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Genetically engineered mucoadhesive spider silk

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Keywords: mucoadhesion, engineered silk, mucus, mucins, galectin

Abstract:

Mucoadhesion is defined as the adhesion of a material to the mucus gel covering the mucous membranes. The mechanisms controlling mucoadhesion include nonspecific electrostatic interactions and specific interactions between the materials and the mucins, the heavily glycosylated proteins that form the mucus gel. Mucoadhesive materials can be used to develop mucosal wound dressings, and noninvasive transmucosal drug delivery systems. Spider silk, which is strong, biocompatible, biodegradable, nontoxic and lightweight would serve as an excellent base for the development of such materials. Here, we investigated two variants of the partial spider silk protein 4RepCT genetically engineered in order to functionalize them with mucoadhesive properties. The pLys-4RepCT variant was functionalized with six cationically charged lysines, aiming to provide nonspecific adhesion from electrostatic interactions with the anionically charged mucins, while the hGal3-4RepCT variant was genetically fused with the Human Galectin-3 Carbohydrate Recognition Domain which specifically binds the mucin glycans Galβ1-3GlcNAc and Galβ1-4GlcNAc. First, we demonstrated that coatings, fibers, meshes, and foams can be readily made from both silk variants. Measured by the adsorption of both bovine submaxillary mucin and pig gastric
mucin, the newly produced silk materials showed enhanced mucin binding properties
compared to materials of wildtype (4RepCT) silk. Moreover, we showed that pLys-4RepCT
silk coatings bind mucins through electrostatic interactions, while hGal3-4RepCT silk
coatings bind mucins through specific glycan-protein interactions. We envision that the two
new mucoadhesive silk variants pLys-4RepCT and hGal3-4RepCT, alone or combined with
other biofunctional silk proteins, constitute useful new building blocks for a range of silk
protein-based materials for mucosal treatments.

I. Introduction

Mucous membranes line various body cavities and orifices, including the mouth, stomach,
and bowels. All mucous membranes are covered with a mucus gel which hydrates, lubricates
and protects the underlying epithelium. The 300-400 m² of mucosal surfaces offer an
opportunity to deliver drugs in a noninvasive fashion while avoiding the first-pass hepatic
metabolism that oral administration requires, and ensuring rapid absorption of the drug due to
the high blood perfusion of the mucous membranes. However, the mucus membranes are
also vulnerable to diseases and wounds including cancer, mucosal lesions, ulcers and
chronic inflammations. These conditions affect millions of individuals each year and are
increasingly prevalent with the rise of chronic diseases such as diabetes. Unfortunately,
mucosal surfaces are a challenging environment for localized treatments. First, the surfaces
are very hydrated and well lubricated, which makes it difficult to anchor any material to
allow treatment in place. The mucus layer is also very dynamic, with a mucus turnover rate
that ranges from seconds on ocular surfaces to hours in the digestive tracts, removing
foreign objects in the process. To address these challenges, many transmucosal drug delivery
systems, mucosal wound dressings and mucosal tissue engineering scaffolds, have
been engineered to adhere to mucosal surfaces to increase their residency times.
Mucins, a family of large and heavily glycosylated proteins, and the main gelling component of mucus, are a key in mediating the interaction between mucus and the surface of implanted materials. The interactions between the material and the mucin molecules define to a great extent its mucoadhesive properties. Mucoadhesive materials can adhere onto mucous membranes through nonspecific electrostatic interactions, hydrogen bonds, molecular entanglements, and through specific binding between receptors and ligands on the mucin molecules. Mucoadhesive micro- and nano-particles, gel slabs, patches, and capsules, have been developed using mucoadhesive synthetic polymers and biopolymers. For instance, chitosan, a well-studied mucoadhesive polysaccharide has been assembled into hydrogels, nanoparticles, tablets, bilayers and colloidal systems for ocular, nasal, vaginal and buccal drug delivery. These polymers rely on their intrinsic chemical properties to bind to mucins, however, they provide no other functionalities, relying on therapeutic molecules embedded in the material to provide anti-inflammatory or wound healing action. A modulatory system that could be mucoadhesive and able to deliver biological signals beneficial to wound healing is thus of interest.

Spider silk is a strong and lightweight protein-based material shown to be biocompatible, nontoxic and biodegradable. It has been previously described that the partial spider silk protein 4RepCT can be produced using recombinant technology and spontaneously self-assemble under physiological conditions, taking different forms such as coatings, fibers, foams and films. Moreover, it is possible to functionalize 4RepCT with bioactive protein domains attached to its N-terminus while retaining its self-assembling ability and the function of the added domain. Choosing the N-terminus of 4RepCT for functionalizing the protein is important, since the C-terminal domain has been proven to be necessary for silk
assembly, promoting spontaneous fiber formation\textsuperscript{24–27}. Previously, 4RepCT has been
functionalized with the antimicrobial peptide magainin (Mag-4RepCT)\textsuperscript{31}, as well as the cell-
binding fibronectin-mimicking FN motif (FN-4RepCT)\textsuperscript{32}, both promoting wound healing by
respectively suppressing infection and supporting proliferation of fibroblasts and extracellular
matrix (ECM) formation. Both Mag-4RepCT and FN-4RepCT variants were able to self-
assemble into coatings on materials such as titanium, hydroxyapatite, stainless steel, and
polystyrene, which are often used in implants, stents, and \textit{in vitro} cell cultures, respectively
\textsuperscript{31}, as well as on superhydrophobic pillar surfaces\textsuperscript{33}. In this study, we aimed to functionalize
4RepCT with mucoadhesive properties. Two different mucoadhesion mechanisms of action
are employed. On one hand, a (poly-Lysine)-4RepCT (pLys-4RepCT) variant, containing six
cationically charged lysines, intended to provide nonspecific adhesion from electrostatic
interactions with the anionically charged mucins\textsuperscript{34}. On the other hand, the human galectin 3-
4RepCT (hGal3-4RepCT) variant, containing the human galectin-3 (hGal3) carbohydrate
recognition domain (CRD), which specifically binds the Galβ1-3GlcNAc and Galβ1-
4GlcNAc mucin glycans\textsuperscript{35}. From these two silk variants we form two- and three-dimensional
materials including coatings, fibers, meshes and foams, and assess their mucoadhesive
properties by measuring the binding of pig gastric mucin (PGM) and bovine submaxillary
mucin (BSM).

II. Materials and Methods

Design and production of recombinant silk proteins

The amino acid sequence representing the carbohydrate recognition domain of the protein
human Galectin-3 (UniProtKB ID: P17931) was selected as residue 113-250\textsuperscript{35,36}. Standard
bioinformatics tools available online were used for reverse translation of the hGal3 amino
acid sequence into nucleotide code, followed by codon-optimization for expression in
Escherichia coli (E. coli) strain B. The amino acid sequence of the codon-optimized hGal3 DNA was verified by nucleotide sequence translation (ExPASy, SIB Bioinformatics Resource Portal). After addition of restriction cleavage sites (NdeI and BamHI) flanking the hGal3 gene, the corresponding DNA sequence was ordered as a synthetic gene (Genewiz LLC). A plasmid harboring the 4RepCT silk gene was used for insertion of the hGal3 sequence in-between a His\textsubscript{6} tag and 4RepCT using standard molecular cloning techniques. The resulting DNA construct His\textsubscript{6}-hGal3-4RepCT was verified for correct insertion of hGal3 by DNA sequencing (Microsynth AG).

Expression of the fusion protein His\textsubscript{6}-hGal3-4RepCT (denoted hGal3-4RepCT for short) was performed for 3 h at 25°C using E. coli BL21(DE3) cells in 1.5 L of TSB+Y medium (30 g Tryptic Soy Broth, 5 g Yeast extract, 1 L de-ionized water), supplemented with 50 µg/ml kanamycin and 300 µM isopropyl β-D-1-thiogalactopyranoside (IPTG). Harvested cells, resuspended in 20 mM Tris (tris(hydroxymethyl)aminomethane) buffer (pH 8), were lysed for 1 h using 0.9 mg/ml lysozyme, after which the supernatant was centrifuged (4648 relative centrifugal force (rcf), 15 min, +4°C), supplemented with 200 mM NaCl and 10 mM imidazole and then loaded onto immobilized metal ion affinity chromatography (IMAC) columns packed with Chelating Sepharose Fast Flow matrix (GE Healthcare) and pre-loaded with Zn\textsuperscript{2+} ions. After elution of the target protein with 20 mM Tris (pH 8) / 200 mM NaCl / 200 mM imidazole, the NaCl and imidazole was removed by overnight dialysis (molecular weight cut-off: 6-8 kDa) at +4°C against 20 mM Tris (pH 8) buffer. The dialyzed hGal3-4RepCT protein (2.1 mg/ml) was stored at -20°C until further use.

Soluble pLys-4RepCT silk protein at 3 mg/ml in 20 mM Tris (pH 8) buffer, supplemented with 150 mM NaCl, was provided by Spiber Technologies AB as frozen aliquots. Briefly,
pLys–4RepCT was recombinantly expressed in *Escherichia coli* cells, followed by purification using immobilized metal ion affinity chromatography, performed essentially as previously stated\textsuperscript{25}. Frozen aliquots of 4RepCT, Z-4RepCT\textsuperscript{28} and FN-4RepCT\textsuperscript{32} were also provided by Spiber Technologies AB at 3 mg/ml in 20 mM Tris (pH 8). All silk proteins were stored at -20°C until further use.

**Handling and preparation of silk solutions**

The frozen silk aliquots were thawed and centrifuged for 2 min (13,000 rcf, 4 °C), prior to dilution in 20 mM Tris (pH 8) buffer (supplemented with an appropriate NaCl concentration) to the desired concentration. Silk coatings, fibers, fiber meshes, and foams were then prepared from the soluble silk solution as described in the following sections.

**Quartz crystal microbalance with dissipation monitoring (QCM-D)**

To investigate in real-time the ability of different silk variants to form surface coatings, as well as the adsorption of mucins or asialofetuin on them, we used QCM-D (Q-Sense E4 series, Q-Sense AB, Sweden). 4RepCT has been shown to spontaneously form coatings on gold surfaces before \textsuperscript{31}, therefore we monitored the adsorption of the silk variants on gold-coated quartz crystals (Q-Sense QSX 301, Biolin Scientific). Previously used sensors were cleaned before each experiment by first submerging them in concentrated formic acid (Sigma) for 10 minutes at room temperature. The crystals were then rinsed with ultrapure water (PURELAB® Option-Q, ELGA) and dried by blowing with N\textsubscript{2} gas, followed by air-plasma treatment for 3 minutes. Finally, the sensors were cleaned with a 5:1:1 mixture of ultrapure water (PURELAB® Option-Q, ELGA), 25% ammonia and 30% hydrogen peroxide at 80 °C for 10 minutes, rinsed again with ultrapure water (PURELAB® Option-Q, ELGA) and dried by blowing N\textsubscript{2} gas. During all QCM-D experiments the flow rate was set at 25
µl/min and the temperature at 25 °C. The buffer used for baseline stabilization, for washes, and to dilute the soluble silks and mucins was 20 mM Tris (pH 8) / 10 mM NaCl (except for the case of NaCl dependency experiments, where higher NaCl concentration was also used). Preceding the adsorption measurements, a stable baseline was obtained in buffer after which the silk was adsorbed to the gold surface from a 0.1 mg/mL solution. The coating was then washed with buffer until a stable signal was obtained, then was subjected to mucin or asialofetuin solutions (1 mg/ml). The experiments were terminated by a wash step to remove weakly-bound molecules. The 7th overtone was chosen to be displayed for all frequency and dissipation results.

**Atomic Force Microscopy (AFM)**

Atomic force microscopy (AFM) was used to image the morphology and coverage of the adsorbed silk material on the gold QCM-D sensors (Q-Sense QSX 301, Biolin Scientific). The surfaces were coated for 30 minutes with a 0.1 mg/ml silk solution, washed with 20 mM Tris (pH 8) / 10 mM NaCl, then with ultrapure water (PURELAB® Option-Q, ELGA) to remove all salts. In order to not alter the morphology of the adsorbed layer by air-drying, the sensors were snap frozen with liquid nitrogen immediately after washing with ultrapure water (PURELAB® Option-Q, ELGA) and subsequently lyophilized. The adsorbed silk material was imaged in air using a Bruker Multimode 8 (Bruker, Santa Barbara, CA) running in the ScanAsyst mode with a cantilever having a nominal tip radius of 2 nm (SCANASYST-AIR, Bruker, Camarillo, CA).

**Preparation of 3D materials: fibers, meshes and foams**

In order to create silk-based wound dressing prototypes from our genetically engineered silk proteins we formed different types of 3D materials, fibers, meshes and foams.
Macroscopic silk fibers of 4RepCT, pLys-4RepCT and hGal3-4RepCT were self-assembled by gentle wagging of the respective silk protein solution (1 mg/ml) at room temperature for 6 days. The fibers were then thoroughly washed in fresh 20 mM Tris (pH 8) buffer, prior to storage immersed in Tris buffer at 4°C.

For making the fiber mesh, a 1:1 mix of pLys-4RepCT and hGal3-4RepCT silk fibers or 4RepCT silk fibers were washed in ultrapure water (PURELAB® Option-Q, ELGA) and cut in small pieces (~4 mm) using a scalpel. The fiber pieces were transferred onto a Teflon sheet one by one, assembling a mesh-like structure. After air drying at room temperature, the mesh was transferred in a well plate and rehydrated in 20 mM Tris (pH 8).

For making the foam, a total volume of 1.5 ml of 3 mg/ml soluble 4RepCT protein (1:1 mix of pLys-4RepCT and hGal3-4RepCT, or 1:1:1 mix of 4RepCT, FN-4RepCT and Z-4RepCT) was placed in a 25 ml glass beaker and frothened for 5 seconds using a conventional milk frother. The formed foam was quickly transferred into a 6-well plate well whose walls were covered with two silicon rings placed on top of each other, and the bottom was covered with a Teflon sheet. The foam was initially placed for an hour at 37 °C with 30% humidity, and after that it was gradually hydrated with 20 mM Tris (pH 8) in a drop-wise fashion. The hydrated foam was then stored at 4 °C.

**Preparation of mucins**

Two different mucin sources were used for the investigation of the mucoadhesive properties of our materials: bovine submaxillary mucin (BSM) and Pig Gastric Mucin (PGM). The BSM used is commercially available from Sigma-Aldrich (product number M3895). It was dissolved at 10 mg/ml in ultrapure water (PURELAB® Option-Q, ELGA), ultracentrifuged
for one hour at 400,000 rcf and 4°C to remove insoluble particles, and the supernatant was snap frozen with liquid nitrogen and lyophilized. The PGM was purified from the mucus of pig stomachs obtained by Lövsta Kött AB in Uppsala. After cutting open and gently rinsing the stomachs, the mucus was scraped and diluted to a 1 to 5 ratio in 10 mM phosphate buffer (pH 7) with 170 mM NaCl containing 0.04% (w/v) NaN₃, and the pH was adjusted to 7.4 with NaOH. Then, 5 mM benzamidine HCl, 1 mM 2,4'-dibromoacetophenone, 1 mM phenylmethylsulfonyl-fluoride, and 5 mM EDTA (pH 7.4) were added and gently stirred overnight at 4°C. The solubilized mucus was centrifuged at 8,300 rcf for 30 minutes at 4°C, the supernatant from which was centrifuged at 15,000 rcf for 45 minutes at 4°C and then ultracentrifuged at 11,800 rcf for 1 hour at 4°C (70 Ti rotor, Beckman Coulter). The mucins in the supernatant were purified using a Sepharose 6 size exclusion chromatography column; the column was equilibrated using Phosphate Buffered Saline (PBS), the absorbance was recorded at 280 nm, and 12 ml fractions were collected. The fractions included in the first excluded peak were pooled and concentrated as well as desalted with ultrapure water (PURELAB® Option-Q, ELGA) using an Amicon Stirred Cell (Merck Millipore) and a 100 kDa cutoff membrane. The final desalted and concentrated PGM was snap frozen with liquid nitrogen, lyophilized and stored at -20°C. The purified mucins were previously shown to be composed mainly of MUC5AC and traces of MUC5B by mass spectrometry.

**Labeling of mucins with fluorescein isothiocyanate**

To investigate the mucoadhesive properties of the 3D materials (fibers, meshes and foams) using fluorescence microscopy, the mucins were fluorescently labeled with fluorescein isothiocyanate (FITC). FITC was first dissolved in anhydrous dimethyl sulfoxide (DMSO) at a concentration of 40 mg/ml. Then, the mucins were FITC-labeled at a final mucin concentration of 5 mg/ml in the presence of 2 mg/ml FITC and 100 mM
carbonate/bicarbonate (pH 9) in ultrapure water (PURELAB® Option-Q, ELGA), on a rocking table for 90 minutes at room temperature. For the elution of FITC labeled BSM a PD10 Size Exclusion Chromatography (SEC) column (GE Healthcare Life Sciences, product number 17085101, 2017) was used and the fluorescence of the elution fractions was measured using a plate reader (CLARIOstar, BMG LABTECH). In the case of FITC labeled PGM, an Amicon Ultra-15 centrifugal filter device with a 50 kDa cutoff (Millipore) was used instead to remove free FITC. Finally, in order to compare the degree of labeling for both FITC-labeled mucin types, the fluorescence intensity was measured using a plate reader (Clario Star, BMG Labtech, excitation 490 nm, emission 525 nm).

**Binding of FITC-labeled mucins to 3D silk materials**

To examine the adhesion of the FITC-labeled mucins to the 3D silk materials, pieces of the materials small enough to fit in a 96-well plate were cut using a scalpel and placed in 100 µl of 1 mg/ml FITC-labeled BSM or PGM in 20 mM Tris (pH 8) / 100 mM NaCl for 30 minutes. The NaCl concentration was here increased to 100 mM in order to better mimic the physiological Na\(^{+}\) concentration in the saliva\(^{38}\). The pieces were then removed from the wells and washed in 20 mM Tris (pH 8) / 100 mM NaCl twice. The 3D materials were visualized using an Inverted Nikon Eclipse Ti fluorescence microscope. Exposure time, binning, and magnification were kept constant to enable the comparison of the samples. From the obtained fluorescence microscopy images, the fluorescence density was calculated by dividing the integrated density of the fiber area with the fiber area itself, both values obtained using ImageJ (NIH). Also, it is important to note that due to variations in the amount of the material and the position of the material in the well, the values we obtain are only rough values of the fluorescence density.
Statistical analysis

All statistical analyses were conducted using GraphPad Prism 5. Data are expressed as means ± standard error of the mean (SEM) of the technical triplicates. Comparison of means was achieved using paired two tailed t-test for pairwise comparisons and one-way analysis for variance (one-way ANOVA) for multiple comparisons using Prism software (GraphPad, version 6). Two datasets were assumed to be statistically different if p<0.05.

III. Results and Discussion

Silk proteins functionalized with pLys and hGal3 can be recombinantly produced

In the present study we aimed at constructing silk-based materials with mucoadhesive properties, applicable in mucosal treatments. The approach to achieve this was to design protein building blocks consisting of two entities with distinct functions. One entity represents a protein domain, or peptide, promoting mucosal adhesion, whereas the other one contributes with the material properties and is herein represented by a silk protein. In this way both the mucoadhesion and the ability to form a material is displayed by each individual protein building block, reducing the time and effort during protein production.

Using the above approach, the gene corresponding to the 4RepCT silk protein (23 kDa) (Figure 1A) was fused at its N-terminal end at the gene level to either a poly-lysine (pLys) peptide with six adjacent lysine residues, or to the human galectin-3 carbohydrate recognition domain (hGal3), resulting in the two mucoadhesive silk proteins pLys-4RepCT (24 kDa) and hGal3-4RepCT (40 kDa), respectively (Figure 1B-C). After recombinant expression and purification using immobilized metal ion affinity chromatography (IMAC), both proteins were recovered as soluble silk fusion proteins with acceptable purity (Figure 1D). Furthermore, given that 4RepCT assembles only with itself, the silk self-assembly process
will exclude the incorporation of impurities in the materials. The difference in molecular size between 4RepCT and hGal3-4RepCT (16 kDa) was evident through SDS-PAGE, while the 1 kDa difference between 4RepCT and pLys-4RepCT was not easily visible due to the small size difference from the six fused lysines. Nonetheless, the verified DNA sequence for pLys-4RepCT, and its need for higher NaCl in order to remain soluble compared to 4RepCT, suggest the successful production of the pLys-4RepCT protein.

Figure 1. Mucoadhesive recombinant silk proteins. Schematic illustrations of the silk protein variants used in the present study. 4RepCT (A) was used to genetically engineer the two mucoadhesive silk variants pLys-4RepCT (B) and hGal3-4RepCT (C). Poly-Lysine (pLys), six consecutive lysine amino acid residues (~1 kDa); hGal3, human galectin-3 carbohydrate recognition domain (~16 kDa) (Protein DataBank ID: 4XBN). All three silk proteins also contained an N-terminal His$_6$-tag for purification purposes (not shown), which was subsequently removed for 4RepCT and pLys-4RepCT proteins. (D) Appearance on SDS-PAGE of 4RepCT (23 kDa), pLys-4RepCT (24 kDa) and hGal3-4RepCT (40 kDa) after IMAC purification.
pLys-4RepCT and hGal3-4RepCT proteins spontaneously form coatings

The successful production of the two new silk variants prompted us to investigate whether the protein would assemble into mucoadhesive materials. We first tested whether the silk solutions could spontaneously form coatings. Such coatings are useful tools to further investigate the properties of the silk variants, and could be used to equip a range of materials with mucoadhesive properties. Using quartz crystal microbalance with dissipation (QCM-D), we were able to monitor the formation of silk surface coatings on gold-coated quartz crystals. The 3rd, 5th, and 7th overtones showed similar results (SI Figure 1), so the 7th overtone was chosen to be presented for all QCM-D experiments. In QCM-D measurements, the decrease in the resonance frequency of the quartz crystal is related to the increase in sensed mass, including water and ions associated with the adsorbed species. The different silk solutions were flowed at a concentration of 0.1 mg/ml over the gold-coated QCM-D sensors. Both new variants, pLys-4RepCT and hGal3-4RepCT, adsorbed strongly, with a -58 Hz (Figure 2C) and -55 Hz (Figure 2E) decrease in frequency after 30 minutes, respectively. Within the same timeframe, 4RepCT adsorbed less, with a response of -37 Hz (Figure 2A). Otherwise the adsorption profiles were similar for the three silk solutions. First, a rapid decrease in frequency is seen, which we hypothesize corresponds to an initial adsorption and coverage of the gold surface with silk. This phase of rapid coating was then followed by a more linear decrease of frequency, which is likely due to steady addition of silk on the initial existing silk coating and the possible formation of silk multilayers that continues until the silk flow is stopped and changed to the buffer solution. These observations are in accordance with previously published data for coatings of 4RepCT silk, where the silk self-assembly continued as long as soluble 4RepCT proteins were provided to the system 31. During
subsequent wash of the silk coatings with a 25 µl/min flow of buffer solution, the frequency signal remained stable, indicating that the coating is stable under these conditions.

While a change in frequency represents a change in mass, a change in dissipation represents a change in the rigidity of the adsorbed material. Indeed, soft and hydrated coatings are able to dampen the acoustic energy which results in high dissipation values, while a denser and more rigid layer will dissipate less energy resulting in low dissipation values. For all three silk variants, the dissipation increased slightly during the formation of the coatings, indicating that the silk coatings are quite dense and rigid (Figure 2A, C, E). Although a decrease in frequency is systematically associated with an increase in dissipation, the extent of the dissipation increase per Hz of frequency decrease can vary from material to material, representing the rigidity of the coating. Thus, the D/F ratio, which serves the purpose of enabling us to compare the silk coatings through their dissipation response per frequency decrease, showed similar values for all three silk coatings (SI Figure 2), suggesting that all silk coatings were characterized by a similar rigidity during the coating process.

We then used Atomic Force Microscopy (AFM) to visualize the coatings formed on the gold-coated QCM-D crystals. We could see evidence of the formation of fibrils on the surfaces for 4RepCT and pLys-4RepCT coatings (Figure 2B, D). However, for hGal3-4RepCT coatings (Figure 2F) no fibrils were seen, but only a rather homogeneous hGal3-4RepCT layer, covering the 9 nm high mounts visible on bare gold surfaces (Figure 2G). In order to get a zoomed-out picture of the hGal3-4RepCT coated sensor, we obtained a bigger scan size AFM image from it, which confirmed that no fibrils were present, but a rather homogenous globular silk layer (SI Figure 3).
As previously mentioned, 4RepCT has already been reported to spontaneously form coatings on surfaces made of gold, titanium, hydroxyapatite, stainless steel, and polystyrene\textsuperscript{31}, with an initial rapid adsorption to the surface followed by a continuous accumulation of material. Here, we observe a similar behavior of the new silk variants, which indicates that the self-assembly on the surface was not perturbed by the addition of the poly-lysine peptide and the galectin domain. However, hGal3-4RepCT does not show the same characteristic nanofibril structure after self-assembly onto the surface as 4RepCT and pLys-4RepCT, which could be due to the relatively large size of the hGal3 domain interfering with self-assembly of 4RepCT.

![Figure 2. Formation of coatings of the different silk variants.](image)

Quartz crystal microbalance with dissipation (QCM-D) was used to monitor the formation of surface coatings for 4RepCT (A), pLys-4RepCT (C) and hGal3-4RepCT (E) on gold-coated quartz crystals in real time. A
solution of 20 mM Tris (pH 8) / 10 mM NaCl was flowed onto the crystal until a stable baseline was established (time points 0-A), coated with 0.1 mg/ml silk solution (time points A-B), and washed again to examine the stability of the coatings (time points B-end). AFM images from measurements of gold-coated quartz crystals coated with 4RepCT (B), pLys-4RepCT (D) and hGal3-4RepCT (F) for 30 minutes. A bare gold sensor surface is shown as a reference (G).

Mucins bind to pLys-4RepCT and hGal3-4RepCT silk coatings

After ensuring the ability of pLys-4RepCT and hGal3-4RepCT to form coatings on the gold coated crystals, we used QCM-D to investigate the interaction of bovine submaxillary mucins (BSM) and pig gastric mucins (PGM) on the silk coatings. After 20 minutes of 1 mg/ml mucin flow, the sensors coated with 4RepCT showed almost no decrease of frequency for either BSM (0.8 Hz increase) or PGM (0.18 Hz increase) (Figure 3 A, D), indicating that the added mucins did not bind to the silk coated surface. The same applied for the dissipation measurements, with scant increase in dissipation observed (0.18 x 10^-6 units and 0.3 x 10^-6 units respectively for BSM and PGM, Figure 3A, D).

For pLys-4RepCT coated sensors we observed a -7.8 Hz and -13.4 Hz decrease in frequency after the addition of BSM and PGM, respectively, indicating a small but significant adsorption of mucins (Figure 3B, E). The hGal3-4RepCT coatings also bound mucins, albeit to a greater extent than pLys-4RepCT with a -9.6 Hz and -21.4 Hz decrease of frequency for BSM and PGM, respectively (Figure 3C, F). We observed no significant change in frequency during the subsequent wash, which suggests that the mucins remained adsorbed to the silk material. We observed that the extent of mucin adsorption could vary between experiments due to differences in thickness of the underlying silk coating (data not shown).
Batch-to-batch variation of the silk is the most probable source for such variations. However, the silk coating and subsequent mucin adsorption were consistent intra-experimentally. The dissipation measurements showed a similar trend to frequency with a larger increase of dissipation for PGM compared to BSM for both pLys-4RepCT ($4.4 \times 10^{-6}$ and $2.7 \times 10^{-6}$ units respectively, Figure 3B, E) and hGal3-4RepCT ($4.6 \times 10^{-6}$ and $1.5 \times 10^{-6}$ units respectively, Figure 3C, F). The mucin adsorbed on pLys-4RepCT showed higher D/F ratios for BSM when compared to hGal3-4RepCT, while the D/F ratios for PGM were similar for pLys-4RepCT and hGal3-4RepCT (SI Figure 4). This suggests that the BSM molecules adopt a more hydrated and extended conformation on the pLys-4RepCT than on the hGal3-4RepCT coatings, while PGM molecules show a similar behavior on both coatings.

The two mucins tested herein share common structural features, with a central protein core decorated with dense glycosylation, but differ in the detail of the protein and glycan composition. BSM is a highly sialylated (up to 30% sialic acid) mucin, with short O-linked glycans, and a relevant model for salivary mucins. PGM is less sialylated (1-3% sialic acid), with more complex glycosylation structures, and a relevant model for gastric and vagino-cervical mucosa. The cause for the significantly higher adsorption of PGM over BSM is not immediately obvious. Although the denser anionic charge of BSM brought by the anionically charged sialic acid sugars should favor its binding to pLys-4RepCT, it does not appear to be the case. The higher molar mass of the PGM (in the MDa range) compared with BSM (hundreds of kDa) in molecular weight could partially explain such differences.
Figure 3. Binding of BSM and PGM on the mucoadhesive silk coatings. QCM-D was used to monitor the binding of BSM on 4RepCT (A), pLys-4RepCT (B) and hGal3-4RepCT (C), as well as the binding of PGM on 4RepCT (D), pLys-4RepCT (E) and hGal3-4RepCT (F) on gold-coated quartz crystals over time. The crystals were coated with 4RepCT, pLys-4RepCT, and hGal3-4RepCT as shown in Figure 2, washed with 20 mM Tris (pH 8) / 10 mM NaCl (beginning-time point A), and subsequently subjected to 1 mg/ml of BSM or PGM (time point A-B), and washed again to examine the stability of the binding (time point B-end).

**Binding of mucins to pLys-4RepCT is affected by the NaCl concentration**

To investigate the role of electrostatic interactions in the binding of mucins to pLys-4RepCT coatings, we increased the NaCl concentration in the mucin solution from 10 mM to 150 mM. Increasing the salt concentration to 150 mM should screen the electric charges and decrease the entropy gain due to the release of counterions from the cationically charged lysines and the anionically charged pig gastric mucins. Indeed, **Figure 4** shows that the increased salt concentration limited mucin adsorption, as seen by a decrease in the frequency shift measured by QCM-D. Adsorption of PGM was affected by the increase from 10 mM to 150 mM NaCl,
limiting the frequency shift to -15 Hz compared to -30 Hz for 10 mM NaCl. The 4RepCT silk coating did not bind mucin regardless of the NaCl concentration. The D/F ratios after PGM adsorption in 10 mM NaCl were lower compared to those observed in 150 mM NaCl (slope of 0.24 x 10^{-6} Hz^{-1} and 0.35 x 10^{-6} Hz^{-1}, respectively, **SI Figure 5**). This suggests that the higher frequency shift measured for the PGM in 10 mM NaCl was not due to a higher hydration level of the mucin, but on the contrary, that the PGM formed a more hydrated layer on the silk in 150 mM NaCl. This can most probably be explained by the presence of salts that weaken the interactions between polyelectrolytes of opposing charges resulting in more hydrated structures as previously shown in similar systems 42.

It thus seems that the charges of pLys-4RepCT are essential for the binding of mucins, which is likely driven by the release of counterions from the cationically charged lysines and the anionically charged sugar residues of the mucins. This does not come as a surprise, since several other mucoadhesive polymers such as chitosan also rely on electrostatic interactions 43. A previous study showed that a concentration of 200 mM NaCl strongly limited the interactions between chitosan and pig gastric mucin 18,19. However, our results do not exclude the role of other nonspecific interactions for the pLys-4RepCT variant. Indeed, in the case of chitosan, urea and ethanol also had an effect on the interaction, which suggests that other types of interactions have an influence on the adsorption.
Figure 4. Salt concentration dependence of the PGM binding to pLys-4RepCT coatings.

QCM-D was used to monitor the binding of PGM in 10 mM NaCl to 4RepCT (A) and pLys-4RepCT (B), and in 150 mM NaCl to 4RepCT (C) and pLys-4RepCT (D). The crystals were coated with 4RepCT and pLys-4RepCT as shown in Figure 2, washed with the appropriate buffer (20 mM Tris (pH 8) / 10 mM NaCl or 20 mM Tris (pH 8) / 150 mM NaCl) to establish a stable baseline (beginning-time point A), subsequently coated with 1 mg/ml of PGM in the respective buffer (time point A-B), and washed again to examine the stability of the binding (time point B-end).

Asialofetuin binds the carbohydrate recognition domain of hGal3-4RepCT

We based the design of the hGal3-4RepCT silk materials on the hypothesis that the CRD of hGal3-4RepCT could bind the mucin-associated glycans and thus lead to enhanced interactions between the mucin and the silk materials. Although we show strong binding of mucins to hGal3-4RepCT coatings (Figure 3), the interaction could be driven by nonspecific
interactions between the mucins and the silk. Indeed mucins have complex chemical structures that can engage with a broad range of molecular partners through entropic driving forces such as the release of water upon adsorption, enthalpic interactions such as the creation of disulfide bonds, and electrostatic interactions. We thus tested whether the CRD in the hGal3-4RepCT coating was indeed functional and could specifically bind its ligand. To do so, we monitored the adsorption of the asialofetuin glycoprotein, a well-known ligand and biological inhibitor of human galectin 3 \(^{44}\). As can be seen in Figure 5, asialofetuin binds the hGal3-4RepCT coating, causing a strong decrease in QCM-D resonance frequency of -40 Hz, while the response in contact with 4RepCT was limited to -18 Hz, which we attribute to nonspecific binding. Complementary experiments using Bio-Layer Interferometry (BLI) also showed a stronger adsorption of asialofetuin to hGal3-4RepCT (0.87 nm shift) compared to 4RepCT coatings (0.42 nm shift) (SI Figure 6, SI Materials and Methods). Given the fact that nonfunctionalized 4RepCT probably has more exposed hydrophobic patches, compared to its functionalized versions, which are prone to nonspecific interactions, some adsorption of asialofetuin to 4RepCT is expected. Thus, we introduced Z-4RepCT as an extra negative control, which is 4RepCT genetically fused with another folded protein domain, the immunoglobulin (IgG) binding Z domain \(^{28}\). Indeed, asialofetuin showed no binding at all to Z-4RepCT coated sensors (SI Figure 6).

Asialofetuin glycosylation is composed of oligosaccharides terminating with Gal-\(\beta\)-1,4GlcNAc (80%) and Gal-\(\beta\)-1, 3-GalNAc (20%), both able to bind the galectin 3 CRD \(^{45}\). The strong binding of asialofetuin to the hGal3-4RepCT coating thus suggests that the CRD of hGal3-4RepCT is functional and accessible for binding when assembled into coatings. This specific affinity for Gal\(\beta\)1-3/4GlcNAc disaccharides is likely to be driving the adsorption of BSM and PGM, which both contain these glycans in large amounts \(^{46}\).
Importantly, the same sugars are found in many glycosylated proteins, including on the surface of cells and in the glycocalyx, implying that the hGal3 variant could be used as a tissue adhesive. Since our findings show that it is possible to integrate a CRD into the silk materials, the design of the silk could be tuned to change the binding specificity. For instance the variation in the abundance of the sialic acid residues content between healthy and disease state and between mucosal tissues could be exploited to target the material’s adhesion to specific tissues.

**Figure 5. Binding of asialofetuin to hGal3-4RepCT coatings.** QCM-D was used to monitor the binding of asialofetuin to 4RepCT (A) and hGal3-4RepCT (B) on gold-coated quartz crystals in real time. The crystals were coated with 4RepCT and hGal3-4RepCT as shown in Figure 2, washed with 20 mM Tris (pH 8) / 10 mM NaCl (beginning-time point A), subsequently coated with 1 mg/ml of asialofetuin (time point A-B), and washed again to examine the stability of the binding (time point B-end).

In this study, we add two new functionalized silk variants, pLys-4RepCT and hGal3-4RepCT, to the repertoire of recombinant silks that can spontaneously form coatings. The new silk variants showed good mucoadhesive properties through different mucoadhesion mechanisms. We envision that such coatings can be useful to engineer the surface of devices
that require good adherence to mucosal tissues. Advantageously, the biofunctional silk coatings form spontaneously under mild conditions, their thickness can be tuned with adsorption time, they form on a wide range of surface, and they are stable in physiological conditions \(^{30,31,48}\). However, it could also be desirable to form free-standing three-dimensional materials that can be more conveniently used as drug delivery vehicles and patches for mucosal wounds. We thus investigated the formation of fibers, fiber meshes and foams of these new silk variants.

**pLys-4RepCT and hGal3-4RepCT silk form fibers onto which mucins can bind**

It has been previously shown that 4RepCT has the ability to self-assemble into fibers under physiological conditions\(^ {26}\). For this reason we first attempted to form fibers from pLys-4RepCT and hGal3-4RepCT silk protein solutions. Silk fibers were formed for both pLys-4RepCT and hGal3-4RepCT (Figure 6A-C). The pLys-4RepCT fibers were elongated and thin, sharing many morphological similarities with the 4RepCT fibers. The hGal3-4RepCT fibers presented less distinct alignment between individual silk fibrils, and with a more "fluffy" microscopic appearance. Interestingly, the hGal3-4RepCT fibers took significantly longer time than 4RepCT and pLys-4RepCT to form from a protein solution. This decrease in the rate of fiber formation has been previously observed with 4RepCT fibers functionalized with the enzyme xylanase (Xyl-4RepCT) \(^ {30}\), where it was suggested that the slower fiber formation process could be due to a combination of high solubility of the xylanase domain and steric hindrance of the silk formation from the relatively large size of xylanase. For the case presented herein, the hGal3 domain (16 kDa) is also relatively large compared to 4RepCT (23 kDa), and might therefore be a key factor to the decreased rate of fiber formation for hGal3-4RepCT.
To test their ability to bind mucins, small pieces of fibers were cut and incubated with FITC-labeled BSM or PGM for 30 minutes, then washed with 20 mM Tris (pH 8) / 100 mM NaCl and observed by fluorescence microscopy. The NaCl concentration used was 100 mM instead of 10 mM, in order to better mimic the physiological Na$^+$ concentration found in saliva, which can range from 75 mM to 200 mM$^{38}$. Furthermore, salivary pH can range from 6.2 to 7.6, with our pH 8 solution being in the upper range of physiological pH values$^{49}$. The fluorescence signal from hGal3-4RepCT showed the highest mucin binding for both BSM and PGM (Figure 6F, J) (5.7 times and 2.7 times more than 4RepCT, respectively, Figure 6G, K), while pLys-4RepCT showed no BSM binding (Figure 6E), and low binding for PGM (Figure 6I) (1.7 times more than 4RepCT, Figure 6K).

The FITC-PGM used in these experiments contained double the amount of attached FITC molecules compared to FITC-BSM (SI Figure 7), making a direct comparison between the two mucin types inaccurate. However, these experiments demonstrate the comparatively low unspecific binding of mucins to 4RepCT, and the good mucoadhesion of hGal3-4RepCT fibers. Similarly to silk coatings, the pLys-4RepCT fibers bound less mucins than the hGal3-4RepCT fibers, the differences being perhaps exacerbated by the presence of 100 mM NaCl in these experiments. Nevertheless, we show that the mucoadhesive functionality was not altered by fiber formation.
Figure 6. Adhesion of fluorescently labeled mucins to silk fibers. Self-assembled silk fibers from 4RepCT (A), pLys-4RepCT (B) and hGal3-4RepCT (C) were visualized using light microscopy. Fiber pieces from 4RepCT (D), pLys-4RepCT (E) and hGal3-4RepCT (F) incubated with FITC-labeled BSM for 30 minutes at room temperature were visualized using fluorescence microscopy. The same applied for fiber pieces from 4RepCT (H), pLys-4RepCT (I) and hGal3-4RepCT (J) incubated with FITC-labeled PGM. The scale bars for the light microscopy images (A, B, C) represent 2 mm, while scale bars for the fluorescence microscopy images represent 1 mm (D, E, F, H, I, J). Exposure used for BSM binding: 100 ms. Exposure used for PGM binding: 10 ms.

The fluorescence density of BSM-FITC labeled fibers (G) and PGM-FITC labeled fibers (K) was calculated from the fluorescence microscopy images by dividing the integrated density with the fiber area. Bar graphs represent mean values of the fluorescent density levels and error bars represent SEM. Statistical significance assessed with one-way ANOVA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. 4RepCT control samples.
Meshes of dried pLys-4RepCT/hGal3-4RepCT fibers retain their mucin binding properties

We then explored whether we could form larger mucoadhesive materials by generating mesh-like structures from the silk fibers. The fibers were cut into pieces and then assembled into a mesh structure by letting them dry together. A mesh of both pLys-4RepCT and hGal3-4RepCT fibers mixed was made in order to make use of both mucoadhesion mechanisms revealed for the silk coatings. We then rehydrated the meshes before imaging them (Figure 7A, B and SI Figure 8A) and checked whether the mucoadhesive properties of the fibers had been affected after the drying and rehydration steps. As with the individual fibers, 4RepCT and pLys/hGal3-4RepCT meshes were incubated with FITC-BSM or FITC-PGM. As can be seen in Figure 7C, D, E, BSM bound the pLys/hGal3-4RepCT mesh approximately 6 times more compared to the 4RepCT mesh, while PGM bound 2 times more on the pLys/hGal3-4RepCT mesh (Figure 7F, G, H), showing that the mucoadhesive effect had also been maintained in these materials.
Figure 7. Meshes of pLys/hGal3-4RepCT retain their mucoadhesive properties. Silk fibers from both pLys-4RepCT and hGal3-4RepCT were assembled into a dry mesh and visualized with macro photography (A) and after rehydration with light microscopy (B). Mesh pieces made from 4RepCT fibers (C), and from pLys-4RepCT and hGal3-4RepCT fibers (pLys/hGal3) (D) were incubated with FITC-labeled BSM for 30 minutes at room temperature and visualized using fluorescence microscopy. The same was applied for mesh pieces from 4RepCT fibers (F) and pLys-4RepCT and hGal3-4RepCT fibers (G) incubated with FITC-labeled PGM. The scale bars for the macroscopic image (A), as well as the light microscopy image (B) represent 2 mm. The scale bars for the fluorescent microscopy images (C, D, F, G) represent 500 µm. Exposure used for BSM binding: 100 ms. Exposure used for PGM binding: 10 ms.

The fluorescence density of BSM-FITC labeled meshes (E) and PGM-FITC labeled meshes (H) was calculated from the fluorescence microscopy images by dividing the integrated density with the mesh area. Bar graphs represent mean values of the fluorescent density levels of three samples and error bars represent SEM. Statistical significance assessed with paired two tailed t-test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs.4RepCT control samples.

Hydrated pLys-/hGal3-4RepCT foams bind mucins

It was previously reported that soluble 4RepCT can also form foams under physiological conditions by frothing a silk solution. Such silk foam materials seem suitable candidates for mucoadhesion applications since they can be made relatively big (>1 cm), are flexible, durable and offer a large surface area for seeding cells or adsorbing therapeutic compounds. We thus generated foam materials also from a mixture of pLys-4RepCT and hGal3-4RepCT proteins with the aim to exploit both mucoadhesion mechanisms (Figure 8A, B and SI...
Contrary to the fiber meshes, this material was kept constantly hydrated. We then investigated the mucin binding by incubating cut out small pieces of the foam with FITC-BSM or FITC-PGM. As can be seen in Figure 8C, D and E, BSM bound the pLys/hGal3-4RepCT foam 8.5 times more compared to the control foam (made from a mixture of 4RepCT, FN-4RepCT and Z-4RepCT), while PGM bound two times more (Figure 8F, G, H), suggesting good mucoadhesive properties of the foam material. A mixture of silk variants were used as negative controls, since the functionalized 4RepCT proteins FN-4RepCT and Z-4RepCT reduces the nonspecific interactions compared to non-functionalized 4RepCT.

Mucoadhesive flexible patches used for buccal drug delivery have received a lot of interest since they propose an alternative promising and noninvasive drug delivery system. Here, we investigated two different mucoadhesive silk material formats which physically resemble such patches; mesh and foam. Both meshes and foams have three-dimensional structures that provide a large surface area for adhesion; mesh provides a flatter surface, while foam a porous surface. An interesting aspect of these materials is that they can easily be made to contain bioactive domains that could stimulate mucosal wound healing. Meshes and foams made from 4RepCT alone have shown good biocompatibility by supporting the growth and expansion of primary fibroblasts *in vitro*. As individual biofunctional silk variants can be combined in solution and thereafter processed into multifunctional silk materials, one could mix the herein presented mucoadhesive silk variants with, for example, silk containing the cell-binding FN motif and silk displaying antimicrobial properties to create strongly mucoadhesive, wound healing materials that support the proliferation of epithelial cells and limit the risks of bacterial infections. Combining good mucoadhesive and cell-culture performances, we envision that pLys/hGal3-4RepCT meshes and foams could also be used...
for mucosal regenerative medicine applications, where the cell-compatible three dimensional scaffold could be applied to damaged mucosal membrane to accelerate its regeneration.

Figure 8. pLys/hGal3-4RepCT mucoadhesive foam shows enhanced mucin binding properties. Soluble pLys-4RepCT and hGal3-4RepCT (1:1) formed into a wet and stable foam were visualized using macro photography (A) and light microscopy (B). Foam pieces made from 4RepCT, Z-4RepCT and FN-4RepCT (1:1:1) (WT/Z/FN) (C), and from pLys-4RepCT and hGal3-4RepCT (pLys/hGal3) (D) incubated with FITC-labeled BSM for 30 minutes at room temperature were visualized using fluorescence microscopy. The same was applied for foam pieces from 4RepCT, Z-4RepCT and FN-4RepCT foam (F) and pLys4RepCT and hGal3-4RepCT foam (G) incubated with FITC-labeled PGM. The scale bars for the macroscopic image (A) represent 1 cm, while the scale bar for the light microscopy image (C) represents 2 mm. The scale bars for the fluorescence microscopy
images represent 1 mm. Exposure used for BSM binding: 100 ms. Exposure used for PGM binding: 10 ms.

The fluorescence density of BSM-FITC labeled foams (E) and PGM-FITC labeled foams (H) was calculated from the fluorescence microscopy images by dividing the integrated density with the mesh area. Bar graphs represent mean values of the fluorescence density levels of three samples and error bars represent SEM. Statistical significance assessed with paired two tailed t-test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. WT/Z/FN-4RepCT control samples.

Conclusions

With this work, we describe the design, production, and characterization of two new mucoadhesive spider silk variants, pLys-4RepCT and hGal3-4RepCT. These new variants constitute useful new building blocks for a range of silk based materials for mucosal treatments, including coatings, meshes, and foams. Although we proved that these new silk materials are mucoadhesive, we did not investigate their strength of the interactions. Mucous membranes can be subject to high mechanical stress and shear forces, so an ideal mucoadhesive material should adhere strongly on the mucous membrane. The binding strengths between mucins would be possible to determine at the microscopic level using AFM, or macroscopically using mucoadhesion models, for example buccal animal tissue. The mucoadhesion will be greatly impacted by the way the silk-based material is designed. For instance, the silk foam materials described herein are especially good candidates for future application, since they offer high surface areas for mucin binding and possible molecular entanglement of the mucins into the foam structure. Application of dry or partially
dehydrated foams to the mucosa would also help adhesion by driving some of the mucus into
the foam structure during the material’s rehydration.

Engineered silk materials can be designed to contain virtually any bioactive domain, which
opens vast possibilities to create bioactive mucoadhesive materials for mucosal wound
regeneration. Indeed the mucoadhesive silk variants can be mixed with silk variants
functionalized with motifs promoting wound healing, and assembled into multifunctional
materials such as foams and meshes that could help heal damaged mucosal membranes. Such
mucoadhesive materials could also be used to carry drugs through the mucus membranes.
There, the intrinsic capacity of 4RepCT and its engineered variants to interact with drugs will
be a determining factor. The understanding of the 4RepCT auto-assembly and fiber
structures, as well as the in vitro and in vivo biocompatibility and biodegradation profiles,
will help design the functional materials required for such applications. In addition, new
strategies such as the combination of engineered spider silk with cost-effective silkworm
fibroins or cellulose nanofibrils\(^{54,55}\) could help drive the production cost down, which will be
an essential step towards the translation of the technology.

Supporting Information

The Supporting Information is available free of charge online at http://pubs.acs.org.

- Comparison between different overtones during QCM-D experiments; D/F ratios of
  QCM-D experiments; AFM image of hGal3-4RepCT coating; Bio-layer
  interferometry results for the binding of asialofetuin on hGal3-4RepCT; Fluorescence
  intensity of the FITC-labeled mucins; 3D materials photographed from different
  angles.
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