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Directed Functionalization of Recombinant Spider Silk Nonwoven Membranes with Antibodies Using Non-Canonical Amino Acids

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ABSTRACT

Natural spider silk fibers are recognized for their outstanding mechanical properties. The production of underlying recombinant spider silk proteins in scalable quantities unlocks their potential for technical and biomedical applications. The recombinant spider silk technology further enables the introduction of new functions via genetic encoding. In the present study, the non-canonical amino acid L-azidohomoalanine is incorporated into the engineered *Araneus diadematus* fibroin 4, eADF4(C16), at positions explicitly defined by methionine codons in an N-terminal peptide tag. The azido-functionalized eADF4(C16) is processed into particles, films, or electrospun into nanofibers. As functional entities, high molecular weight molecules, such as antibodies, are modified with bio-orthogonal groups suitable for strain-promoted cycloaddition to the exposed azido-groups on the spider silk supports. The antibody-activated spider silk nonwoven meshes exemplarily show their applicability as bio-capturing membranes.

1 | Introduction

In 400 million years of evolution, spiders have spread to dwell in virtually every corner of the world, on the ground, in the air, and even under water [1]. Their evolutionary success is intricately connected to the spinning of silk threads with extraordinary mechanical properties [2]. Unlike most other natural or artificial fibers [1, 3], spider silk combines strength and elasticity, which renders it two to five times tougher than nylon and Kevlar, depending on the spider species [2, 3]. Most spiders are cannibalistic and territorial, hence, harvesting silk directly from spiders is laborious, and the silk quality suffers from batch-to-batch

variations. Consequently, biotechnological routes to produce spider silk proteins have been explored in recombinant hosts such as *Escherichia coli*. Here, we focused on major ampullate spider silk protein *Araneus diadematus* fibroin 4 (ADF4) representing one constituent of the dragline and the frame in webs produced by one of the most studied spider species, the European Garden Spider *Araneus diadematus*. Scheibel et al. developed a simplified engineered recombinant version eADF4(C16) [4] (Figure S1a) comprising 16 repeats of the ADF4 consensus sequence (C-module). This C-module is composed of a repeating GPGXX motif (X can be tyrosine, leucine or glutamine) providing elasticity and flexibility, as well as a poly-A sequence folding into β -sheets,

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which form a crystalline structure causing the outstanding tensile strength of spider silk fibers. The full-versions of recombinant spider silk proteins, comprising the repetitive core as well as the non-repetitive N- and C-terminal domains (NTD and CTD, respectively), are required only for spinning processes in order to mimic natural spider silk formation [5, 6]. In contrast, the simplified eADF4(C16) is not useful for biomimetic fiber spinning, but can be easily processed into various non-natural morphologies such as hydrogels, films, coatings, electrospun fibers, mesoporous foams, microcapsules, and solid spheres [7]. Moreover, materials based on this protein are biodegradable, non-cytotoxic, and non-inflammatory [8]. This outstanding combination of properties and processability provides a promising basis for applications ranging from cosmetics to pharmaceuticals and biomedicine [8, 9].

Advanced applications (beyond biomimetic fiber spinning) of spider silk scaffolds require a controllable modification of the underlying spider silk proteins. In contrast to synthetic polymers, recombinant spider silk proteins can be tailored using two approaches: genetic engineering resulting in protein fusions or tagged variants [10–12] and chemical modification through functionalizable groups, such as accessible amino residues of the N-terminus [13, 14], or various side chains of canonical amino acids (cAAs), such as those of lysine [15], cysteines [16, 17], glutamic acid or tyrosine residues [18]. Such modifications are rather site-unspecific, since typically several of such residues are distributed along a protein sequence, which leads to heterogeneous bioconjugations and consequently to sub-optimal stability and tolerability [19–21]. In order to target those obstacles, it is most efficient to combine both approaches, genetic and chemical engineering, by site-specifically modifying the underlying spider silk protein.

Non-canonical amino acids (ncAAs) with bio-orthogonal reactive side chains, e.g. the methionine analog L-azidohomoalanine (Aha, Figure S1b), are a valuable possibility to functionalize proteins, especially in case the cAA is found in explicit sequences only, which allows for homogeneous bioconjugation. The azido group specifically couples either to terminal alkyne groups in a Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) or with cyclooctynes in strain-promoted azide-alkyne cycloaddition (SpAAC) [22]. Although Aha is not encoded by the standard genetic code, it can replace methionine under controlled conditions [23, 24]. This incorporation method exploits a methionine-auxotrophic host which is grown in synthetic minimal medium containing limiting amounts of methionine (Met). At Met depletion, as indicated by a plateau phase of bacterial growth, Aha can be added along with the induction of gene-of-interest expression [25]. The promiscuity of the host methionyl-tRNA synthetase leads to charging of the cognate tRNA_{CAU}^{Met} with Aha and its insertion during the polypeptide biosynthesis, hence, allowing residue-specific incorporation of the bio-orthogonally reactive ncAA.

The functionalization of silk proteins with azido-groups for bio-orthogonal coupling has been demonstrated on *Bombyx mori* and spider silk proteins with fluorophores, polyethylene glycol (PEG) chains and antibiotics [26–28] as reviewed recently [24]. However, the site-specific covalent modification of silk-based scaffolds using click-chemistry with high-molecular-weight biomolecules,

such as antibodies, whose activity is tightly associated with their sensitive tertiary structures and appropriate accessibility of the binding epitopes, has not been shown yet.

In the present study, we developed a scalable process to produce gram quantities of the azido-functionalized spider silk protein in a methionine-auxotrophic *Escherichia coli* strain in which Aha was introduced into the T7-tag of eADF4(C16) (eADF4(C16)[Aha]) at three explicit positions. This novel spider silk variant was used to immobilize engineered rabbit monoclonal anti-green fluorescent protein (αGFP) immunoglobulin G (IgG) antibody in a site-specific manner to be used in bioactive membranes.

2 | Results and Discussion

2.1 | Controlled Introduction of Azido-Moieties In eADF4(C16)

The underlying sequence of the C-module in eADF4(C16) encodes for both protein self-assembly [29] as well as its physicochemical properties in processed materials [30]. Therefore, we avoided modifying the repetitive core [4] sequence and targeted an artificially introduced amino-terminal (N-terminal) T7-tag instead (Figure S1a), which contains four methionine residues and which has been previously used for tracking of the protein during purification. The T7-tag was introduced without compromising the assembly of eADF4(C16) [4]. Since the first N-terminal Met is typically excised post-translationally when followed by small amino acid residues (here alanine and serine) [31], the remaining three Met residues are available for substitution with Aha to accomplish the multivalent incorporation of azido-groups in the T7-tag. The unique position of the Met residues in eADF4(C16) allowed the convenient and scalable introduction of Aha using the supplementation-based approach [23, 32].

The Met auxotrophic *E. coli* strain B834(DE3), transformed with the gene expression construct for eADF4(C16), was used to optimize the production of Aha-labeled eADF4(C16) and to test the scalability of the bioprocess in fed-batch fermentation. The intracellular availability of Met was controlled by growing *E. coli* in synthetic complete medium supplemented with yeast extract as the limiting Met source [25]. Depletion of Met was indicated by a plateau phase in bacterial growth at OD₆₀₀ = 40 (Figure S1c). At this point, the cells were supplemented with Aha, and gene expression was induced. eADF4(C16)[Aha] was purified from the soluble protein fraction using a column-free strategy as described previously [4].

First, the yield of pure protein (μM) per concentration of supplemented ncAA (in mM) was optimized. Since Aha is considerably expensive [33], first trials in fed-batch bioreactor cultures were performed with cheaper norleucine (Nle) [34] (Figure S1b), an inert Met analog [25]. We compared the Nle supplementation with or without an additional supplementation with glycine, alanine, proline, and serine, the four most abundant amino acid residues in the sequence of eADF4(C16) (in total 84% of the AAs). A potential shortage of these highly abundant amino acids during gene expression could limit the spider silk protein production. The addition of 0.25 mM of the most abundant cAAs led to an increase of 38% in the protein titer of eADF4(C16)[Nle]. The

increase in the Nle supplementation from 2.5 to 7.5 mM raised the protein yield from 1.8 to 2.9 μM of pure eADF4(C16)[Nle] per mM of Nle in fed-batch bioreactor cultures. This finding confirmed that the intracellular ncAA accumulation depended on the supplementation in the medium [35]. Based on these results, the productivity was assessed with the supplementation of 7.5 and 10 mM Aha in the medium in fed-batch bioreactor cultures, resulting in 1.1 and 1.2 μM of eADF4(C16)[Aha] per mM of Aha, respectively, indicating a saturation in the process productivity. Hence, we used 10 mM Aha and 0.25 mM of the four cAAs to produce eADF4(C16)[Aha] in fed-batch cultivation. At a cell density of 16.9 g L⁻¹ cell dry weight (CDW) and supplementation with 10 mM Aha and 0.25 mM of the four cAAs, we obtained up to 40 mg of eADF4(C16)[Aha] per gram CDW (0.6 g L⁻¹ of culture). To further increase the production yield, the bioreactor fermentation strategy was adopted to drive eADF4(C16) mainly into the insoluble protein fraction. Purification from inclusion bodies yielded 5.4 μM of pure eADF4(C16)[Aha] per mM Aha, or 233 mg per gram CDW (2.6 g L⁻¹ of culture).

The incorporation of Aha in eADF4(C16) was confirmed using mass spectrometry. Met could be exchanged in the T7-tag at all three positions (Figure S2), and the most abundant variant was the triple azide-labelled protein. The secondary structure of the modified spider silk protein was characterized using circular dichroism spectroscopy, showing a minimum at approximately 200 nm in the spectra, which is characteristic for random coil structures and identical to the structure of non-modified eADF4(C16) in aqueous buffers (Figure S3).

2.2 | Bio-Orthogonal Labeling of the Azido-Functionalized Spider Silk Protein

Azide groups are not naturally present in biomolecules and are chemically relatively inert [36]. Their selective CuAAC or SpAAC reactions termed “click chemistry” have been used extensively to modify proteins [22, 37, 38]. Since the Cu(I) catalyst can selectively damage proteins containing azido groups [39], we employed SpAAC (Figure 1a) with different concentrations of dibenzocyclooctyne (DBCO)-Cy3 to assess the availability of the azido groups in soluble eADF4(C16)[Aha] and analyzed the results using SDS-PAGE (Figure 1b). The fluorescence of the Cy3-labeled protein was equivalent to 40–60 μM of fluorophore implying the accessibility of two to three Aha residues per spider silk protein for conjugation. Like eADF4(C16), the Aha-modified variant could be processed into particles (Figure S4) [40], and the exposure of the azido groups on the particle surface was demonstrated upon fluorescence labeling with DBCO-Cy3 (Figure 1c,d).

2.3 | Furnishing an Antibody with a Bio-Orthogonal Reactive Handle

As a biological entity, we wanted to couple an active antibody to the modified spider silk protein. To introduce a bio-orthogonal handle on the surface of monoclonal αGFP antibody, we targeted its asparagine-linked glycan (N-glycan) at the conserved position N297 in its Fc part [41]. The N-glycan on IgGs displays *cis*-glycol groups of sialic acid and galactose moieties, which were

here specifically oxidized with sodium metaperiodate to reactive aldehyde groups [42] and coupled to an aminooxy-PEG₃-N₃ linker via an oxime ligation [43] (Figure 1e, step 1). We chose the hydrophilic PEG₃ linker to improve the accessibility of the bio-orthogonal azido group [44].

First, we intended to conjugate eADF4(C16)[Aha] to αGFP -PEG₃-N₃ with a bifunctional DBCO-PEG₄-DBCO linker, which has been established previously to crosslink small carbohydrate-binding proteins [45]. However, this attempt was not successful (data not shown). We hypothesized that the antibody's ten-fold larger size (MW 150 kDa) in comparison to the previously used small carbohydrate-binding proteins might obstruct the conjugation via the bifunctional linker [46–48]. As an alternative, DBCO-labeled complementary oligonucleotides [13] were attached to eADF4(C16)[Aha] and αGFP -PEG₃-N₃ (Figure 1e, step 2), which enabled DNA-guided hybridization of the spider silk protein and the functional antibody (Figure 1e, steps 3 and 4). Figure S5 shows a detailed native PAGE analysis of the corresponding modification steps. αGFP -PEG₃-(DNA)-eADF4(C16)[Aha] conjugates (Figure S5, lanes 6, 8, 10) displayed heterogenous bands, likely originating from the heterogeneity of the conjugation based on three Aha residues in the T7-tag as well as in the galactosyl and sialyl residues of the antibody. To test the versatility of the DNA-guided approach, α -fibronectin IgG was successfully conjugated to eADF4(C16) [Aha] as well (Figure S6). Notably, the oligonucleotide design permits the fine-tuning of the binding architecture and avidity [14, 49–51] depending on the application of the antibody-functionalized spider silk material. Since the hybridization of the oligonucleotides can be reversed [52], detachment of the antibody from the spider silk scaffold would be possible. To prevent accidental disassembly of the DNA duplex, nucleotides containing reactive moieties, such as furan [53], could be incorporated to introduce covalent interstrand cross-links.

To improve the site-specificity of the conjugation, the single glutamine (Gln) residue Q295 of the Fc region of the antibody was specifically targeted using microbial transglutaminase (MTGase). This enzyme catalyzes the isopeptide formation between the γ -carboxamide of Gln and a variety of primary amines [54–59]. Since the position is sterically hindered by the proximal N297 glycan, the antibody was first deglycosylated using the enzyme peptide-N-glycosidase F (PNGase F). This enabled the transglutamination with DBCO-PEG₄-amine as a linker for the subsequent SpAAC with eADF4(C16)[Aha]. Whereas the glycosylated αGFP was only slightly modified with the DBCO-PEG₄-amine, the deglycosylation resulted in a much higher yield of DBCO modified antibody, as demonstrated using SDS-PAGE analysis (Figure S7).

2.4 | Antibody-Functionalized Spider Silk Particles and Films

Next, particles [40, 60] and films [30, 61, 62] made of eADF4(C16)[Aha] were functionalized with the antibody (Figure 1f; Figure S8, respectively). The spider silk protein was modified after the assembly (i.e. in solid-state) using the oligonucleotide cap-DBCO and SpAAC, similarly as described in the previous section for the solution-based approach (Figure 1e). Figure 1f confirms the hybridization-based immobilization

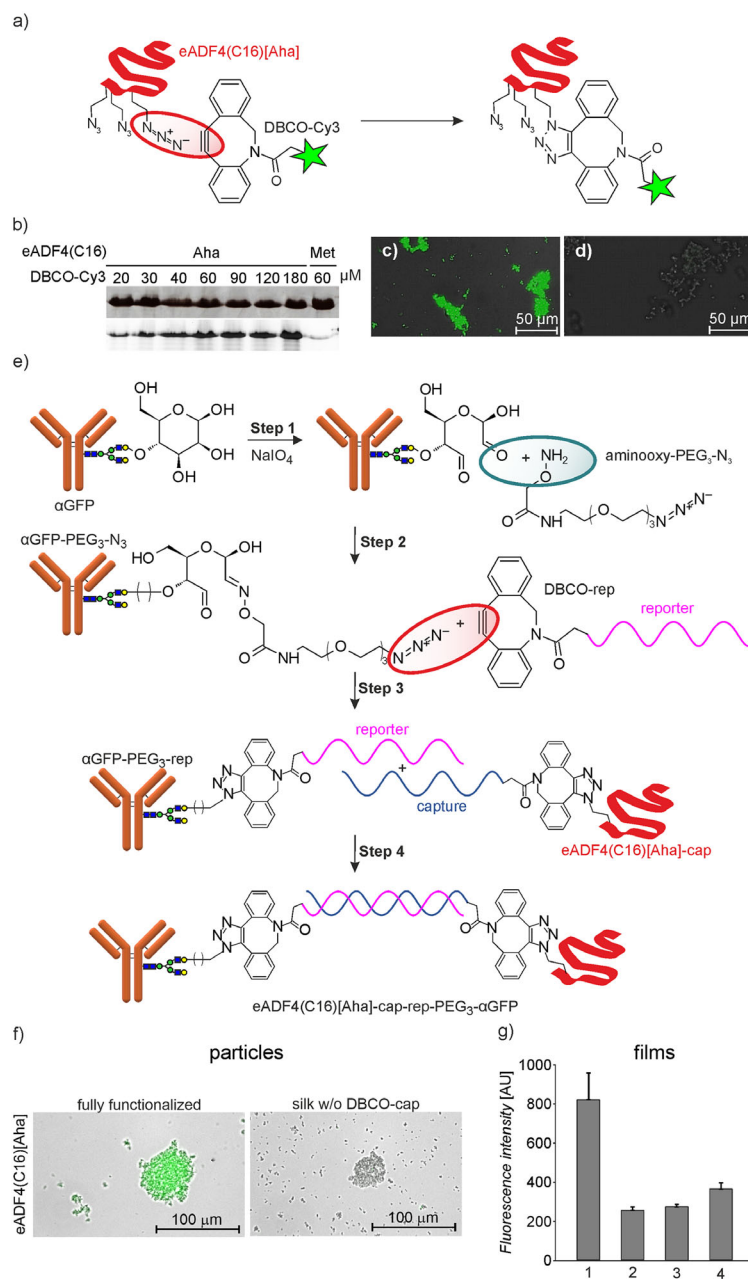


FIGURE 1 | Site-specific modification of spider silk particles and films with antibodies using DNA hybridization. (a) Schematic illustration of the site-selective labeling of eADF4(C16)[Aha] with dibenzocyclooctyne-Cy3 (DBCO-Cy3) at the Aha residues using SpAAC. (b) Functionalization of 20 μM soluble eADF4(C16)[Aha] at the indicated concentrations of DBCO-Cy3. The SDS-PAGE gel was silver stained (top), and the fluorescence was detected using a fluorescence imager, confirming the selectivity of the coupling reaction (bottom). Particles made of eADF4(C16)[Aha] (c) or eADF4(C16)[Met] (d) were labeled with DBCO-Cy3 and visualized using fluorescence microscopy. (e) Schematic illustration of the DNA-based conjugation strategy between eADF4(C16)[Aha] and an antibody. To functionalize the antibody with a compatible group, the glycan at N297 of the $\text{C}_\text{H}2$ chain of rabbit monoclonal αGFP IgG was modified. The cis-glycol groups of the N-linked oligosaccharides were oxidized (Step 1), and the resulting aldehyde-modified αGFP was conjugated with aminooxy-PEG₃-N₃ (Step 2) to introduce an azide bio-orthogonal handle (αGFP -PEG₃-N₃). Single stranded DBCO-modified nucleic acid captures and reporters were used in SpAAC (Step 3) with αGFP -PEG₃-N₃ and eADF4(C16)[Aha], respectively. The products eADF4(C16)[Aha]-cap and αGFP -PEG₃-rep were linked (Step 4) using hybridization. Immobilization of αGFP was tested on eADF4(C16) particles (f) and on films (g), including fully functionalized spider silk with antibody (eADF4(C16)[Aha]-cap-rep-PEG₃- αGFP) as well as controls without cap sequence (eADF4(C16)[Aha] + αGFP -PEG₃-rep) or with spider silk protein only, as indicated. All samples were incubated with GFP. f) GFP binding to αGFP -functionalized spider silk particles was visualized using fluorescence microscopy. g) GFP binding to αGFP -functionalized spider silk films was visualized using a microplate reader to quantify the emitted fluorescence. 1: fully functionalized eADF4(C16)[Aha]-cap-rep-PEG₃- αGFP ; 2: eADF4(C16)[Aha] + αGFP -PEG₃-rep; 3: blank non-modified eADF4(C16)[Met] and antibody αGFP -PEG₃; 4: GFP binding to eADF4(C16)[Aha] in the absence of antibody. Mean values of three replicates are shown in (g), the error bars indicate the standard deviation.

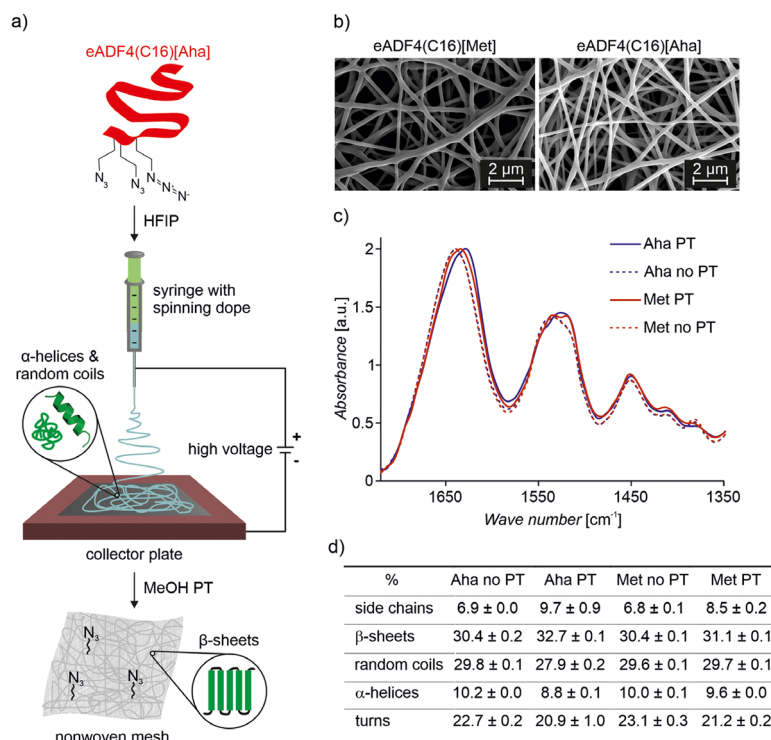


FIGURE 2 | Aha-modified eADF4(C16) fibers are indistinguishable from unlabeled fibers concerning all tested physicochemical properties. (a) Schematic illustration of the production of functionalized nonwoven meshes. (b) SEM images of MeOH post-treated electrospun eADF4(C16) nonwoven meshes. The exchange of Met with Aha had no influence on fiber assembly or fiber morphology. (c) ATR-FTIR spectra of post-treated (PT) and untreated (no PT) eADF4(C16)[Aha] and eADF4(C16)[Met] nonwoven meshes revealed no differences. (d) Percentages of secondary structure elements in post-treated and untreated eADF4(C16)[Aha] and eADF4(C16)[Met] nonwoven meshes determined using Fourier self-deconvolution (FSD) of the amide I band. Mean values of three replicates are shown. HFIP, hexafluoroisopropanol. MeOH PT, post-treatment using methanol steam.

of active reporter-modified α GFP-rep on the cap-modified particles, since GFP was only detected when the silk protein was functionalized with the complementary capture nucleic acid. No unspecific binding of GFP to non-functionalized eADF4(C16)[Aha] occurred. Likewise, eADF4(C16)[Aha] films were prepared using a drop-casting approach into well plates [62] and were modified with cap-DBCO. α GFP-rep was hybridized with the films, and antibody-functionalized spider silk films showed clear immunoreactivity toward GFP (Figure 1g; Figure S8). These findings emphasize that the processing of the azido-modified spider silk protein into particles or films did not compromise the accessibility of the azido group for chemical functionalization, and exposed nucleic acid strands, which hybridized with DNA- α GFP, preserved their epitope binding capability.

2.5 | Processing of eADF4(C16)[Aha] into Nonwoven Membranes

Spider silk nonwoven meshes are suitable, e.g., for high performance air filtration applications [63], where spinning from organic solvents eliminates the need for the CTD and NTD, as these domains are functional only under aqueous spinning conditions [5, 6] and may even be detrimental during electrospinning. The modified eADF4(C16)[Aha] and the non-modified eADF4(C16)[Met] were electrospun from hexafluoroisopropanol (HFIP) solutions to yield nonwoven meshes (Figure 2a). The

stability of the nanofiber membranes in water is a prerequisite for their applicability as substrates for the immobilization of antibodies. However, HFIP generates water-soluble fibers containing α -helical and random coil structures, hence, a post-treatment with methanol vapor was used to induce the formation of stabilizing β -sheet structures, leading to water stability [63].

The nanofiber morphology was visualized using scanning electron microscopy (SEM). The implemented electrospinning parameters resulted in continuous fibers for both, the Aha-modified and the non-modified spidroin variants (Figure 2b). After post-treatment, overlapping fibers appeared partially connected, providing physical crosslinks enabling higher mechanical stability and reduced slipping of the fibers [63]. The fiber diameters and, thus, the pore sizes of the mesh, were adjustable by variation of the protein concentration, solvent, distance to the collector plate, temperature, and relative humidity [63]. The fiber diameters exhibited a slight variance, as determined for eADF4(C16)[Aha] (223 ± 51 nm) and eADF4(C16)[Met] (214 ± 62 nm) (Figure S9). The exchange of Met with Aha, however, had no influence on fiber morphology.

Attenuated total reflection (ATR)-Fourier transformed infrared (FTIR) spectroscopy was used to analyze the protein secondary structures. Absorption maxima of the amide I band in a range of $1600\text{--}1700\text{ cm}^{-1}$ showed a slight shift to lower wave numbers

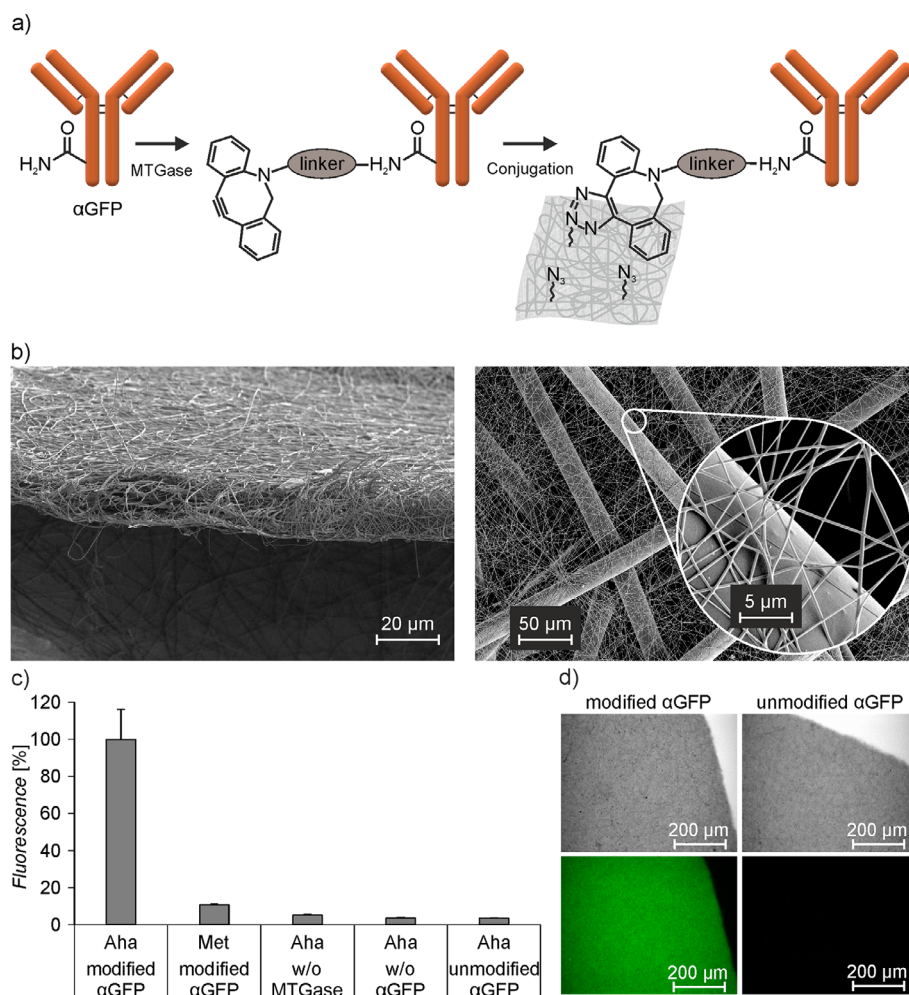


FIGURE 3 | Activity of antibody-functionalized spider silk nonwoven membranes. (a) Schematic illustration of the modification of anti-GFP IgG with a DBCO-PEG₄-amine linker using MTGase and subsequent immobilization of the DBCO-modified antibody on azide-functionalized spider silk fibers using SpAAC. (b) SEM images of electrospun eADF4(C16) nonwoven meshes; left panel—cross section of the nonwoven mesh used for antibody conjugation, right panel—spider silk nanofibers on a polyamide mesh. (c) GFP binding to αGFP-functionalized eADF4(C16) nonwoven meshes was quantified using a plate reader. Mean values of three replicates are shown, and the error bars indicate the standard deviation. (d) αGFP-functionalized and non-functionalized eADF4(C16) nonwoven meshes were visualized using bright-field microscopy (upper panels) and fluorescence microscopy (lower panels) after GFP binding.

in the post-treated nonwoven meshes, suggesting an increased β -sheet content (Figure 2c) [16, 64]. For quantification, Fourier self-deconvolution (FSD) of the amide I band was performed, since the deconvoluted distinct C=O stretching frequencies could be assigned to the secondary structure elements of the spider silk proteins (Figure S10) [14, 65]. The highest portion of secondary structures in untreated nanofibers represented random coils and β -sheets with 30% each (Figure 2d). The portion of β -sheets increased only by 1%–3% in the post-treated samples. Interestingly, already this slight change in β -sheet content was sufficient to render the nanofibers water stable, as confirmed in the solubility tests shown in Figure S11. Previously, Lang et al. measured a much higher β -sheet content in untreated eADF4(C16) nonwoven meshes [66]. This discrepancy might be caused by different relative humidity levels during the electrospinning processes, since a high relative humidity, as applied in so-called water annealing post-treatments, also generated higher β -sheet content in silk fibers [67].

2.6 | Antibody-Functionalized Spider Silk Nonwoven Membranes

Nonwoven meshes were functionalized with αGFP or rabbit α-goat antibodies using covalent coupling via SpAAC. Here, instead of the oxime coupling (Figure 1), αGFP was modified using MTGase to introduce the bio-orthogonal DBCO-PEG₄ moiety (Figure 3a; Figure S7). eADF4(C16)[Aha] was processed into filter-like nonwoven membranes with a thickness of approximately 12 μm, as determined by SEM (Figure 3b, left panel). The immobilization and activity of the αGFP antibodies on the spider silk nonwoven membranes was detected using the antibody's target GFP (Figure 3c,d; Figure S12). αGFP-functionalized eADF4(C16) meshes revealed a 10- to 30-fold higher fluorescence signal than the control eADF4(C16)[Met] nanofibers with modified αGFP and eADF4(C16)[Aha] nanofibers with non-modified αGFP, respectively. eADF4(C16)[Aha] nonwoven meshes incubated with the ligation reaction mixture excluding MTGase or

α GFP also demonstrated low background signals derived from unspecific coupling of components in the reaction mixture. The fluorescence microscopy images of α GFP-functionalized nonwoven meshes revealed homogeneous fluorescence, suggesting no aggregation of α GFP on the meshes (Figure 3d). The detection of the MTGase-modified α GFP using the antigen confirmed its successful immobilization and the accessibility of the antigen-binding sites on the membrane's surface. Moreover, in contrast to the relatively small model protein target GFP (~28 kDa), we also evaluated the capture of bulkier antibodies. Therefore, rabbit α -goat antibodies were immobilized onto nonwoven meshes (Figure 3b), again using the MTGase-based approach (Figure 3a). In this case, binding of a substantially larger α -rabbit FITC-conjugated antibody (~150 kDa) was targeted (Figure S13). Notably, the functionalized nonwoven meshes exhibited binding capabilities comparable to those observed for the anti-GFP system (compare Figure 3c,d with Figure S13). The increased molecular complexity of the targeted α -rabbit antibody, however, was reflected in higher levels of nonspecific binding to the filter material (Figure 3c vs. Figure S13a). Importantly, binding experiments for both GFP (Figure 3c,d) and the α -rabbit antibody (Figure S13) were conducted under conditions in presence of bovine serum albumin as serum protein known for unspecific interactions with other proteins, reflecting to some extent possible competitive binding conditions.

The residue-specific incorporation of Aha into silk proteins was previously developed to attach fluorophores or antibiotics to the conserved, globular CTD [28]. However, the CTD's native structure is required for the pre-assembly of natural spider silk proteins during the natural spider silk fiber spinning [68, 69]. In the present study, azido groups were incorporated into an unstructured amino-terminal T7-tag used in eADF4(C16) solely for immunostaining-based detection. The T7-tag does not interfere with protein self-assembly into diverse morphologies, including nanofibrils, particles, and films, as shown previously [7]. Accordingly, substituting Met residues with Aha within the short, unstructured T7-tag preserves both the self-assembly behavior of the protein and the formation of spider silk typical β -sheet-rich structures. This strategy ensures that Aha-modified eADF4(C16) retains its processability into the diverse morphologies without significantly altering its properties as shown in this study as well as previously [7]. The T7-tag was sufficiently exposed in assembled spider silk materials to render the Aha moieties accessible for click chemistry. Our tests confirmed the applicability of antibody-functionalized spider silk-based membranes, which could be used, e.g., in biofiltration applications. Processing a thin layer of nonwoven mesh with a defined pore size on, for example, support filter meshes made of polyamide (Figure 3b, right panel) and stacking thereof represents a reasonable approach toward ready-to-use products.

3 | Conclusion

The availability of recombinantly produced spider silk proteins with bio-orthogonal groups opens new possibilities to tailor the properties of a respective spider silk material, as demonstrated by its selective functionalization with antibodies. The antibodies were attached to different morphologies of the spider silk protein after processing and fully retained their antigen binding activity.

Two different approaches for attachment were used, adding to the versatility of the new hybrid material. The first used covalent bonding of an azide-silk with a strained alkyne on the antibody. The second approach employed a strained alkyne modification on a pair of complementary oligonucleotide handles attached correspondingly to the spider silk and the antibody surface, thus, allowing reversible loading of the spider silk materials with the active agent. Compared with previously reported silk functionalization strategies that rely on site-unspecific modifications [70–72], harsh protein modification conditions [73], binding of small organic molecules such as antibiotics [28], or genetically fused binding systems [74, 75], our work offers a fundamentally different and more versatile approach. By enabling residue-specific azide incorporation into a structurally and functionally inert T7-tag and employing a scalable fermentation process, we produced an azide-modified spider silk variant that can be processed into both micro- and macroscopic morphologies. Subsequent site-specific and chemically modular click conjugation allowed the covalent attachment of large and sensitive biomolecules, such as antibodies, without compromising their bio-affinity. This strategy overcomes the limitations of earlier methods and establishes a robust platform for the design of multifunctional spider silk-based hybrid biomaterials with precisely controlled biological specificity. Along with the well-established scalable production of the nonwoven meshes [63, 76–78] and their long-term stability in a biological environment [79], the new bio-based materials can be used, e.g., for filtration and binding of active biological agents, e.g. in bio-separation applications.

4 | Experimental Section

All standard chemicals were purchased from Roth (Karlsruhe, Germany), Sigma (St. Louis, MO) or Merck KGaA (Darmstadt, Germany). Aha and Nle were obtained from IRIS Biotech GmbH (Marktredwitz, Germany).

4.1 | Transformation of E. Coli

E. coli B834(DE3) (B F[−]ompT hsdS(r_B−m_B−) dcm⁺ gal λ(DE3) met; Merck KGaA) was used as Met auxotrophic host for spider silk biosynthesis. Transformation was carried out using the plasmid encoding the T7-tagged eADF4(C16) under the T7 promoter. The plasmid was constructed as published before [12].

4.2 | Fed-Batch Fermentation Strategy

The pre-cultivation procedure was performed in shake flasks in LB medium (Roth). A 1.5 L minifors bioreactor (Infors HT, Bottmingen, Switzerland) was inoculated with the preculture at an OD₆₀₀ of 0.1 in 0.6–1.3 L synthetic complete medium (16.15 g L^{−1} tryptone from casein, 20 g L^{−1} yeast extract (Roth), 2.15 g L^{−1} KH₂PO₄, 5.38 g L^{−1} (NH₄)₂HPO₄, 0.11 g L^{−1} CaCl₂ × 2 H₂O, 1.08 g L^{−1} MgSO₄, 1.5 g L^{−1} α-D-(+)-glucose monohydrate). The amount of yeast extract limited the Met supply to facilitate efficient Aha incorporation. To maintain the expression plasmid, we supplemented the medium with 50 μg mL^{−1} kanamycin. The stirrer speed was set to 900 rpm. Prior to inoculation, 1 mL of antifoam agent (5% antifoam

Lube AF12, ILCO Chemikalien, Erkelenz, Germany) was added. During the fermentation, antifoam was automatically added if foaming occurred. The cells received continuous nutrient feed with 30% (w/v) α -D-(+)-glucose monohydrate at 0.32 mL min⁻¹ once the pO₂ dropped to 40%. The pH was maintained at 6.7 by automated addition of 8% (v/v) NH₄OH and 1 M H₃PO₄ using a pH sensor (Hamilton, Bonaduz AG, Switzerland). The oxygen partial pressure was controlled by a pO₂-Sensor (Hamilton) and was maintained at 40% using pure gas inlet. Aha was incorporated into eADF4(C16) using the residue-specific method. The cells were cultivated until Met was depleted and the growth plateaued. The OD₆₀₀ was measured offline to monitor cell growth using a DU800 Spectrometer (Beckman Coulter Inc., Brea, USA). At Met depletion, silk gene expression was induced with 1 mM IPTG. 0.25 mM each of alanine, glycine, proline, and serine, and 2.5 to 10 mM of Met, Aha, or Nle were supplemented at the time of induction. To produce eADF4(C16) in soluble form [4], expression was carried out for 3.5 h at 30°C. To drive eADF4(C16) mainly into inclusion bodies, production was carried out overnight at 34°C. The cells were harvested by centrifugation for 20 min at 6,000 rpm, 4°C (Lynx 6000, Thermo Fisher Scientific, Waltham, USA), washed in 50 mM Tris/HCl, 150 mM NaCl, pH 7.5, resuspended in 5 mL g⁻¹ cells of buffer, and stored at -20°C. The cell dry mass (CDM) was assessed as follows: 2 mL polypropylene tubes (Eppendorf SE, Hamburg, Germany) were dried in a baking oven for 48 h at 105°C and cooled for 4 h at RT in a desiccator before weighing. 1 mL culture was harvested each in three previously weighed 2 mL Eppendorf tubes using a tabletop centrifuge (Eppendorf, 14,000 rpm, 10 min, 4°C). The cell pellets were washed with 1 mL deionized H₂O, centrifuged as described, and the supernatants were discarded. The pellets were incubated for 48 h at 105°C, cooled for 4 h in a desiccator, and weighed.

4.3 | Cell Lysis

Thawed cells were first lysed enzymatically using 2 mg mL⁻¹ lysozyme and addition of 5 mM EDTA, 10 mM sodium ascorbate, and 1 mM phenylmethylsulfonylfluorid under stirring for 100 min at 4°C. Subsequently, cells were further lysed using an ultrasonic treatment (Sonopuls HD 3200, Bandelin, Berlin, Germany) with the TT13 probe, 90% amplitude, 45 s pulse, and 2 s on /1 s off. This treatment was repeated until more than 10 kJ energy per 100 mL suspension was transferred. After centrifugation of the cell lysate (Eppendorf, 14,000 rpm, 45 min, 4°C), the target protein was either purified from the soluble protein fraction in the supernatant or the inclusion bodies in the pellet.

4.4 | Purification Strategy

Purification of eADF4(C16) from the soluble protein fraction was performed as described previously [4]. For the purification from inclusion bodies, the pellet was resuspended in 10 mL of 60 mM EDTA, 2% (v/v) Triton X-100, 1.5 M NaCl per gram of pellet and homogenized in a blender. After removing the foam by centrifugation (Multifuge 3SR+, Heraeus, Hanau, Germany, 4,000 rpm, 5 min, 4°C), the suspension was incubated for 15 min at 4°C under stirring. The suspension was subjected to an ultrasonic treatment as described above until a total energy input of 5 kJ per 100 mL was exceeded. The suspension was centrifuged

(Eppendorf, 14,000 rpm, at least 15 min, 4°C), and the pellet was washed in 100 mM Tris/HCl, 100 mM NaCl, 20 mM EDTA, pH 7.5 by shaking and centrifugation (Eppendorf, 14,000 rpm, at least 15 min, 4°C). For the subsequent ammonium sulfate treatment, the pellet was resuspended in 100 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5, and the pulverized ammonium sulfate corresponding to 30% (w/v) was slowly added while stirring until complete dissolution. After gentle agitation overnight at 4°C and a subsequent centrifugation (Eppendorf, 14,000 rpm, 45 min, 4°C), the pellet was washed 5x in 8 M urea by shaking, stirring for 1 h (4x) or overnight (1x) at RT, followed by centrifugation (14,000 rpm, at least 20 min, RT). Subsequently, the pellet was washed 3x in MilliQ-H₂O by shaking, stirring at RT for at least 30 min, followed by centrifugation (Eppendorf, 14,000 rpm, at least 20 min, 4°C). After resuspension in MilliQ-H₂O and centrifugation (Multifuge 3SR+, Heraeus, 9,000 rpm, at least 20 min, 4°C), the purified protein pellets were frozen in liquid N₂, lyophilized, and stored at -20°C. Importantly, there was no apparent physicochemical difference between the proteins purified from the supernatant or from inclusion bodies.

4.5 | Accessibility of Aha Bio-Orthogonal Handles

Lyophilized Aha modified eADF4(C16) was solubilized in 6 M guanidinium thiocyanate at 2 mg mL⁻¹ and dialyzed against 10 mM Tris/HCl pH 7.5 at RT. For functionalization, 20 μ M protein was mixed with 1–9 equivalent of DBCO-Cy3 (Lumiprobe, Hannover, Germany; dissolved in DMSO), in a total of 20% (v/v) DMSO. The reaction mixtures were incubated at 600 rpm for 1 h at RT and analyzed using SDS-PAGE. The products in the 10% polyacrylamide gel were visualized using an Ettan DIGE fluorescence imager (GE Healthcare, Chicago, USA) followed by silver staining. For the formation of eADF4(C16)[Aha] particles, aqueous protein solution at 1.0 mg mL⁻¹ was mixed with 2 M potassium phosphate pH 8 in the volumetric ratio of 1:1 for 5 min. The precipitated particles were washed 3x with MilliQ-H₂O and incubated with 100 μ M DBCO-Cy3 for 1 h at 600 rpm and 22°C. The fluorescent labeling was visualized using a DMi8 fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

4.6 | Introduction of Aldehydes In α GFP

The rabbit monoclonal α GFP antibody (Sino Biological, Beijing, China, Catalog No. 13105-S07E) was used for the site-specific modification. Vicinal diols on the glycan at the position N297 were oxidized with sodium metaperiodate (Thermo Fisher Scientific) to aldehydes as described previously [80]. The aldehyde-functionalized IgG was ultrafiltrated 5x against 100 mM sodium acetate pH 5.5 using Amicon ultra centrifugal filters with a 50 kDa molecular weight cutoff (Merck Millipore).

4.7 | Oxime Coupling of Aminoxy-PEG₃-N₃

3–5 mg mL⁻¹ aldehyde-functionalized α GFP IgG in 100 mM NaOAc pH 5.5 was treated with aminoxy-PEG₃-azide (Conju-probe, San Diego, USA) at a molar ratio of 1:100 in presence of 100 mM aniline as a nucleophilic catalyst [43] overnight at

RT. Afterward, the buffer was exchanged to 100 mM sodium phosphate, 250 mM NaCl, pH 7.6 using ultrafiltration as described above.

4.8 | Conjugation of IgG-PEG₃-N₃ and eADF4(C16)[Aha] with Oligonucleotides

IgG-PEG₃-N₃ and eADF4(C16)[Aha] were conjugated with the DBCO-functionalized single-stranded nucleic acids reporter (rep, 5'-DBCO-ttt ttt gac agg cga gga at-3') and capture (cap, 5'-DBCO-ttt ttt att cct cgc ctg tc-3'), respectively. In particular, IgG-PEG₃-N₃ was coupled to DBCO-rep at 1:5 molar ratio for 48 h at RT, and the conjugate IgG-PEG₃-rep was purified using dialysis against 100 mM sodium phosphate, 250 mM NaCl, pH 7.6. Aha-modified spider silk protein at 1 mg mL⁻¹ was mixed with 2 equivalent of DBCO-cap in 50 mM HEPES-Na, 4 M urea, pH 8 for 30 h at RT. The excess of oligonucleotides was removed using precipitation of eADF4(C16)[Aha]-cap conjugate in 1 M potassium phosphate, pH 8 and five rounds of centrifugation and washing with 100 mM Tris/HCl, 300 mM NaCl, pH 7.0.

4.9 | Modification of IgG Antibody using MTGase

1 mg mL⁻¹ IgG (monoclonal rabbit αGFP IgG, Sino Biological, Beijing (China) or polyclonal rabbit α-goat IgG; Jackson ImmunoResearch, West Grove, PA, (US)) was deglycosylated with 500 U mg⁻¹ PNGase F (Promega, Madison, USA). For linker ligation, 0.5 mg mL⁻¹ of the deglycosylated IgG was incubated with 80 equivalents of DBCO-PEG₄-amine (Jena Bioscience, Jena, Germany; dissolved in DMSO) and 10 U mg⁻¹ MTGase (Zedira, Darmstadt, Germany). Both reaction mixtures were prepared in 20 mM Tris/HCl, 100 mM NaCl, pH 7.5 and incubated for 1 day at 37°C, respectively. The amount of DMSO in the ligation mixture was kept at 8% (v/v) to prevent activity loss of the MTGase.

4.10 | Preparation of Nonwoven Meshes

The lyophilized spider silk proteins were solubilized in HFIP at a concentration of 15% (w/v) by incubation for 3–6 days under rotation. Upon use, the protein solution was diluted to 12.5% with HFIP and filled into a 1 mL syringe with a 21G blunt cannula, which was then connected to the voltage source in the electrospinning chamber. The collector plate, representing the counter electrode, was positioned at 15 cm from the needle tip. The spinning process started by activating the syringe pump with a flow rate of 420 μL h⁻¹ and a voltage of +15 kV at the needle tip and −2.5 kV at the collector plate. The spinning duration and the substrate were adapted to the respective application: the fibers were spun for 20 min on aluminum foil for FTIR spectroscopy, functionalization with antibodies, and SEM imaging (Figure 3b, left panel). Additionally, fibers were spun for 5 min on RCA-cleaned silicon wafers (according to the Radio Corporation of America-developed RCA cleaning protocol) (Figure 2) or on a polyamide mesh (Figure 3b, right panel) for SEM characterization. The relative humidity was in the range of 33 ± 7% in all electrospinning experiments. The as-spun nonwoven meshes were post-treated using saturated methanol vapor overnight at RT.

4.11 | SEM

Nonwoven meshes fixed on SEM specimen stubs were sputter-coated with platinum at a thickness of 1.3 nm in a high vacuum sputter coater (Leica Microsystems, Wetzlar, Germany). The samples were imaged in a FEI Apreo VS scanning electron microscope (Thermo Fisher Scientific, Waltham, USA) using an ETD secondary electron detector and an acceleration voltage of 5 kV (Figure 2) or 2 kV (Figure 3b).

4.12 | ATR-FTIR Spectroscopy and FSD

Untreated and post-treated nonwoven meshes were analyzed on three different spots using a Bruker Tensor 27 FT-IR-spectrometer coupled with a Hyperion FTIR microscope (Bruker Corporation, Billerica, USA) with a 20x ATR-objective. Absorption spectra in a range of 800–4000 cm⁻¹ were obtained after accumulation of 100 scans at a resolution of 4 cm⁻¹ and subtraction of the background using the software OPUS 8.1 (Bruker Corporation, Billerica, USA). For FSD of the amide I band (1590–1705 cm⁻¹), functions implemented in the OPUS software were used. The spectra were baseline-corrected, smoothed, and synthetically narrowed using Lorentzian line shapes with a half-bandwidth of 23 cm⁻¹ and a noise-reduction factor of 0.3 within the 1590–1705 cm⁻¹ region. The resulting amide I envelopes were used for peak fitting with Gaussian functions, with peak positions centered and assigned according to the literature (side chains: 1595–1615, β-sheets: 1616–1637, 1697–1703, random coils: 1638–1655, α-helices: 1656–1662, turns: 1663–1696) [65]. Fitting was performed first using a local least-squares method with fixed bandwidths, followed by refinement using the Levenberg–Marquardt algorithm with variable bandwidths. The FSD procedure was performed on three independent samples prepared from non-modified and Aha-modified spider silk proteins, both before and after methanol post-treatment.

4.13 | Functionalization of Spider Silk Particles and Films with αGFP Antibody

Films [16, 62] and particles [40, 60] were prepared as described in previous studies, which also provide details on microscopic analysis of the morphologies. The particles were processed using precipitation (see chapter 4.5), whereas the eADF4(C16)[Aha] films were processed using drop-casting into 48-well plates out of an HFIP solution (10 mg/mL) to obtain films at 0.5 mg/cm² after solvent evaporation. After drying the films, post-treatment using methanol was employed to induce insoluble, β-sheet-rich proteins [16]. To functionalize the particles and films, 10 μM DBCO-cap in PBS was added for 24 h at room temperature and washed with PBS to remove unreacted oligonucleotide excess. Unspecific binding sites of the spider silk particles and films were blocked with 1% (w/v) bovine serum albumin in phosphate buffered saline (PBS) pH 7.4 overnight at 4°C, followed by washing 2x with PBS. For the hybridization of αGFP-PEG₃-rep, cap-modified spider silk particles and films were incubated under gentle shaking with 1 or 3.5 μg of the rep-modified or non-modified αGFP (*c* = 0.07 μg μL⁻¹) for 1 h at RT. For the films, unbound αGFP was washed off twice in PBS, 0.05% (v/v) Tween 20. Another five washing steps were conducted after 5 min

of agitation. Particles were washed 5x with the same buffer using centrifugation. For the α GFP bioactivity test, $0.02 \mu\text{g } \mu\text{L}^{-1}$ GFP was added for 2 h at RT. Again, the same washing steps were conducted. GFP binding to the particles was visualized using fluorescence microscopy at a magnification of 40x. The binding of GFP to the films was quantified using GFP fluorescence ($\lambda_{\text{ex}} = 485 \text{ nm}$; $\lambda_{\text{em}} = 535 \text{ nm}$) in a Mithras LB 940 plate reader (Berthold, Bad Wildbad, Germany) with a counting time of 0.1 s.

4.14 | Functionalization of Spider Silk Nanofibers with IgG Antibody

Nonwoven meshes were fixed in CellCrown™ 24NX inserts (Scaffdex Oy, Tampere, Finland) and incubated overnight with $200 \mu\text{L}$ of $20 \mu\text{g mL}^{-1}$ MTGase-modified α GFP and α -goat IgG, respectively. The α GFP- (Figure 3) and α -goat antibody functionalized (Figure S13) nonwoven meshes were washed $3 \times 10 \text{ min}$ with $200 \mu\text{L}$ buffer and subsequently incubated with $100 \mu\text{L}$ of $10 \mu\text{g mL}^{-1}$ of GFP (Chair of Biomaterials, University of Bayreuth, Germany) [11] or secondary α -rabbit FITC-Ab (polyclonal, fluorescein (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch)), respectively, for 1 h in the dark. After washing, the fluorescence of the nonwoven meshes was determined using a SpectraMax iD5 plate reader (Molecular Devices, San José, USA) with $\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$, and scans with 21 data points per sample. In addition, the nonwoven mesh fluorescence was detected using an Ettan DIGE fluorescence imager and a fluorescence microscope. All steps were performed in PBS, 300 mM NaCl, 1% (w/v) bovine serum albumin, 0.05% (v/v) Tween 20, pH 7.4.

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Conflicts of Interest

Thomas Scheibel is co-founder and shareholder of AMSilk GmbH, Germany.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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