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D. HUEMMERICH
U. SLOTTA
T. SCHEIBEL✉

Processing and modification of films made from recombinant spider silk proteins

Department Chemie, Lehrstuhl für Biotechnologie, Technische Universität München, Lichtenbergstr. 4, 85747 Garching, Germany

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ABSTRACT Protein films represent an interesting class of materials with various possibilities for applications. We investigated films made of two different synthetic spider silk proteins derived from the garden spider's (Araneus diadematus) two dragline silk proteins ADF-3 and ADF-4. Protein films cast from hexafluoroisopropanol solutions displayed a predominantly α-helical secondary structure. Processing such films with potassium phosphate or methanol resulted in a transition to a β-sheet rich structure. While as-cast films could be dissolved in water, processed β-sheet rich films were water insoluble. The chemical stability of processed films depended on the amino acid sequence of the respective protein employed. As a proof of principle, fluorescent probes or enzymes were covalently attached to the film surface. The presented approach provides a basis for designing tailor-made protein films using silk proteins as scaffold, in which the film properties can be controlled by genetic engineering of the underlying silks.

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1 Introduction

Silk proteins belong to a fascinating group of structural proteins. Because of their excellent mechanical properties, biocompatibility, and biodegradability, silks and silk-based materials are suitable for biomedical applications such as suture materials, wound dressings, and scaffolds for cell adhesion and differentiation [1–3]. For several of these applications, natural silk has to be dissolved and reassembled into other morphologies such as films.

Most investigations concerning films made of silk proteins have been performed using silk fibroin, the main protein component of the silk from the silkworm Bombyx mori. Silk fibroin films have been cast from aqueous solutions [4–6] or from solutions containing hexafluoroisopropanol (HFIP) [4], formic acid [5], and trifluoroacetic acid [6]. Before film casting, silk fibroins tended to adopt helical or random coil conformations in solution, depending on the solvent used. After casting into films, proteins either maintained the conformation of the soluble state or adopted a more β-sheet rich conformation. In most cases processing of the films with methanol led to an increase of β-sheet content and crystallinity in the respective proteins [4–6]. Besides silk fibroins, other silk proteins have been employed to cast films. Vollrath and co-workers investigated films made of spider silk proteins extracted from major ampullate glands of the spider Nephila senegalensis. The proteins mainly showed a random coil conformation in aqueous solution, which was maintained in the cast film. Their structure changed into β-sheet upon addition of potassium chloride [7]. Finally, films have been made from a synthetic silk protein derived from the dragline silk protein MaSp1 of the spider Nephila clavipes. In HFIP the protein adopted an α-helical structure which changed into a more β-sheet rich conformation when cast into a film [8].

While investigations of spider silk based films concerning their potential application as a biological material are low numbered, there are several examples demonstrating the usefulness of films made of silk fibroins. Wound dressings made of silk fibroin films revealed superior support to the healing processes in comparison to conventional wound dressings [9]. In another set-up, silk fibroin films have been employed as scaffold to immobilize enzymes [10].

One restriction in generating functional films of silk fibroins is based on their amino acid composition. Selective chemical modification of silk fibroin is limited due to the low abundance ($< 1.5\%$) of chemically reactive amino acid side chains containing thiols, amino, or carboxyl groups [11]. Since genetic manipulations within silkworms are tedious, silk fibroins are hard to engineer and thus it is complicated to generate specific film properties. In order to avoid such complications, a recombinant spider silk technology has been employed, which allows adjustments of protein sequences to specific requirements. In our study, we have employed two recombinant spider silk proteins to cast films and further investigated the ability of these films to be processed and modified.

2 Experimental

2.1 *Preparation of protein solutions*

Production and purification of $(AQ)_{24}NR3$ and C_{16}, which are derived from the dragline silk proteins ADF-3 and ADF-4 from the garden spider Araneus diadematus was

✉ Fax: +49-89-28913345, E-mail: thomas.scheibel@ch.tum.de

performed as described previously [12]. $(AQ)_{24}NR3$ consists of 24 repeats of the amino acid sequence GPYGPGASAAAAAAGGYGPGSGQQGPGQQGPGQQGPGQQGPGQQ, and the carboxyl terminal non-repetitive region of ADF-3 (= NR3). Its molecular mass is 106 kDa. C_{16} comprises 16 repeats of the sequence GSSAAAAAAAASGPGGYGPENQGPSGPGGYGPGGP, resulting in a molecular mass of 48 kDa. Both proteins were synthesized in E. coli. Protein purification included a heat step and ammonium sulphate precipitation (for details see [12]). To obtain aqueous solutions of $(AQ)_{24}NR3$ and C_{16}, lyophilized protein was dissolved in 6 M guanidinium thiocyanate at a concentration of 10 mg/ml and subsequently dialyzed against 5 mM potassium phosphate pH 8.0. Aggregates were removed by sedimentation at $15\,000 \times g$ for 10 min. Protein concentrations were determined photometrically in a 1 cm path length quartz cuvette at 276 nm using calculated extinction coefficients of $73\,950\,M^{-1}cm^{-1}$ for $(AQ)_{24}NR3$ and $46\,400\,M^{-1}cm^{-1}$ for C_{16}. Alternatively, lyophilized silk proteins were dissolved directly in 100% HFIP.

2.2 *Secondary structure analysis*

Far-UV circular dichroism (CD) spectra were obtained using a Jasco 715 spectropolarimeter (Jasco, Japan). Spectra of soluble proteins were taken at a protein concentration of 200 µg/ml in 5 mM potassium phosphate (pH 8.0) or 100% HFIP in a 0.1 cm path length quartz cuvette at 20 °C. For measuring films, 100 µl of a 2 mg/ml protein solution in HFIP were cast on a plain quartz glass of 4 cm^2 and air-dried before CD-measurement. Scan speed was 20 nm/min, step size was 0.2 nm, integration time was set to 1 s and band width was 1 nm. Four scans were averaged.

2.3 *Film modification*

2.3.1 Coupling of fluorescein to C_{16} *film surfaces.* Films were prepared by spreading 15 µl per well of 20 mg/ml C_{16} in HFIP on the bottom of a 24-well plate (Nunc, Denmark). After evaporation of HFIP, films were incubated for 5 minutes with 1 M potassium phosphate pH 8.0. After rinsing with water, carboxyl groups were activated by incubating, 15 min with 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) (Roth) pH 5.0, 100 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma-Aldrich) and 20 mM N-hydroxysulfosuccinimide (NHS) (Sigma-Aldrich). Subsequently ethylenediamine was added to yield a final concentration of 500 mM. After 2 h of incubation, films were thoroughly rinsed with water. Finally, films were incubated for 1 h with 1 mg/ml fluorescein-5-isothiocyanate (FITC) (Molecular Probes) in 100 mM sodium carbonate pH 9.0, followed by rinsing with water and air-drying.

2.3.2 Coupling of β-galactosidase to C_{16} *film surfaces.* Films were prepared and activated with EDC/NHS as described in Sect. 2.3.1. After rinsing with water, activated films were incubated for 2 h with 100 µg/ml β-galactosidase from E. coli (Roche), 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl (PBS). After thorough rinsing with PBS, enzymatic activity was tested on the film surface (see Sect. 2.4).

2.4 *β-galactosidase assay*

β-galactosidase coupled films were incubated for 16 h at room temperature with a solution containing 100 mM sodium phosphate pH 7.0, 10 mM potassium chloride, 1 mM magnesium sulfate, 50 mM β-mercaptoethanol, and 2 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Roth, Germany).

3 Structural state of two different recombinant spider silk proteins

In order to cast films we employed the two synthetic silk proteins, $(AQ)_{24}NR3$ and C_{16} [12], which are derived from the dragline silk proteins ADF-3 and ADF-4 from the garden spider Araneus diadematus. We chose these proteins based on previous observations that ADF-3 and ADF-4 as well as their derivatives display a markedly different solubility and assembly behaviour [12, 13].

Measuring circular dichroism (CD) of $(AQ)_{24}NR3$ and C_{16} solutions revealed a different influence of aqueous buffer and HFIP on the secondary structure. In aqueous solution both proteins displayed a CD-spectrum with a single minimum at a wavelength below 200 nm which is indicative of a mainly random coiled protein (Fig. 1). In contrast, the spectra of both proteins in HFIP displayed one minimum at 201–202 nm and an additional minimum ($(AQ)_{24}NR3$) or shoulder (C_{16}) at 220 nm which is indicative of an increased α-helical content (Fig. 1). Such an effect of fluorinated alcohols on proteins and

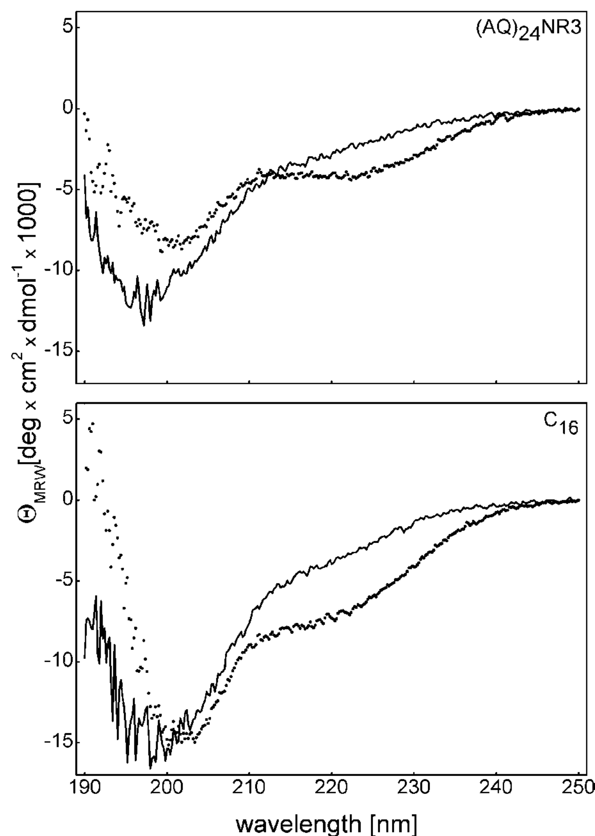

FIGURE 1 CD-spectra of synthetic silk proteins $(AQ)_{24}NR3$ and C_{16} dissolved in 6 M guanidinium thiocyanate followed by dialysis against 5 mM potassium phosphate pH 8.0 (*straight line*) or dissolved in HFIP (*dotted line*)

peptides has been reported previously [14, 15] and has also been observed for silk fibroin [4] and a synthetic silk protein derived from the dragline silk protein MaSp1 from the spider Nephila clavipes [8].

4 Film formation

Films were cast from 200 µl HFIP solutions containing 2% w/v protein on a polystyrene surface (or on quartz glass for CD-measurements). After evaporation of the solvent, $(AQ)_{24}NR3$ and C_{16} both formed transparent films that could easily be peeled off the surface (Fig. 2 and data not shown). Assuming complete evaporation of the solvent and the density of the protein film to be identical with the reported value of 1.3 g/cm^3 for spider dragline silk [16], the thickness of the films was calculated to range from 0.5 to 1.5 µm. As-cast (freshly prepared) films made of either protein dissolved upon contact with water. Since water insolubility is a prerequisite for most applications of protein films, we searched for a processing method in order to render films insoluble. Potassium phosphate is known to induce aggregation and formation of chemically stable structures of the employed silk proteins [12 and data not shown]. Also methanol has been used to obtain insoluble silk morphologies [17]. Accordingly, processing (incubating) of as-cast films with 1 M potassium phosphate or methanol resulted in the conversion of water-soluble films into water-insoluble ones.

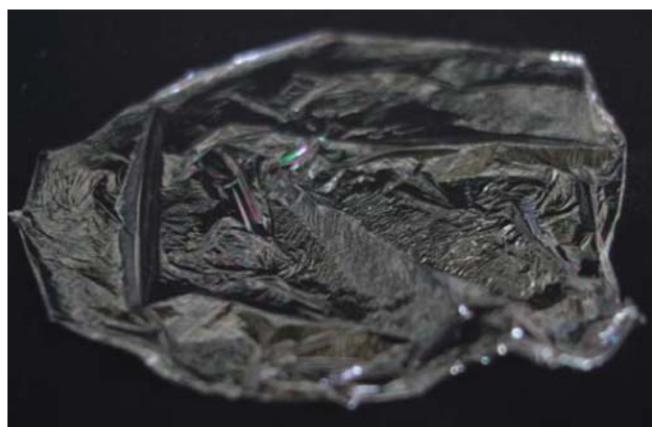

FIGURE 2 C_{16} film cast from a 2% w/v C_{16} solution in HFIP

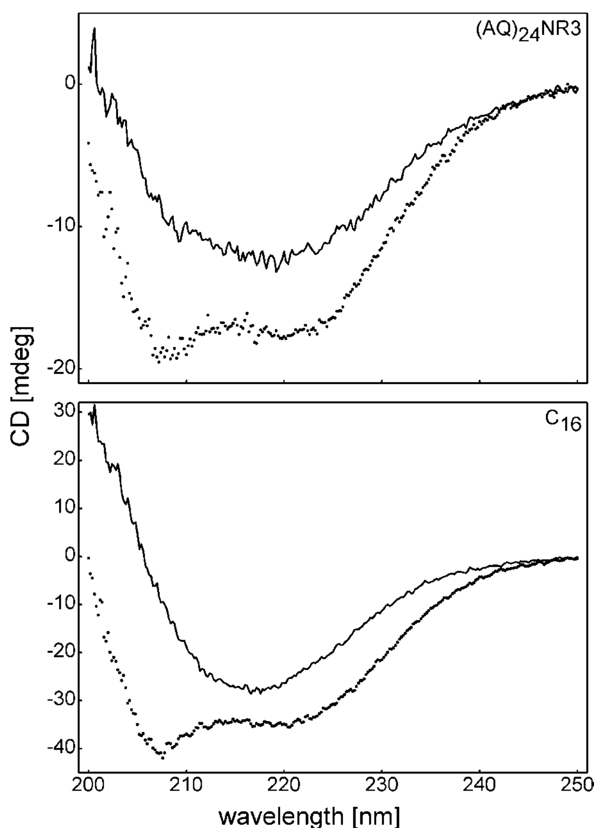

FIGURE 3 CD-spectra of protein films made from $(AQ)_{24}NR3$ and C_{16}. Films were cast from a protein solution in HFIP directly on a plain quartz glass and analyzed by CD-spectroscopy (*dotted line*). The film was subsequently processed with 1 M potassium phosphate and re-analyzed. Due to inaccuracies in defining the thickness of the films, Θ_{MRW} could not be determined

5 Secondary structure of silk films

To investigate the structural properties of the films, the secondary structure of the underlying proteins was investigated by CD-spectroscopy. As-cast films revealed a spectrum with two pronounced minima at 208 nm and 220 nm indicative of an α-helical content higher than that of soluble proteins. After processing with 1 M potassium phosphate, films revealed spectra with a single minimum at 218 nm, which is typical for a β-sheet rich protein structure (Fig. 3a). Similar results were obtained after processing films with methanol (data not shown). Thus, the transition from water-solubility to water-insolubility was paralleled by a conversion of the protein's secondary structure from α-helix to β-sheet.

6 Chemical stability

To test their chemical stability, films were submerged for 24 hours in 8 M urea, 6 M guanidinium hydrochloride and 6 M guanidinium thiocyanate (Table 1). As-cast films of both proteins as well as $(AQ)_{24}NR3$ films processed with potassium phosphate or methanol were soluble in all of these denaturants. In contrast, C_{16} films processed with potassium phosphate or methanol could only be dissolved in guanidinium thiocyanate. This remarkable chemical stability of C_{16} films is identical to that of natural dragline silk and to

	8 M urea	6 M guanidinium hydrochloride	6 M guanidinium thiocyanate
$(AQ)_{24}NR3$ as-cast	+	+	+
$(AQ)_{24}NR3$ processed	+	+	+
C_{16} as-cast	+	+	+
C_{16} processed	−	−	+

TABLE 1 Solubility of protein films in denaturants. Films were regarded as insoluble (−), where complete immersion in the respective agent for 24 hours did not result in a change of optical appearance. In contrast, solubility (+) was marked by the complete disintegration of the film under the same conditions

that of recombinantly produced and assembled ADF-4 [13]. Previous studies could correlate assembly properties and stabilities of assembled structures directly with the amino acid sequences of the silk proteins [12, 13]. Thus, properties of spider silk films can directly be modified by altering the primary structure of the silk protein via manipulation of the corresponding silk gene.

7 Film modification

Many applications of protein films require the presence of specific functionalities on the film's surface. In order to demonstrate that the employed spider silk films can be modified with small organic molecules, as well as with biological macromolecules such as proteins, the chromophor fluorescein and the enzyme β-galactosidase were chemically coupled to C_{16} films processed with potassium phosphate as a proof of principle. The coupling was achieved by activating surface exposed carboxyl groups of C_{16} using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The films were further incubated with ethylenediamine leading to the formation of an amide. The remaining free amino group of ethylenediamine was subsequently coupled to fluoresceinisothiocyanate resulting in the efficient covalent linkage of fluorescein via formation of a stable thiourea derivative (Fig. 4a). Similarly, incubation of β-galactosidase with EDC-activated C_{16} films led to the formation of amide bonds between carboxyl groups of C_{16} and primary amines (e.g. from lysine residues) of β-galactosidase which were accessible at the enzyme's surface. After repeated washing of such modified films, β-galactosidase activity could be detected using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as a substrate (Fig. 4b).

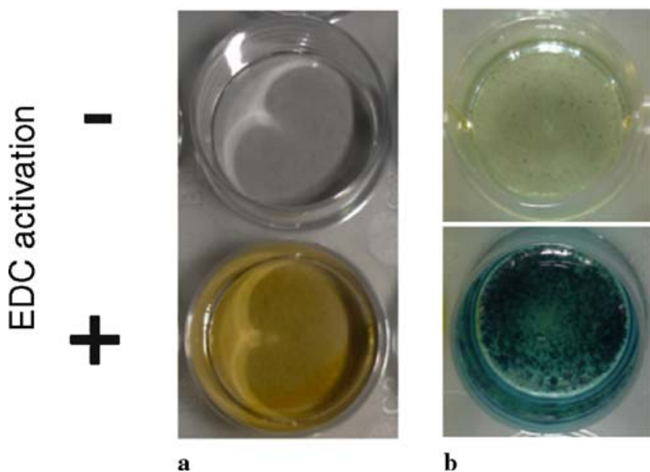

FIGURE 4 Modification of C_{16} films cast from a HFIP solution and processed with potassium phosphate. (**a**) Efficient coupling of fluorescein (*yellow colour*) only occurred when the carboxyl groups of C_{16} were activated (+) using EDC. In contrast almost no fluorescein bound to films without EDC activation (−). (**b**) Activity of coupled β-galactosidase was monitored using X-Gal as the substrate. The occurrence of a blue precipitate indicated enzyme activity only on films that had been activated with EDC (+), while non-activated films only showed residual enzymatic activity (−)

8 Conclusion

We have demonstrated that protein films can be obtained from synthetic spider silk proteins. The films, which initially were water soluble, can be processed with potassium phosphate or methanol leading to water-insolubility, which is a major requirement for many applications. Comparison of the chemical stabilities of films made from two different synthetic spider silk proteins suggested that the properties of the films were based on the primary structure of the proteins. On employing our previously established cloning strategy for spider silk genes, it will be possible to generate silk proteins that form films displaying specific properties. Since different functional molecules can be covalently attached to the film's surface, a great variety of technical or medical applications can therefore be approached.

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