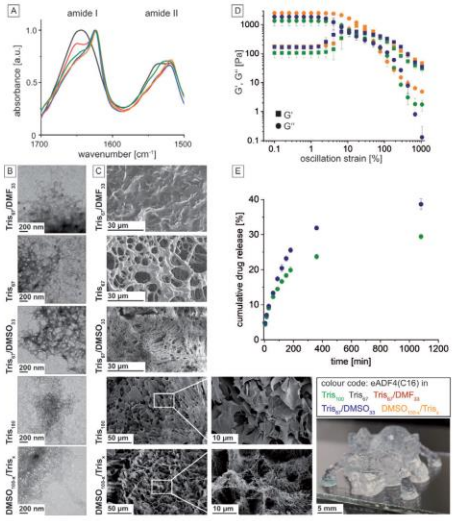


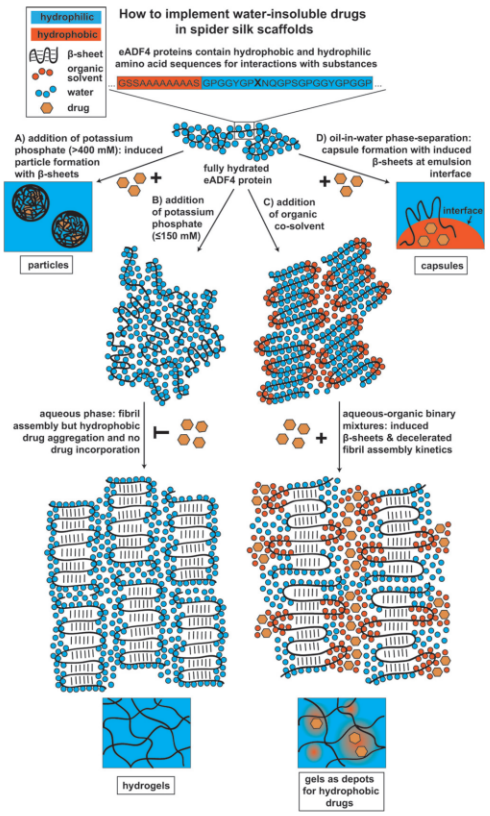
Figure 2. Impact of organic additives on spider silk gels: Comparison of gels from initial 3% (w/v) eADF4(C16) silk solutions in 10 mM Tris pH 7.5 ($Tris_{100}$) diluted with one third volume ratio of the co-solvents water ($Tris_{67}$), DMF ($Tris_{67}/DMF_{33}$), or DMSO ($Tris_{67}/DMSO_{33}$), and gels made upon dialysis against DMSO ($DMSO_{100-x}/Tris_x$). A) Mean ATR-FTIR spectra of the co-solvent gels. B) TEM images of silk fibrils in the co-solvent gels. C) SEM images of the respective co-solvent gels after freeze-drying. D) Mean amplitude sweep rheological measurements of co-solvent gels. E) Normalised cumulative release of 6-mercaptopurine from 2% (w/v) eADF4(C16) $Tris_{100}$ gel (green data points) and from 2% (w/v) eADF4(C16) $Tris_{67}/DMSO_{33}$ gel (blue data points) at 37°C. F) Photo of a 3D printed tarantula from DMSO blend gels ($Tris_{67}/DMSO_{33}$). Scale bar as indicated.

Far fewer β-sheets were found in the presence of water (24 ± 5 %) or in case of blends with DMF (22 ± 4 %). The formation of fibrillar networks was pronounced in the presence of DMSO, indicated by intertwined fibrils as found in TEM images (Figure 2B). In presence of DMF or water, significantly shorter fibrils were formed. SEM imaging showed a sheet-like structure for lyophilised co-solvent gels with DMF. Fused pore structures were found upon water addition. Strikingly, a highly fibrillary and porous structure was present in case of DMSO-triggered gelation. At higher magnification, pore walls showed fibrillar sub-structures in Tris/DMSO-gels after organo-dialysis (Figure 2C). Sharp pore structures were found in Tris-gels with smooth, sheet-like pores as reported previously.[7a] Fibrils in Tris-hydrogels might have collapsed into these sheet structures upon freeze-drying. Rheological characterisation (Figure 2D) showed slightly higher storage and loss moduli for 2 % (w/v) eADF4(C16) Tris/DMSO-gels in comparison to 3 % (w/v) eADF4(C16) Tris-hydrogels. Nonetheless, for Tris/DMSO-gels and Tris-hydrogels, moduli were in the same range, and similar yield points were detected, indicating comparable visco-elastic properties. 3 % (w/v) eADF4(C16) Tris/DMSO-gels from organo-dialysis showed the highest storage modulus and a slight shift in the yield point towards higher oscillation strain, indicating higher resistance of the material until break.

Nonetheless, the new gels showed typical spider silk hydrogel shear-thinning behaviour and stability (Figure S1A,B). Accordingly, using a RegenHU bioplotter, multilayer scaffolds with high shape stability could be 3D printed using the DMSO blend gels (Figure 2F, Figure S3, and Video S4).

To test the application of such gels as drug depots, fluorescein (FITC) was loaded as a first model substance into Tris/DMSO-gels, and a comparison was made to Tris-hydrogels regarding loading and release at 37 °C. Both gel types showed the same release profile (Figure S2), indicating the possibility of generating injectable or transdermal drug-loaded gel depots. Further, the poorly water-soluble 6-mercaptopurine, as clinically relevant cytostatic drug, was loaded into the DMSO-phase of 2 % (w/v) eADF4(C16) blend gels. The drug was incorporated by non-covalent interactions, but in case a reversible coupling is intended, a recently published system with a different spider silk variant can be used.[20] Upon non-covalent introduction in Tris-hydrogels, the water-insoluble drug aggregated during the gelation process and accumulated at the bottom of the gel. In the DMSO blend gel, however, loading and release could be accomplished. These results confirmed the suitability of co-solvent-produced spider silk gels with clinically relevant substances as injectable and even 3D printable drug depots.

Finally, we wanted to unravel the driving force of spider silk assembly in presence of co-solvents. The herein reported route towards spider silk gel formation in aqueous–organic binary mixtures is driven in part by structure formation at organic–water interfaces (Scheme 1). eADF4(C16) was found to form water-insoluble β-sheet structures in microcapsules[21] with barrier function[22] upon adsorption at aqueous–organic interfaces. In case of aqueous–organic binary mixtures, micro-



Scheme 1. Illustration of routes towards drug depots made of recombinant spider silk proteins. A) Potassium phosphate-induced co-precipitation of microparticles and any type of drugs. B) Hydrogels cannot incorporate hydrophobic but only hydrophilic drugs during gelation. C) New route of gel formation, allowing the incorporation of hydrophilic as well as hydrophobic drugs. D) Microcapsules can incorporate hydrophilic, hydrophobic, and amphiphilic (at the aqueous–organic interface) drugs. Aqueous–organic binary mixtures as seen in (C) represent intermediate conditions between hydrogel formation in one homogenous (aqueous) phase (B) and phase-separation-induced β-sheet formation at the water–oil interface (D). **X** = selected amino acids of the silk variants, that is, glutamic acid, glutamine, or lysine.

emulsions can form in presence of organic co-solvents such as DMSO[23] or DMF,[13] which are miscible in water.

The formed interphases are micro-heterogeneous[24] and are based on the interaction of hydrogen bond[25] forming molecules. These interphases can be described by a side-by-side arrangement of both solvents in molecular clusters.[25] DMSO and DMF are amphiphilic molecules, which can form hydrogen bonds, for example, with water but also enable hydrophobic interactions among them or with other molecules such as proteins. Similarly, eADF4 exhibits amphiphilic properties due to its amino acid sequence[26] and can therefore

interact with hydrophobic as well as hydrophilic drugs/molecules. Charged amino acid residues can form hydrogen bonds with DMSO to a varying degree, and negatively charged molecules are favoured over uncharged or positively charged ones.[27]

Spider silk gels made out of aqueous–organic co-solvents enlarge the range of applications of spider silk-based materials since material properties can be controlled by the choice of the co-solvent and of the used spider silk variant. DMSO is FDA approved[28] in topical formulations at concentrations at approximately 30% (v/v). Based on our results it can be applied in spider silk gels in pharmaceutical formulations, e.g. for transdermal applications. These gels allow formulation of water-insoluble drugs along with adjustable organic content to yield biocompatible, biodegradable, non-toxic, non-inflammatory, transdermal, injectable, and even 3D printable drug depots. Depending on the application, the drug–silk interaction can be further controlled, for example, by reversible covalent bonds.[20]

Experimental Section

The engineered spider silk protein eADF4(C16) comprises 16 repeats of the consensus sequence GSSAAAAAAAASGPGGYG-PENQGPSGPGGYGPGGP (named C-module). The C-module is based on the consensus sequence of the repetitive core domain of the MaSp2 dragline silk fibroin 4 of the European garden spider *Araneus diadematus*.[6] eADF4(C16) was purchased from AMSilk GmbH (Planegg, Germany). In variant eADF4(κ16),[17] all glutamic acid residues are substituted with lysine and in eADF4(Ω16)[18a] with glutamine ones. Both proteins were produced and purified as reported earlier.[6,17,18] Spider silk proteins were dissolved in 6 M guanidinium thiocyanate (Carl Roth, Karlsruhe, Germany) and dialysed against 10 mM Tris buffer, pH 7.5 for several hours, whereas the dialysis of eADF4(Ω16) was done at 4 °C. Concentration adjustment was conducted, if necessary, by follow-up dialysis in 20% (w/v) poly(ethylene glycol) (PEG, 20 kDa, Carl Roth, Karlsruhe, Germany) as reported previously[7a] or using a high vacuum concentrator (Speedvac, Eppendorf). For biphasic gels, co-solvents or co-solute buffers were blended with 3% (w/v) silk solutions and gelled at 37 °C. For organo-dialysis gels, 3% (w/v) silk solutions were dialysed against small volumes (1:100 volume ratio) of DMSO (Carl Roth, Karlsruhe, Germany) at RT for 4 h without further thermal treatment.

For analysis of the gelation kinetics of recombinant spider silk proteins, turbidity changes were monitored at 570 nm using a microplate reader (Mithras LB 940, Berthold Technologies, Germany). Triplicates of 100 µL aliquots were prepared for all solutions (3% (w/v) eADF4(C16), 2% (w/v) eADF4(κ16), and 1% (w/v) eADF4(Ω16)). Spider silk solutions were diluted by addition of different volumes of DMSO to reach final concentrations of 5, 15, and 30% (v/v) DMSO in the blend. As a control, 150 mM potassium phosphate was used as fibrillisation trigger, and data were normalised to this sample's endpoint.

Gel samples were transferred on glass slides for image collection using a Leica M205C stereomicroscope (Wetzlar, Germany) with Leica LAS software and light reflection from dark field mode. The microscope was equipped with a polarisation lens and a 0.63× objective. For scanning electron microscopy (SEM), lyophilised gel samples were investigated after platinum sputtering (2 nm). Images were recorded using a Thermo Scientific (FEI) Apreo VS with a Field Emission Gun at 2 kV and a SE2-detector. Transmission electron microscopy (TEM) images of stained (2% uranyl acetate) spider silk fibrils immobilised on Pioloform-coated 100-mesh copper grids (Plano GmbH, Germany) were recorded using a JEM-2100 TEM (JEOL, Japan), operated at 80 kV, and imaging was carried out using a 4000 × 4000 charge-coupled device camera (UltraScan 4000, Gatan, USA) and Gatan Digital Micrograph software (version 1.83.842).

Rheological behaviour was investigated using a Discovery Hybrid Rheometer 3 (TA, USA) with a plate–plate geometry (diameter 25 mm) at 25 °C. To prevent drying effects on the gels, a wet sponge adapter was used. Amplitude sweeps ($n = 3$) were recorded as triplicates at 31.4 rad s^{-1} and a strain of 0.1–1000%.

Attenuated total reflectance-Fourier transformation infrared spectroscopy (ATR-FTIR) was conducted with lyophilised gel samples. Spectra ($n = 3$) were recorded using a Bruker Tensor 27 (Ettlingen, Germany) with a germanium crystal at a spectral resolution of 2 cm^{-1} with 100 scans. Atmospheric compensation algorithm was applied in OPUS 8.0 software to correct water vapour and carbon dioxide fluctuations during the measurement. Fourier self-deconvolution was carried out as reported previously[29] with band assignment for partial secondary structure determination.[30]

To study the release of 6-mercaptopurine (Sigma, USA) from 2% (w/v) $Tris_{100}$ and 2% (w/v) $Tris_{67}$/$DMSO_{33}$ eADF4(C16) gels, the drug was dissolved in 20 µL DMSO and added to the spider silk solutions or the DMSO-phase. Triplicate release measurements were conducted in 1:1 blends of MilliQ water:DMSO. UV absorbance was monitored using a UV spectrometer (Genesys 10S UV/Vis, Thermo Scientific). Spectra were recorded between 200 and 600 nm, and peak maxima at 328 nm were used to determine cumulative release curves.

3D dispense plotting was carried out using a RegenHU 3D Discovery Gen1 (Switzerland) bioplotter with cartridges size 3cc and according pistons. The printing speed was pre-set to 10 mm s^{-1}. 2% (w/v) $Tris_{67}$/$DMSO_{33}$ eADF4(C16) gels were printed with Luer lock plastic needles with an inner diameter of 0.41 mm and an applied pressure of 0.3 bar.

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Conflict of interest

TS is founder and share-holder of AMSilk GmbH.

Keywords: binary mixtures · co-solvent · hydrophobic effect · micro-heterogeneity · self-assembly

[1] J. L. Drury, D. J. Mooney, *Biomaterials* **2003**, *24*, 4337 – 4351.
[2] a) W.-H. Tan, S. Takeuchi, *Adv. Mater.* **2007**, *19*, 2696 – 2701; b) A. G. Tabriz, M. A. Hermida, N. R. Leslie, W. Shu, *Biofabrication* **2015**, *7*, 045012.
[3] a) N. Bhattarai, J. Gunn, M. Zhang, *Adv. Drug Delivery Rev.* **2010**, *62*, 83 – 99; b) Y. Hong, H. Song, Y. Gong, Z. Mao, C. Gao, J. Shen, *Acta Biomater.* **2007**, *3*, 23 – 31.
[4] N. Wu, H. Yu, M. Sun, Z. Li, F. Zhao, Y. Ao, H. Chen, *ACS Appl. Biol. Mater.* **2020**, *3*, 721 – 734.
[5] T. Zehnder, B. Sarker, A. R. Boccaccini, R. Detsch, *Biofabrication* **2015**, *7*, 025001.
[6] D. Huemmerich, C. W. Helsen, S. Quedzuweit, J. Oschmann, R. Rudolph, T. Scheibel, *Biochemistry* **2004**, *43*, 13604 – 13612.

[7] a) K. Schacht, T. Scheibel, *Biomacromolecules* **2011**, *12*, 2488 – 2495; b) E. DeSimone, K. Schacht, T. Scheibel, *Mater. Lett.* **2016**, *183*, 101 – 104; c) E. DeSimone, K. Schacht, T. Jüngst, J. Groll, T. Scheibel, *Pure Appl. Chem.* **2015**, *87*, 737 – 749.

[8] a) K. Schacht, T. Juengst, M. Schweinlin, A. Ewald, J. Groll, T. Scheibel, *Angew. Chem. Int. Ed.* **2015**, *54*, 2816 – 2820; *Angew. Chem.* **2015**, *127*, 2858 – 2862; b) E. DeSimone, K. Schacht, A. Pellert, T. Scheibel, *Biofabrication* **2017**, *9*, 044104.

[9] S. Kumari, H. Bargel, M. U. Anby, D. Lafargue, T. Scheibel, *ACS Biomater. Sci. Eng.* **2018**, *4*, 1750 – 1759.

[10] F. Hofmeister, *Arch. Exp. Pathol. Pharmakol.* **1888**, *24*, 247 – 260.

[11] U. K. Slotta, S. Rammensee, S. Gorb, T. Scheibel, *Angew. Chem. Int. Ed.* **2008**, *47*, 4592 – 4594; *Angew. Chem.* **2008**, *120*, 4668 – 4670.

[12] a) L. Eisoldt, A. Smith, T. Scheibel, *Mater. Today* **2011**, *14*, 80 – 86; b) E. Doblhofer, A. Heidebrecht, T. Scheibel, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 9361 – 9380; c) M. Heim, D. Keerl, T. Scheibel, *Angew. Chem. Int. Ed.* **2009**, *48*, 3584 – 3596; *Angew. Chem.* **2009**, *121*, 3638 – 3650.

[13] D. R. Canchi, A. E. García, *Annu. Rev. Phys. Chem.* **2013**, *64*, 273 – 293.

[14] C. N. Pace, S. Treviño, E. Prabhakaran, J. M. Scholtz, *Philos. Trans. R. Soc. London Ser. B* **2004**, *359*, 1225 – 1235.

[15] N. Javid, K. Vogtt, C. Krywka, M. Tolan, R. Winter, *ChemPhysChem* **2007**, *8*, 679 – 689.

[16] A. Lammel, M. Schwab, U. Slotta, G. Winter, T. Scheibel, *ChemSusChem* **2008**, *1*, 413 – 416.

[17] E. Doblhofer, T. Scheibel, *J. Pharm. Sci.* **2015**, *104*, 988 – 994.

[18] a) S. Kumari, G. Lang, E. DeSimone, C. Spengler, V. T. Trossmann, S. Lücker, M. Hudel, K. Jacobs, N. Krämer, T. Scheibel, *Data Brief* **2020**, *32*, 106305; b) S. Kumari, G. Lang, E. DeSimone, C. Spengler, V. T. Trossmann, S. Lücker, M. Hudel, K. Jacobs, N. Krämer, T. Scheibel, *Mater. Today* **2020**, *41*, 21 – 33.

[19] M. R. Harpham, N. E. Levinger, B. M. Ladanyi, *J. Phys. Chem. B* **2008**, *112*, 283 – 293.

[20] H. M. Herold, A. Döbl, S. Wohlrab, M. Humenik, T. Scheibel, *Biomacromolecules* **2020**, *21*, 4904 – 4912.

[21] K. D. Hermanson, D. Huemmerich, T. Scheibel, A. R. Bausch, *Adv. Mater.* **2007**, *19*, 1810 – 1815.

[22] K. D. Hermanson, M. B. Harasim, T. Scheibel, A. R. Bausch, *Phys. Chem. Chem. Phys.* **2007**, *9*, 6442 – 6446.

[23] J. Hansen, F. Platten, D. Wagner, S. U. Egelhaaf, *Phys. Chem. Chem. Phys.* **2016**, *18*, 10270 – 10280.

[24] D. N. Shin, J. W. Wijnen, J. B. F. N. Engberts, A. Wakisaka, *J. Phys. Chem. B* **2002**, *106*, 6014 – 6020.

[25] a) S. Roy, S. Banerjee, N. Biyani, B. Jana, B. Bagchi, *J. Phys. Chem. B* **2011**, *115*, 685 – 692; b) A. Wakisaka, H. Abdoul-Carime, Y. Yamamoto, Y. Kiyozumi, *J. Chem. Soc. Faraday Trans.* **1998**, *94*, 369 – 374; c) A. Wakisaka, T. Ohki, *Faraday Discuss.* **2005**, *129*, 231 – 245; d) D. B. Wong, K. P. Sokolowsky, M. I. El-Barghouthi, E. E. Fenn, C. H. Giammanco, A. L. Sturlaugson, M. D. Fayer, *J. Phys. Chem. B* **2012**, *116*, 5479 – 5490.

[26] C. B. Borkner, S. Lentz, M. Müller, A. Fery, T. Scheibel, *ACS Appl. Polym. Mater.* **2019**, *1*, 3366 – 3374.

[27] K. L. Shaw, G. R. Grimsley, G. I. Yakovlev, A. A. Makarov, C. N. Pace, *Protein Sci.* **2001**, *10*, 1206 – 1215.

[28] K. Capriotti, J. A. Capriotti, *J. Clin. Aesthet. Dermatol.* **2012**, *5*, 24 – 26.

[29] J. Petzold, T. B. Aigner, F. Touska, K. Zimmermann, T. Scheibel, F. B. Engel, *Adv. Funct. Mater.* **2017**, *27*, 1701427.

[30] X. Hu, D. Kaplan, P. Cebe, *Macromolecules* **2006**, *39*, 6161 – 6170.

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Rekombinante Spinnenseidengele aus wässrig-organischen Mischphasen als Wirkstoffdepots

*Vanessa J. Neubauer, Vanessa T. Trossmann, Sofia Jacobi, Annika Döbl und Thomas Scheibel**

Abstract: *Hydrogele finden weitreichend Anwendung in der Biomedizin, wo sie nicht nur als Material in der Biofabrikation, sondern u. a. auch als Wirkstoffdepots verwendet werden. Die Möglichkeiten, wasserunlösliche Wirkstoffe in Hydrogelen zu formulieren, sind jedoch begrenzt. In dieser Studie konnten rekombinante Spinnenseidenproteine durch ein neuartiges Herstellungsverfahren in wässrig-organischen Mischphasen zu Gelen selbstassemblieren. Deren Eigenschaften konnten dabei durch die Wahl der Lösungsmittel kontrolliert werden. Die Gegenwart einer organischen Phase erlaubte das Einbringen hydrophober Wirkstoffe, was exemplarisch anhand von 6-Mercaptopurin gezeigt wurde. Die entwickelten Gele zeigten scherverdünnende Eigenschaften und konnten injiziert (zum Beispiel für Anwendungen als Wirkstoffdepot) und sogar 3D-verdruckt werden, um Gerüste für die Geweberegeneration herzustellen. Mithilfe dieser neuen Herstellungsroute können nun wasserunlösliche Wirkstoffe in Spinnenseidendepots formuliert und damit gängige pharmazeutische Löslichkeitsgrenzen überwunden werden.*

Hydrogele werden für biomedizinische Anwendungen, wie Geweberegeneration, Wirkstofftransport und neuerdings auch Biofabrikation, genutzt.[1] Neben Polysacchariden, wie Alginat[2] oder Chitosan,[3] wurden bereits auch Proteine der Seidenspinnerseide[4] oder Gelatine[5] mithilfe verschiedener Herstellungsmethoden zu Hydrogelen verarbeitet. Die Hydrogel-Bildung des rekombinanten Spinnenseidenproteins eADF4(C16), welches auf Basis der repetitiven Kerndomäne eines Proteins des Abseilfadens von *Araneus diadematus* designt wurde,[6] ist bereits intensiv untersucht worden.[7] Spinnenseidenhydrogele besitzen scherverdünnende Eigenschaften,[7a] was eine essentielle Voraussetzung für den 3D-Druck und die Biofabrikation[8] oder den Einsatz als Wirkstoffdepot darstellt.[9] Anpassungen während der Herstellung erlaubten die Formulierung wasserlöslicher Biomoleküle in eADF4(C16)-Hydrogelen und deren nachhaltige Freisetzung.[9] Einige pharmazeutisch interessante Agenzien sind jedoch nur begrenzt wasserlöslich oder -stabil und können daher nicht aus Hydrogelen freigesetzt werden. Aus diesem Grund war Ziel dieser Studie, Gel-basierte Wirkstoffdepots zur Formulierung von sowohl wasserlöslichen als auch wasserunlöslichen Wirkstoffen herzustellen.

Kalium- oder Phosphat-Ionen können Faltungseffekte[10] in Spinnenseidenproteinen[11] hervorrufen. Diese durch kosmotrope Ionen induzierte Strukturbildung findet ebenfalls während des natürlichen Assemblierungsverhaltens von Spinnenseide statt.[12] Faltungseffekte können jedoch auch durch nicht-physiologische, organische Co-Lösungsmittel erzielt werden, vorausgesetzt sie sind vollständig mit Wasser mischbar.[13] Die Anwesenheit organischer Co-Lösungsmittel mit variierender Polarität kann die Löslichkeitszustände verändern und somit in vielen Fällen Konformationsänderungen von Proteinen hervorrufen.[13] Es ist eine weithin akzeptierte Annahme, dass besonders hydrophobe Wechselwirkungen und Wasserstoffbrückenbindungen eine generell treibende Kraft der Proteinfaltung darstellen.[14] Neben intramolekularer Faltung kann in wässrig-organischen Mischphasen auch intermolekulare Strukturbildung erreicht werden, die letztlich zur Assemblierung und Fibrillisierung von Proteinen führt.[15]

Aus diesem Grund wurden neuartige Gelierungsprozesse rekombinanter Spinnenseidenproteine durch Fibrillisierung in mikro-heterogenen, wässrig-organischen Mischphasen untersucht, die zugrunde liegenden Assemblierungsmechanismen aufgeklärt und praktische Anwendungsbeispiele der Gele als Wirkstoffdepots und ihre Verarbeitung mittels 3D Drucker gezeigt.

Rekombinante Spinnenseidenproteine wurden bereits in Abhängigkeit von der Kaliumphosphat-Konzentration zu Fibrillen und/oder Partikeln assembliert, wobei die erhaltene Morphologie von der Konzentration des kosmotropen Salzes abhängt.[6,11,16] In dieser Studie wurde die Assemblierung in Gegenwart von Co-Lösungsmitteln und Salzen, wie bei-

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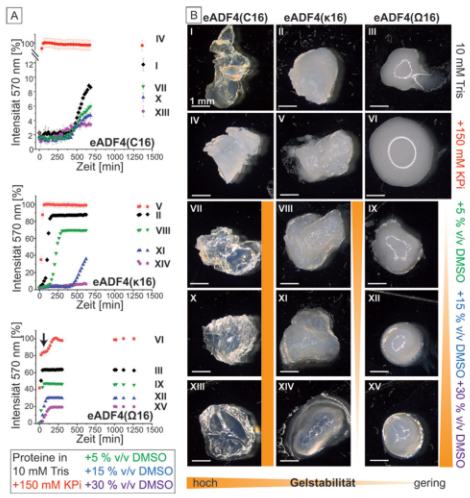


Abbildung 1. Assemblierung von drei eADF4-Varianten in Mischphasen mit unterschiedlichen Tris/DMSO-Volumenverhältnissen im Vergleich zur Assemblierung in 150 mM Kaliumphosphat (KPi). I–III in 10 mM Tris pH 7,5, IV–VI in Gegenwart von 150 mM KPi, VII–IX in Gegenwart von 5 % (v/v) DMSO, X–XII in Gegenwart von 15 % (v/v) DMSO und XIII–XV in Gegenwart von 30 % (v/v) DMSO. A) Trübungsmessungen von eADF4(C16), eADF4(κ16) und eADF4(Ω16) als Indikator der Fibrillenbildung. Die Fibrillisierung wurde auf die Werte für KPi normiert. Der Pfeil zeigt Partikelbildung im Falle von eADF4(Ω16). B) Stereomikroskopische Aufnahmen der Gele unter allen untersuchten Bedingungen wie angegeben; Maßstab 1 mm. Teilbild VI zeigt ringförmige Reflexionen der Lichtquelle. Qualitative Eindrücke der Gelstabilität sind durch Balken dargestellt.

spielsweise DMSO und Kaliumphosphat, untersucht. Dabei konnten charakteristische, sigmoidale Trübungskurven aufgenommen werden, die auf Fibrillisierung zurückzuführen waren[7a] (Abbildung 1 A). Um nähere mechanistische Einblicke in dieses Verhalten zu erlangen, wurden drei Spinnenseidenproteinvarianten, die sich zwar in ihrer Nettoladung unterscheiden, sonst aber identische Aminosäurezusammensetzung aufweisen (siehe Experimentelles), untersucht. Kaliumphosphat-induzierte Fibrillisierung der negativ geladenen Variante eADF4(C16)[6] wurde als Positivkontrolle genutzt, da dieser Fibrillisierungsmechanismus bereits im Vorfeld aufgeklärt worden war.[11] Die positiv geladene Variante eADF4(κ16)[17] zeigte in Anwesenheit von Kaliumphosphat eine beschleunigte Proteinaggregation und Phasenseparation, während die ungeladene Variante eADF4(Ω16)[18] Partikelbildung zeigte. Dadurch erfolgte eine Verschiebung hin zu einer niedrigeren, kritischen Kaliumphosphatkonzentration für eine Partikelbildung für diese Variante. Der Einfluss von DMSO wurde daraufhin in wässrig-organischen, binären Mischungen[19] mit unterschiedlichen Volumenanteilen untersucht. Die ungeladene Variante wies durch die geringe elektrostatische Abstoßung, ähnlich wie mit Kaliumphosphat, auch in DMSO die schnellste Keimbildung und somit Fibrillenwachstum auf, was dementsprechend kaum kontrollierbar war. Die positiv geladene Variante zeigte eine rasche Trübung, welche vermutlich auf Aggregationsprozesse zurückzuführen war, und sie bildete nur gallertartige Morphologien, aber keine stabilen Gele. Die negative Variante wies die längste Latenz-Phase auf und bildete somit Gele in einer kontrollierbaren Art und Weise. Da dieses Protein auf der natürlich vorkommenden Konsensussequenz der Spinnenseide basiert, wurde bereits im Vorfeld das am besten kontrollierbare Verhalten erwartet und hier experimentell bestätigt. Dabei muss erwähnt werden, dass die Zugabe von DMSO bei allen Varianten zu einer verringerten Trübung führte. Weiterhin konnten im Fall von eADF4(Ω16) eine langsamere Fibrillisierung und bei eADF4(κ16) eine verlängerte Latenz-Phase beobachtet werden.

Interessanterweise war die Gelstabilität, wie bereits erwähnt, sowohl von der Nettoladung der eADF4-Variante, als auch vom organischen Additiv abhängig (Abbildung 1 B). Dies ließ auf ladungsabhängige intra- und intermolekulare Strukturbildung schließen, da sich die Varianten nur in einer Aminosäure pro repetitives Modul unterscheiden. Wasserstoffbrückenbindungen zwischen eADF4(C16)-Glutaminsäureseitenketten und DMSO, unabhängig von der DMSO-Konzentration, waren in diesem Fall wahrscheinlich die Ursache für die gesteigerte Gelstabilität. Im Gegensatz dazu wurden die eADF4(Ω16)-Gele erst mit ansteigender DMSO-Konzentration stabiler, was ebenfalls auf Wasserstoffbrücken, dieses Mal aber zwischen DMSO und Glutaminseitenketten, basiert. Die Stabilität der gelartigen eADF4(κ16)-Morphologien nahm dagegen mit steigender Konzentration von DMSO ab, welches offenbar mit Lysinseitenketten weniger Wasserstoffbrücken bildet.

Da sich eADF4(C16) als am besten kontrollierbar erwiesen hat, wurden wässrige Lösungen aus 3 % (w/v) eADF4(C16) mit Wasser und, im Vergleich dazu, mit weniger polarem DMF und dem am wenigsten polaren DMSO versetzt, um den Einfluss der Lösemittelpolarität weiter zu untersuchen. Um auch einen graduellen Anstieg der DMSO-Konzentration zu ermöglichen, wurden wässrige 3 % (w/v) eADF4(C16)-Lösungen gegen DMSO dialysiert. Diese so genannte „Organo-Dialyse“ erlaubte einen schnellen Austausch des Lösungsmittels und lieferte gleichzeitig hoch transparente Gele.

Der Einfluss auf Proteinstrukturbildung wurde mittels ATR-FTIR-Spektroskopie untersucht. Die Sekundärstrukturanteile in den Gelen wurden anhand der Amid-I- und -II-Banden zwischen 1720 und 1490 cm^{-1} abgeleitet (Abbildung 2 A) und über Fourier-Selbstdekonvolution quantifiziert. Den größten Anteil an β-Faltblattstrukturen (39 ± 1 %) wiesen Tris-Hydrogele und Gele mit DMSO-Anteil auf. Tris/DMSO-Gele, welche durch Organo-Dialyse hergestellt wurden, zeigten mit 28 ± 5 % geringere Anteile an β-Faltblattstrukturen, was auf eine schnellere Gelierung zurückzuführen sein könnte. Deutlich weniger β-Faltblätter wurden bei Mischverhältnissen mit Wasser (24 ± 5 %) oder mit DMF (22 ± 4 %) gefunden. Die Ausbildung fibrillärer Netzwerke war in Gegenwart von DMSO stark ausgeprägt und wurde durch verschlungene Fibrillen in TEM-Aufnahmen sichtbar (Abbildung 2 B). In Gegenwart von DMF oder Wasser wurden dagegen signifikant kürzere Fibrillen gebildet. REM-

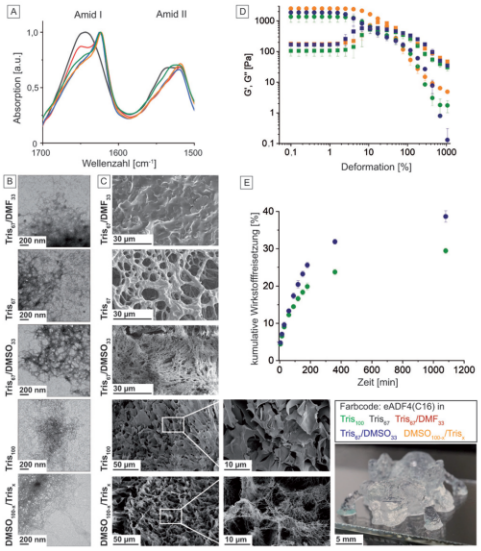


Abbildung 2. Einfluss organischer Additive auf Spinnenseidengele: Gele aus einer 3% (w/v) eADF4(C16)-Spinnenseiden-Ausgangslösung in 10 mM Tris pH 7,5 ($Tris_{100}$) stehen im Vergleich zu Gelen, bei denen die Ausgangslösung zu einem Volumendrittel aus Wasser ($Tris_{67}$), DMF ($Tris_{67}/DMF_{33}$) oder DMSO ($Tris_{67}/DMSO_{33}$) bestand, sowie zu Gelen nach Dialyse gegen DMSO ($DMSO_{100-x}/Tris_x$). A) Gemittelte ATR-FTIR-Spektren der Mischphasengele. B) TEM-Aufnahmen der Seidenfibrillen in Mischphasengelen. C) REM-Aufnahmen der entsprechenden Mischphasengele in gefriergetrocknetem Zustand. D) Gemittelte Rheologiemessungen mittels Amplitudenscan der Mischphasengele. E) Normierte, kumulative Freisetzungsmessungen für 6-Mercaptopurin aus 2% (w/v) eADF4(C16)-$Tris_{100}$-Gelen (grüne Messpunkte) und aus 2% (w/v) eADF4(C16)-$Tris_{67}/DMSO_{33}$-Gelen (blaue Messpunkte) bei 37°C. F) Foto einer 3D-gedruckten Tarantel aus einem DMSO-Mischphasengel ($Tris_{67}/DMSO_{33}$). Maßstäbe wie angegeben.

Aufnahmen hingegen offenbarten schichtartige Strukturen in lyophilisierten DMF-Mischphasen. Nach Wasserzugabe bildeten sich fusionierte Porenstrukturen aus. Für die Gelproben mit DMSO wurden unerwarteterweise hoch fibrilläre, aber gleichzeitig poröse Strukturen sichtbar. Vor allem bei höherer Vergrößerung zeigten sich in Gelen aus der Organo-Dialyse fibrilläre Unterstrukturen an den Porenwänden (Abbildung 2C). In Tris-Gelen hingegen zeigte sich der bereits bekannte schichtartige Aufbau mit scharf definierten Poren und glatten Wänden.[7a] Ein Grund dafür könnte das Kollabieren der Fibrillen in diese Schichtstrukturen während des Gefriertrocknens sein.

Eine rheologische Charakterisierung (Abbildung 2D) zeigte leicht erhöhte Speicher- und Verlustmoduln für 2% (w/v) eADF4(C16)-Tris/DMSO-Gele im Vergleich zu 3% (w/v) eADF4(C16)-Tris-Hydrogelen. Nichtsdestotrotz lagen die Modulwerte für Tris/DMSO-Gele und Tris-Hydrogele in derselben Größenordnung. Ferner wurden auch ähnliche Fließpunkte erreicht, wodurch sich vergleichbare viskoelastische Eigenschaften ergaben. Gele aus 3% (w/v) eADF4(C16)-Tris/DMSO, die mittels Organo-Dialyse hergestellt wurden, zeigten dabei die höchsten Werte für den Speichermodul und gleichzeitig eine Verschiebung der Fließgrenze hin zu höheren Oszillationen, was als eine höhere Materialsteifigkeit interpretiert werden kann. Die neuen Gele zeigten die für Spinnenseidenhydrogele typische Scherverdünnung und Stabilität (Abbildung S1A,B). Dementsprechend konnten mehrlagige Gerüste mit hoher Formstabilität aus DMSO-Mischphasengelen mit einem RegenHU Bioplotter gedruckt werden (Abbildung 2F, Abbildung S3 und Video S4).

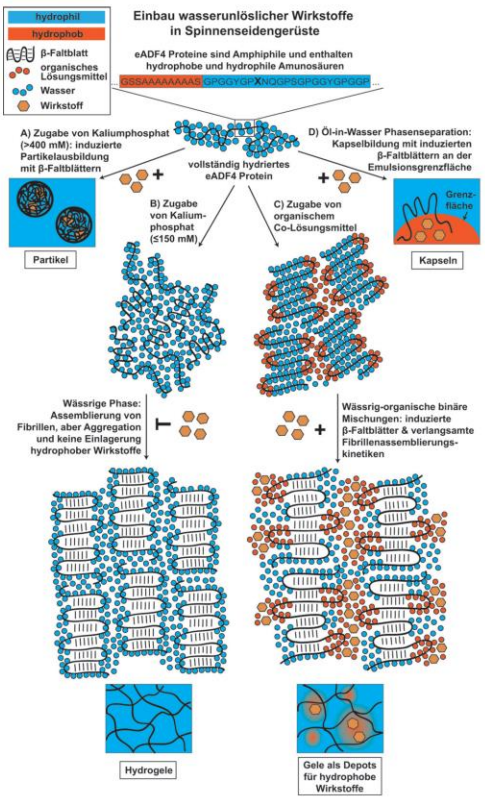
Um die Anwendung solcher Gele als Wirkstoffdepot zu testen, wurde zunächst Fluorescein (FITC) als Modellsubstanz in Tris/DMSO-Gele eingebracht und hinsichtlich seiner Beladung und Freisetzung daraus mit Tris-Hydrogelen bei 37°C verglichen. Beide Gel-Typen zeigten ein nahezu identisches Freisetzungsprofil (Abbildung S2), was die Möglichkeit einer Anwendung für injizierbare oder transdermale Wirkstoffdepots in Gel-Form eröffnet.

Weiterhin konnte der klinisch relevante, jedoch gering wasserlösliche Wirkstoff 6-Mercaptopurin über die DMSO-Phase in 2% (w/v) eADF4(C16)-Mischphasengele eingebracht werden. Der Wirkstoff wurde hier mittels nicht-kovalenter Wechselwirkungen eingebunden. Wäre hingegen eine reversible, kovalente Kupplung nötig, könnte diese über eine andere, kürzlich veröffentlichte Spinnenseidenvariante erfolgen.[20]

Da der wasserunlösliche Wirkstoff nicht-kovalent in Tris-Hydrogele eingebracht wurde, aggregierte er während der Gelierung und sammelte sich am Boden des Gefäßes. Im DMSO-Mischphasengel war eine Beladung und Freisetzung hingegen erfolgreich. Diese Ergebnisse bestätigten, dass Spinnenseidengele aus wässrig-organischen Mischphasen mit klinisch relevanten Substanzen als injizierbare und sogar 3D-druckbare Geldepots genutzt werden können.

Letztendlich sollte auch die Triebkraft hinter der Assemblierung von Spinnenseide in Gegenwart von Co-Lösungsmitteln aufgedeckt werden. Die hier beschriebene Route zur Bildung von Spinnenseidengelen in wässrig-organischen, binären Mischungen wird zum Teil durch Strukturbildung an der organisch-wässrigen Grenzfläche vorangetrieben (Schema 1). Es wurde bereits beobachtet, dass eADF4(C16) durch Adsorption an wässrig-organischen Grenzflächen wasserunlösliche β-Faltblattstrukturen in Form von Mikrokapseln[21] mit Barrierefunktion[22] ausbildet. Im Fall von wässrig-organischen, binären Mischungen können sich durch die Anwesenheit von organischen Co-Lösungsmitteln, wie DMSO[23] oder DMF,[13] Mikroemulsionen bilden, da diese mit Wasser mischbar sind.

Die gebildeten Mischphasen sind mikro-heterogener Natur[24] und basieren auf der Wechselwirkung von Molekülen, die Wasserstoffbrücken[25] ausbilden. Diese Mischphasen zeigen somit ein gemeinsames Arrangieren beider Lösungsmittel in molekularen Clustern.[25] DMSO und DMF können als amphiphile Moleküle Wasserstoffbrücken z. B. mit Wasser bilden, ermöglichen aber auch hydrophobe Interaktionen untereinander oder mit anderen Molekülen, wie Proteinen. Auch eADF4-Proteine weisen aufgrund ihrer Aminosäuresequenz[26] amphiphile Eigenschaften auf und können dem-



Schema 1. Illustration der verschiedenen Herstellungsrouten für Wirkstoffdepots aus rekombinanten Spinnenseidenproteinen. A) Kaliumphosphat-induzierte Cofällung von Mikropartikeln mit jeglicher Art von Wirkstoffen. B) Hydrogele können hydrophile, aber keine hydrophoben Wirkstoffe während der Gelierung einschließen. C) Neue Gelbildungsroute, die das Einbringen von sowohl hydrophilen als auch hydrophoben Wirkstoffen ermöglicht. D) Mikrokapseln können sowohl hydrophile als auch hydrophobe und amphiphile Wirkstoffe an der wässrig-organischen Grenzfläche einschließen. Wässrig-organische, binäre Mischungen, wie in (C) gezeigt, stellen Zwischenbedingungen zwischen einer Hydrogel-Bildung in vollständig wässriger Phase (B) und Phasenseparations-induzierter β-Faltblattbildung an der Wasser-Öl-Grenzfläche (D) dar. **X** = ausgewählte Aminosäure in den Spinnenseidenvarianten, z. B. Glutaminsäure, Glutamin oder Lysin.

entsprechend sowohl mit hydrophoben als auch mit hydrophilen Wirkstoffen oder Molekülen interagieren. Geladene Aminosäureseitenketten können Wasserstoffbrücken mit DMSO in unterschiedlichem Ausmaß bilden, wobei negativ geladene Aminosäureseitenketten gegenüber positiv geladenen oder gar ungeladenen bevorzugt sind.[27]

Spinnenseidengele aus wässrig-organischen Mischungen erweitern das Anwendungsspektrum der Spinnenseidenmaterialien, da ihre Materialeigenschaften durch die Wahl des Co-Lösungsmittels und der Spinnenseidenvariante gesteuert werden können. DMSO ist von der FDA[28] in topischen Formulierungen mit bis zu ca. 30 % (v/v) zugelassen. Durch die vorliegenden Ergebnisse können Spinnenseidengele nun in pharmazeutischen Formulierungen z. B. für transdermale Anwendungen verwendet werden. Diese Gele erlauben die Formulierung wasserunlöslicher Wirkstoffe bei gleichzeitiger Kontrolle über den Gehalt des organischen Lösungsmittels, um letztlich biokompatible, bioabbaubare, nicht-toxische, nicht entzündliche, transdermale, injizierbare oder sogar 3D-druckbare Wirkstoffdepots zu erhalten. Von der jeweiligen Anwendung abhängig, kann ebenfalls die Wechselwirkung zwischen Seide und Wirkstoff z. B. über reversible, kovalente Bindungen[20] gesteuert werden.

Experimentelles

Das rekombinante Spinnenseidenprotein eADF4(C16) enthält 16 Wiederholeinheiten der Konsensussequenz GSSAAAAAAAA SGPGGYGP**E**NQGPSGPGGYGPGGP (das so genannte C-Modul). Dem C-Modul liegt die Konsensussequenz der repetitiven Kerndomäne des Großen Ampullendrüsen-Spidroins 2 des Abseilfadenfibroins 4 der Europäischen Gartenkreuzspinne *Araneus diadematus* zugrunde.[6] eADF4(C16) wurde von AMSilk GmbH (Planegg, Deutschland) bezogen. In der eADF4(κ16)-Variante[17] wurden alle Glutaminsäurereste gegen Lysin ausgetauscht, in eADF4(Ω16)[18a] dagegen mit Glutamin ersetzt. Beide Proteine wurden wie bereits veröffentlicht hergestellt und gereinigt.[6,17,18] Die Spinnenseidenproteine wurden in 6 M Guanidiniumthiocyanat (Carl Roth, Karlsruhe, Deutschland) gelöst und gegen 10 mM Tris-Puffer, pH 7,5 für mehrere Stunden dialysiert, wobei die Dialyse der eADF4(Ω16)-Variante bei 4 °C durchgeführt wurde. Die Konzentrationen wurden, falls notwendig, durch eine anschließende Dialyse gegen 20 % (w/v) Polyethylenglykol (PEG, 20 kDa, Carl Roth, Karlsruhe, Deutschland), wie bereits bekannt,[7a] angepasst oder im Hochvakuum aufkonzentriert (Speedvac, Eppendorf). Für Zweiphasengele wurden 3 % (w/v) Spinnenseidenlösungen mit Co-Lösungsmitteln oder Salzlösungen versetzt und bei 37 °C geliert. Zur Organo-Dialyse wurden 3 % (w/v) Spinnenseidenlösungen gegen geringe Mengen (1:100 Volumenverhältnis) an DMSO (Carl Roth, Karlsruhe, Deutschland) bei RT für 4 h ohne weitere thermische Behandlung dialysiert.

Änderungen in der Trübung bei 570 nm wurden genutzt, um Gelierungskinetiken für die Spinnenseidenproteine mittels Mikroplattenleser (Mithras LB 940, Berthold Technologies, Deutschland) zu verfolgen. Es wurden Triplikatmessungen mit 100 µL Aliquots für alle Seidenlösungen (3 % (w/v) eADF4(C16), 2 % (w/v) eADF4(κ16) und 1 % (w/v) eADF4(Ω16)) durchgeführt. Die Spinnenseidenlösungen wurden durch Zugabe unterschiedlicher Volumina an DMSO verdünnt, um Endkonzentrationen von 5, 15 und 30 % (v/v) DMSO zu erhalten. Als Kontrolle und als Auslöser der Fibrillisierung diente Kaliumphosphat-Puffer (150 mM) pH 8,0; daher wurden die anderen Messkurven auf diesen Endwert normiert.

Für stereomikroskopische Aufnahmen der Gele mittels eines Leica-M205C-Stereomikroskops (Wetzlar, Deutschland) und zugehöriger Software wurden Proben auf Glasobjektträger übertragen und im Dunkelfeld betrachtet, um Reflexionen zu vermeiden. Das Mikroskop war mit einem Polfilter und einem 0,63 ×-Objektiv ausgestattet. Für die Rasterelektronenmikroskopie (REM) wurden die Gelproben lyophilisiert und mit Platin (2 nm) beschichtet. Die Bilder wurden mit einem „Thermo Scientific (FEI) Apreo VS"-Gerät mit einer Elektronenquelle mit 2 kV und einem SE2-Detektor aufgenommen. Für transmissionselektronenmikroskopische (TEM-)Aufnahmen an einem JEM-2100 TEM (JEOL, Japan) wurden Spinnenseidenfibrillen auf Pioloform-beschichteten 100er Kupfer-Netzträgerchen (Plano

GmbH, Deutschland) immobilisiert und mit 2% Uranylacetat angefärbt. Die Bildgebung erfolgte bei 80 kV mit einer 4000×4000 ladungsabhängigen, geräteinternen Kamera (UltraScan 4000, Gatan, USA) und der Gatan Digital Micrograph Software (Version 1.83.842).

Das rheologische Verhalten wurde mit einem Discovery Hybrid Rheometer 3 (TA, USA) mit einer Platte-Platte-Geometrie (Durchmesser 25 mm) bei 25 °C untersucht. Um Trocknungseffekte der Gele zu vermeiden, wurden Adapter mit nassen Schwämmen angebracht. Amplitudenscan-Messungen ($n=3$) wurden als Triplikate bei 31,4 rad s^{-1} und Scherung von 0,1–1000% durchgeführt.

Attenuated-Total-Reflectance-Fourier-Transformations-Infrarot-Spektroskopie (ATR-FTIR) wurde an lyophilisierten Gelproben durchgeführt. Die Spektren ($n=3$) wurden mit einem Bruker Tensor 27 (Ettlingen, Deutschland) auf einem Germaniumkristall mit einer spektralen Auflösung von 2 cm^{-1} und 100 Durchläufen aufgenommen. Eine Kompensation des atmosphärischen Hintergrunds wurde in der OPUS-8.0-Software aktiviert, um Wasserdampf- und Kohlendioxid-Schwankungen während der Messung zu korrigieren. Eine Fourier-Selbstdekonvolution, wie bereits beschrieben,[29] diente der Bandenzuordnung, um die Anteile der Sekundärstrukturen zu bestimmen.[30]

Die Freisetzungsstudie von 6-Mercaptopurin (Sigma, USA) aus 2% (w/v) $Tris_{100}$- und 2% (w/v) $Tris_{67}/DMSO_{33}$-eADF4(C16)-Gelen erfolgte, indem der Wirkstoff zunächst in 20 µL DMSO gelöst und entweder zur Spinnenseidenlösung oder der DMSO-Phase zugegeben wurde. Dann wurden Triplikat-Messungen der Freisetzung in 1:1-Mischungen aus MilliQ-Wasser:DMSO durchgeführt. Die UV-Absorption wurde mit einem UV-Spektrometer (Genesys 10S UV-Vis, Thermo Scientific) verfolgt. Die Spektren wurden zwischen 200 und 600 nm aufgenommen, und das Kurvenmaximum bei 328 nm wurde genutzt, um kumulative Freisetzungskurven zu bestimmen.

Der 3D-Dispensdruck wurde mit einem „RegenHU 3D Discovery Gen1"-Bioplotter (Schweiz) mit 3cc-Kartuschen und zugehörigen Stopfen durchgeführt. Die Druckgeschwindigkeit wurde auf 10 mm s^{-1} voreingestellt. 2% (w/v) $Tris_{67}/DMSO_{33}$-eADF4(C16)-Gele wurden mit Luer-lock-Plastiknadeln (Innendurchmesser 0,41 mm) und einem angelegten Druck von 0,3 bar verdruckt.

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Stichwörter: Binäre Mischungen · Co-Lösungsmittel · Hydrophobe Effekte · Mikro-Heterogenität · Selbstassemblierung

[1] J. L. Drury, D. J. Mooney, *Biomaterials* **2003**, *24*, 4337 – 4351.
[2] a) W.-H. Tan, S. Takeuchi, *Adv. Mater.* **2007**, *19*, 2696 – 2701; b) A. G. Tabriz, M. A. Hermida, N. R. Leslie, W. Shu, *Biofabrication* **2015**, *7*, 045012.
[3] a) N. Bhattarai, J. Gunn, M. Zhang, *Adv. Drug Delivery Rev.* **2010**, *62*, 83 – 99; b) Y. Hong, H. Song, Y. Gong, Z. Mao, C. Gao, J. Shen, *Acta Biomater.* **2007**, *3*, 23 – 31.
[4] N. Wu, H. Yu, M. Sun, Z. Li, F. Zhao, Y. Ao, H. Chen, *ACS Appl. Biol. Mater.* **2020**, *3*, 721 – 734.
[5] T. Zehnder, B. Sarker, A. R. Boccaccini, R. Detsch, *Biofabrication* **2015**, *7*, 025001.
[6] D. Huemmerich, C. W. Helsen, S. Quedzuweit, J. Oschmann, R. Rudolph, T. Scheibel, *Biochemistry* **2004**, *43*, 13604 – 13612.
[7] a) K. Schacht, T. Scheibel, *Biomacromolecules* **2011**, *12*, 2488 – 2495; b) E. DeSimone, K. Schacht, T. Scheibel, *Mater. Lett.* **2016**, *183*, 101 – 104; c) E. DeSimone, K. Schacht, T. Jüngst, J. Groll, T. Scheibel, *Pure Appl. Chem.* **2015**, *87*, 737 – 749.
[8] a) K. Schacht, T. Juengst, M. Schweinlin, A. Ewald, J. Groll, T. Scheibel, *Angew. Chem. Int. Ed.* **2015**, *54*, 2816 – 2820; *Angew. Chem.* **2015**, *127*, 2858 – 2862; b) E. DeSimone, K. Schacht, A. Pellert, T. Scheibel, *Biofabrication* **2017**, *9*, 044104.
[9] S. Kumari, H. Bargel, M. U. Anby, D. Lafargue, T. Scheibel, *ACS Biomater. Sci. Eng.* **2018**, *4*, 1750 – 1759.
[10] F. Hofmeister, *Arch. Exp. Pathol. Pharmakol.* **1888**, *24*, 247 – 260.
[11] U. K. Slotta, S. Rammensee, S. Gorb, T. Scheibel, *Angew. Chem. Int. Ed.* **2008**, *47*, 4592 – 4594; *Angew. Chem.* **2008**, *120*, 4668 – 4670.
[12] a) L. Eisoldt, A. Smith, T. Scheibel, *Mater. Today* **2011**, *14*, 80 – 86; b) E. Doblhofer, A. Heidebrecht, T. Scheibel, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 9361 – 9380; c) M. Heim, D. Keerl, T. Scheibel, *Angew. Chem. Int. Ed.* **2009**, *48*, 3584 – 3596; *Angew. Chem.* **2009**, *121*, 3638 – 3650.
[13] D. R. Canchi, A. E. García, *Annu. Rev. Phys. Chem.* **2013**, *64*, 273 – 293.
[14] C. N. Pace, S. Treviño, E. Prabhakaran, J. M. Scholtz, *Philos. Trans. R. Soc. London Ser. B* **2004**, *359*, 1225 – 1235.
[15] N. Javid, K. Vogtt, C. Krywka, M. Tolan, R. Winter, *ChemPhysChem* **2007**, *8*, 679 – 689.
[16] A. Lammel, M. Schwab, U. Slotta, G. Winter, T. Scheibel, *ChemSusChem* **2008**, *1*, 413 – 416.
[17] E. Doblhofer, T. Scheibel, *J. Pharm. Sci.* **2015**, *104*, 988 – 994.
[18] a) S. Kumari, G. Lang, E. DeSimone, C. Spengler, V. T. Trossmann, S. Lücker, M. Hudel, K. Jacobs, N. Krämer, T. Scheibel, *Data Brief* **2020**, *32*, 106305; b) S. Kumari, G. Lang, E. DeSimone, C. Spengler, V. T. Trossmann, S. Lücker, M. Hudel, K. Jacobs, N. Krämer, T. Scheibel, *Mater. Today* **2020**, *41*, 21 – 33.
[19] M. R. Harpham, N. E. Levinger, B. M. Ladanyi, *J. Phys. Chem. B* **2008**, *112*, 283 – 293.
[20] H. M. Herold, A. Döbl, S. Wohlrab, M. Humenik, T. Scheibel, *Biomacromolecules* **2020**, *21*, 4904 – 4912.
[21] K. D. Hermanson, D. Huemmerich, T. Scheibel, A. R. Bausch, *Adv. Mater.* **2007**, *19*, 1810 – 1815.
[22] K. D. Hermanson, M. B. Harasim, T. Scheibel, A. R. Bausch, *Phys. Chem. Chem. Phys.* **2007**, *9*, 6442 – 6446.
[23] J. Hansen, F. Platten, D. Wagner, S. U. Egelhaaf, *Phys. Chem. Chem. Phys.* **2016**, *18*, 10270 – 10280.
[24] D. N. Shin, J. W. Wijnen, J. B. F. N. Engberts, A. Wakisaka, *J. Phys. Chem. B* **2002**, *106*, 6014 – 6020.
[25] a) S. Roy, S. Banerjee, N. Biyani, B. Jana, B. Bagchi, *J. Phys. Chem. B* **2011**, *115*, 685 – 692; b) A. Wakisaka, H. Abdoul-Carime, Y. Yamamoto, Y. Kiyozumi, *J. Chem. Soc. Faraday Trans.* **1998**, *94*, 369 – 374; c) A. Wakisaka, T. Ohki, *Faraday Discuss.* **2005**, *129*, 231 – 245; d) D. B. Wong, K. P. Sokolowsky, M. I. El-Barghouthi, E. E. Fenn, C. H. Giammanco, A. L. Sturlaugson, M. D. Fayer, *J. Phys. Chem. B* **2012**, *116*, 5479 – 5490.
[26] C. B. Borkner, S. Lentz, M. Müller, A. Fery, T. Scheibel, *ACS Appl. Polym. Mater.* **2019**, *1*, 3366 – 3374.

[27] K. L. Shaw, G. R. Grimsley, G. I. Yakovlev, A. A. Makarov, C. N. Pace, *Protein Sci.* **2001**, *10*, 1206 – 1215.
[28] K. Capriotti, J. A. Capriotti, *J. Clin. Aesthet. Dermatol.* **2012**, *5*, 24 – 26.
[29] J. Petzold, T. B. Aigner, F. Touska, K. Zimmermann, T. Scheibel, F. B. Engel, *Adv. Funct. Mater.* **2017**, *27*, 1701427.
[30] X. Hu, D. Kaplan, P. Cebe, *Macromolecules* **2006**, *39*, 6161 – 6170.

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Supporting Information

Recombinant Spider Silk Gels Derived from Aqueous–Organic Solvents as Depots for Drugs

*Vanessa J. Neubauer, Vanessa T. Trossmann, Sofia Jacobi, Annika Döbl, and Thomas Scheibel**

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Experimental section

Rheological characterization of the co-solvent spider silk gels

Rheological behaviour was investigated using a Discovery Hybrid Rheometer 3 (TA, USA) with a plate-plate geometry (diameter 25 mm) at 25 °C. To prevent drying effects, a wet sponge adapter was used. Frequency sweep experiments (n=3) were recorded at angular frequencies between 0.1-100 rad/s and 100-0.1 rad/s for recovery at 50 % strain. Time sweep experiments (n=3) were recorded at angular frequencies of 31.4 rad/s and 0.5 % strain for 120 s.

Release studies of FITC as model substance from co-solvent spider silk gels

To study the release of 5(6)-Carboxy-fluorescein (FITC, Thermo Scientific, Germany) from 2 % w/v Tris- and 2 % w/v $Tris_{67}/DMSO_{33}$ eADF4(C16) gels, FITC was dissolved in 20 µL DMSO and added to the spider silk solutions. Samples without FITC loading served as reference. Triplicate release measurements were done in 1:1 blends of MilliQ water:DMSO. Fluorescence detection was conducted using a fluorescence spectrometer FP-6300 (JASCO, Germany) at an excitation wavelength of 495 nm. Spectra were recorded between 500 and 600 nm, and peak maxima at 517 nm were used to determine release curves. Error bars were calculated according to the law of error propagation.

3D dispense plotting of co-solvent spider silk gels

For 3D dispense plotting, the printing speed of the RegenHu Bioplotter was pre-set to 20 mm/s. 2 % w/v $Tris_{67}/DMSO_{33}$ eADF4(C16) gels were printed with Luer lock plastic and blunt steel needles with an inner diameter of 0.41 mm and an applied pressure of 0.3 - 0.5 bar. 3 % w/v Tris/DMSO-gels after organo-dialysis were printed using Luer lock steel blunt needles with 0.51 mm inner diameter and an applied pressure of 1.06 bar.

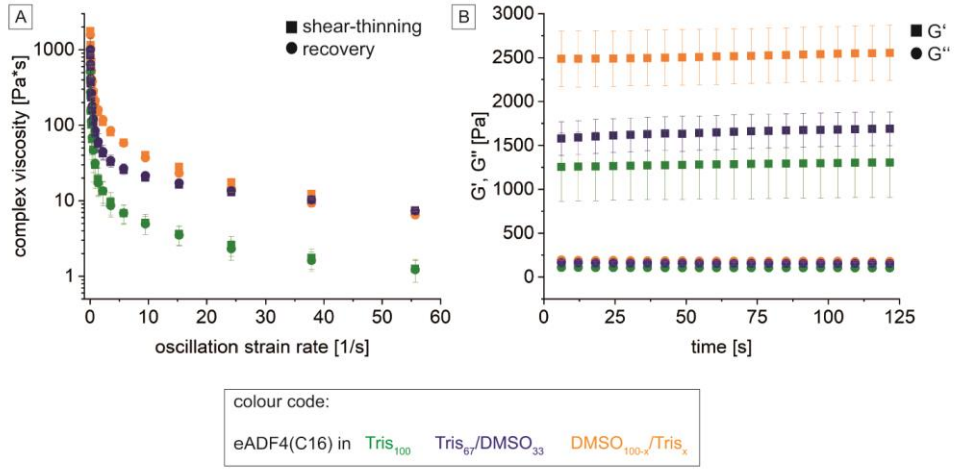

Figure S1: Gels from initial 3 % w/v eADF4(C16) silk solutions in 10 mM Tris pH 7.5 ($Tris_{100}$, green), diluted with 33% v/v of the co-solvent DMSO ($Tris_{67}/DMSO_{33}$, blue), and gels made upon dialysis against DMSO ($DMSO_{100-x}/Tris_x$, orange) were tested concerning the impact of organic additives. A) Mean frequency sweep rheological measurements of co-solvent gels showed shear-thinning behaviour and recovery properties of the gels. B) Mean time sweep rheological measurements of co-solvent gels showed gel stability over time.

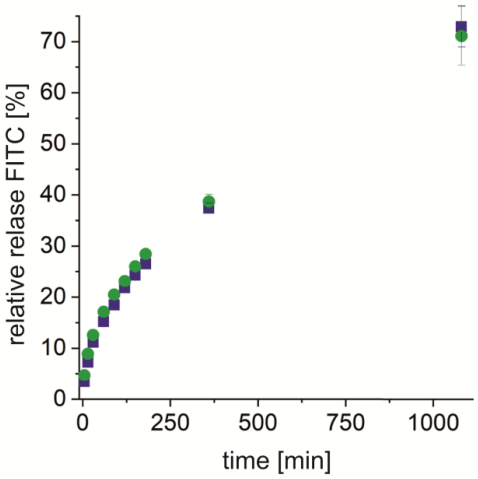

Figure S2: Normalized release of the model drug 5(6)-Carboxy-fluorescein (FITC) from gels at 37 °C: Gels from 2 % w/v eADF4(C16) silk solutions in 10 mM Tris pH 7.5 ($Tris_{100}$, green) and in presence of the co-solvent DMSO ($Tris_{67}/DMSO_{33}$, blue).

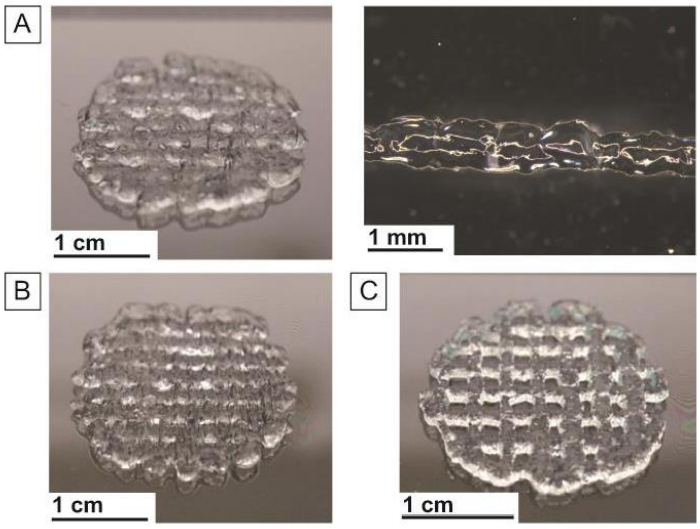

Figure S3: Images of 3D printed scaffolds of A) 2 % w/v $Tris_{67}/DMSO_{33}$ eADF4(C16) gels printed using a conical needle (0.41 mm diameter) on a RegenHU Bioplotter (left) and stereomicroscopic image of one single strand (right); B) 2 % w/v $Tris_{67}/DMSO_{33}$ eADF4(C16) gels printed using a steel needle (0.41 mm diameter); C) 3 % w/v Tris/DMSO-gels from organo-dialysis printed using a steel needle (0.51 mm diameter) and larger line spacing. Scale bars as indicated.

A Video of 3D dispense plotting of a tarantula scaffold is available as Supporting Information Video S4.

Experimentalteil

Rheologische Charakterisierung der Spinnenseiden-Mischgele

Das rheologische Verhalten wurde mit einem Discovery Hybrid Rheometer 3 (TA, USA) mit einer Platte-Platte-Geometrie (Durchmesser 25 mm) bei 25 °C untersucht. Um Trocknungseffekte der Gele zu vermeiden, wurden Adapter mit nassen Schwämmen angebracht. Frequenzscan-Messungen (n=3) wurden als Triplikate bei Winkelfrequenzen von 0,1-100 rad/s und 100-0,1 rad/s für die gegenläufige Wiederherstellungsmessung je mit einer 0,5 %-igen Scherung für 120 s durchgeführt.

Freisetzungsstudie von FITC als Modellsubstanz aus Spinnenseiden-Mischgelen

Eine Freisetzungsstudie bezüglich 5(6)-Carboxyfluorescein (FITC, Thermo Scientific, Deutschland) aus 2 % w/v $Tris_{100}$ und 2 % w/v $Tris_{67}/DMSO_{33}$ eADF4(C16)-Gelen erfolgte, indem FITC in 20 µL DMSO gelöst und entweder zur Spinnenseidenlösung oder der DMSO-Phase zugegeben wurde. Proben ohne FITC dienten als Referenz. Dann wurden Triplikat-Messungen der Freisetzung in 1:1 Mischungen aus MilliQ Wasser:DMSO durchgeführt. Fluoreszenzsignale wurde mit einem Fluoreszenzspektrometer FP-6300 (JASCO, Deutschland) bei einer Anregungswellenlänge von 495 nm detektiert. Spektren wurden zwischen 500 und 600 nm aufgenommen und das Kurvenmaximum bei 517 nm genutzt, um kumulative Freisetzungskurven zu bestimmen. Die Fehlerbalken wurden mittels Fehlerfortpflanzung ermittelt.

3D Dispensdruck von Spinnenseiden-Mischgelen

Für den 3D Dispensdruck mit einem RegenHU 3D Discovery Gen1 (Schweiz) Bioplotter wurde die Druckgeschwindigkeit auf 20 mm/s voreingestellt. 2 % w/v $Tris_{67}/DMSO_{33}$ eADF4(C16)-Gele wurden mit Luer-lock Plastik- und stumpfen Stahlnadeln (Innendurchmesser je 0,41 mm) und einem angelegten Druck von 0,3-0,5 bar verdruckt. 3 % w/v Tris/DMSO-Gele nach der Organo-Dialyse wurden mit stumpfen Luer-lock Stahlnadeln mit einem Innendurchmesser von 0,51 mm und einem angelegten Druck von 1,06 bar verdruckt.

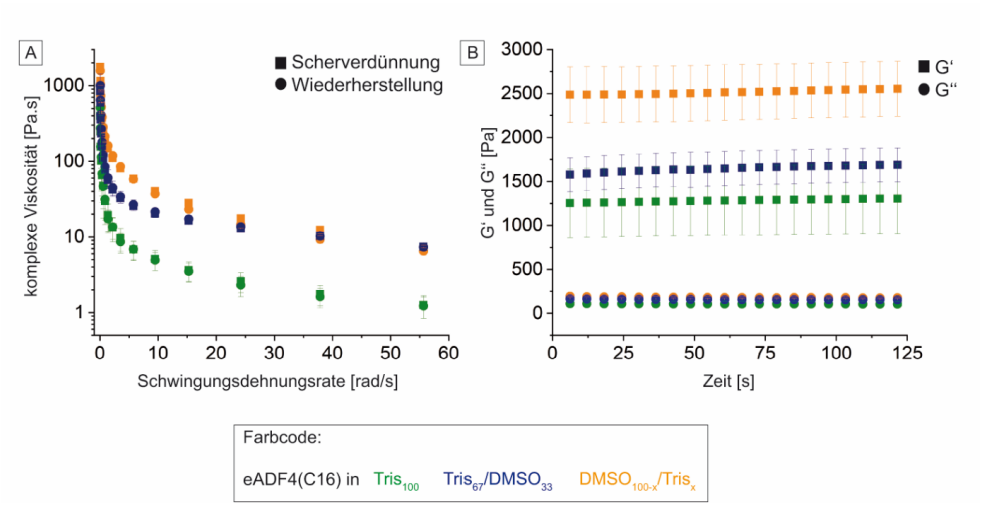

Abbildung S1: Einfluss organischer Additive auf Spinnenseidengele: Gele aus einer 3 % w/v eADF4(C16) Spinnenseiden-Ausgangslösung in 10 mM Tris pH 7,5 ($Tris_{100}$, grün) stehen im Vergleich zu Gelen, bei denen die Ausgangslösung zu einem Volumendrittel mit DMSO ($Tris_{67}/DMSO_{33}$, blau) versetzt wurde, sowie Gele aus Organo-Dialysen gegen DMSO ($DMSO_{100-x}/Tris_x$, orange). A) Gemittelte Rheologiemessungen mittels Frequenzscan der Mischphasengele zeigten scherverdünnendes Verhalten der Gele. B) Gemittelte Rheologiemessungen mittels Zeitscan der Mischphasengele zeigten die zeitliche Stabilität der Gele.

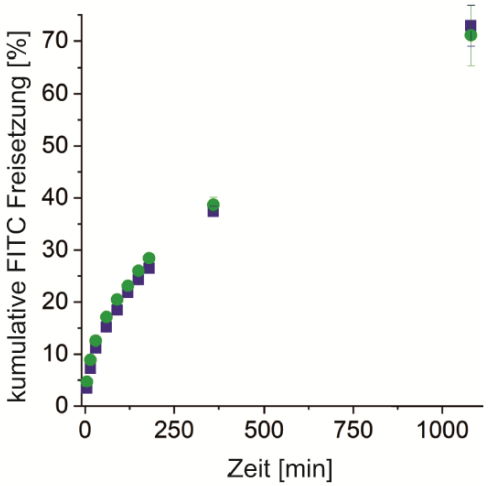

Abbildung S2: Normalisierte kummulative Freisetzung des Modellwirkstoffes 5(6)-Carboxyfluorescein (FITC) aus Gelen bei 37 °C: Gele aus 2 % w/v eADF4(C16) Seidenlösungen in 10 mM Tris pH 7,5 ($Tris_{100}$, grün) und in Gegenwart von DMSO als Co-Lösungsmittel ($Tris_{67}/DMSO_{33}$, blau).

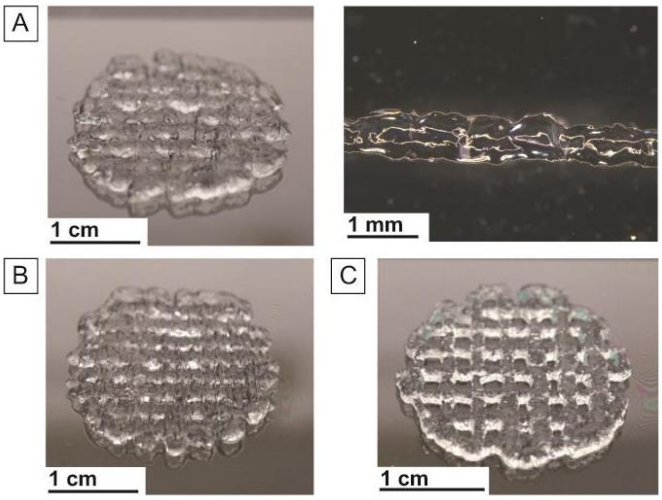

Abbildung S3: Aufnahmen der 3D gedruckten Gerüste aus A) 2 % w/v $Tris_{67}/DMSO_{33}$ eADF4(C16) Gelen, welche mit einer konischen Plastiknadel (Innendurchmesser 0,41 mm) mit einem RegenHU Bioplotter gedruckt wurden (links) und dazugehörige stereomikroskopische Aufnahmen eines Einzelstrangs (rechts); B) 2 % w/v $Tris_{67}/DMSO_{33}$ eADF4(C16) Gele, welche mit einer Stahlnadel (0,41 mm Innendurchmesser) gedruckt wurden; C) 3 % w/v Tris/DMSO-Gele aus Organo-Dialysen, welche mit einer Stahlnadel (0,51 mm Innendurchmesser) und größerem Druckabstand gedruckt wurden. Maßstäbe wie angegeben.

Das Video des 3D Dispensdrucks in Form einer Vogelspinne ist als Supporting Information Video S4 erhältlich.

Danksagung

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Eine Promotion am Lehrstuhl Biomaterialien ist ein Komplettpaket, das sich neben herausragender Forschung auch durch stark interdisziplinäres Arbeiten auszeichnet. Doch

im Kern sind es die interessanten Menschen und die Zusammenarbeit, die die spezielle Atmosphäre der „BioMat“ ausmachen und die die Zeit hier unvergesslich machen.

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Eidesstattliche Versicherungen und Erklärungen

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Hiermit versichere ich eidesstattlich, dass ich die Arbeit selbstständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe (vgl. Art. 64 Abs. 1 Satz 6 BayHSchG).

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Bayreuth,_____ _____

Vanessa J. Neubauer