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Avidin Adsorption to Silk Fibroin Films as a Facile Method for Functionalization

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Abstract

Silk fibroin biomaterials are highly versatile in terms of materials formation and functionalization, with applications in tissue engineering and drug delivery, but necessitate modifications for optimized biological activity. Herein, a facile, avidin-based technique is developed to non-covalently functionalize silk materials with bioactive molecules. The ability to adsorb avidin to silk surfaces and subsequently couple biotinylated macromolecules via avidin-biotin interaction is described. This method better preserved functionality than standard covalent coupling techniques using carbodiimide crosslinking chemistry. The controlled release of avidin from the silk surface was demonstrated by altering the adsorption parameters. Application of this technique to culturing human foreskin fibroblasts (hFFs) and human mesenchymal stem cells (hMSCs) on arginine-glycine-aspartic-acid-modified (RGD-modified) silk showed increased cell growth over a seven-day period. This technique provides a facile method for the versatile functionalization of silk materials for biomedical applications including tissue engineering, drug delivery, and biological sensing.

Keywords

silk fibroin; avidin; surface modification; adsorption

INTRODUCTION

Silk fibroin (silk) isolated from *Bombyx mori* silkworm cocoons is an abundant, biocompatible, FDA approved biomaterial that can be used for a wide range of applications

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Notes

The authors declare no competing financial interest.

SUPPORTING INFORMATION

Colorimetric characterization of avidin and B-HRP adsorption (Figure S1), raw data of kinetic curves of adsorbed or covalently coupled HRP moiety functionalized silk films (Figure S2), raw data of kinetic curves of A-HRP released from surface of silk films (Figure S3), raw data of kinetic curves of A-HRP remaining on surface of silk films after release studies (Figure S4), metabolic activity of hFFs on avidin-modified silk surfaces (Figure S5), metabolic activity of hMSCs on modified silk films at Day 1 and 3 of culture (Figure S6). Slope of linear region of A-HRP remaining on surface of silk films after release studies (Table S1).

including tissue engineering, regenerative medicine, and drug delivery.¹⁻² Silk is a glycine-rich polymer that exhibits many favorable material features including tunable mechanical properties, controllable degradation rates, minimal immune response, and the ability to be processed into many different formats.^{1, 3} At physiological pH, the silk is negatively charged, providing sites for initial electrostatic interactions with cationic macromolecules and small molecules.⁴⁻⁶ Macromolecules and small molecules that promote cell attachment, proliferation, and differentiation may be attached to silk for tissue engineering/regenerative medicine purposes.⁷⁻⁸ Additionally, therapeutic small molecules and antibodies may be attached for drug delivery purposes.^{5-6, 9-10}

Multiple covalent modification techniques have been developed for silk. However, due to the limited functional amino acid side groups found within silk, these can be time consuming, requiring multiple chemical reaction steps, are challenging to control with a consistent level or degree of substitution, and often utilize toxic reagents to facilitate a high degree of modification.¹¹⁻¹⁶ Diazonium coupling has been used to quickly functionalize silk through tyrosine side chains, but requires basic conditions and is limited to, at most, 277 amino acids modified out of approximately 5,263 when considering silk fibroin heavy chain, the predominant protein in silk.¹⁵ Serine residues in silk have also been carboxylated through reaction with chloroacetic acid. However, this process requires an initial step under basic conditions which degrades the silk.¹⁶ In one case, production of a silk fibroin scaffold functionalized with the cell binding domain arginine-glycine-aspartic acid (RGD) was created by dissolving the components in formic acid for one day to incorporate the RGD into the silk solution which was then spun into scaffolds.¹⁴ In addition to avoiding toxic reagents from a leachable component standpoint, reactions requiring toxic reagents may also alter the activity and function of the terminal molecule, a particular concern for functional protein modifications.^{4, 17} A commonly used functionalization method that is less time consuming and requires only slightly acidic conditions for silk surface modification is carbodiimide chemistry with an 1-ethyl-3-(3 dimethylaminopropyl)carbodiimide hydrochloride/ N-hydroxysuccinimide (EDC/NHS) coupling mechanism. This method is commonly used to functionalize silk with RGD.¹⁸⁻²⁰ This technique utilizes free carboxylate groups activated with an O-acylisourea to covalently couple with primary amines.²¹ However, the necessary chemical groups may not be available or by utilizing these groups the molecular activity decreases.^{9, 22}

Silk is an amphiphilic protein that possess the ability to interact with hydrophobic, hydrophilic, and charged molecules. The net negative charge of silk at physiological pH can be utilized to non-covalently immobilize positively charged molecules. Avidin is a tetrameric protein isolated from egg whites that is positively charged at physiological pH. When avidin interacts with biotin, a water-soluble vitamin, the two form the strongest known non-covalent bond.²³ Avidin-biotin systems have been widely used in biotechnology, therapeutic, and tissue engineering applications.^{9, 24-26} This interaction can be utilized for surface immobilization of functional biotinylated molecules. For example, biotin may be conjugated to the peptide RGD, resulting in biotinylated-RGD (B-RGD) for cell attachment. Commonly in these systems, either avidin or biotin is covalently attached to a surface.^{9, 27-28} Avidin and NeutrAvidin, a deglycosylated avidin derivative, have previously been coupled to silk opal films or silk microspheres, respectively, through EDC/NHS coupling as a means of

functionalizing the silk surface.^{9, 29} Utilizing non-covalent modification methods allows for desorption of the functional molecules for release and potential long-range biological effects.³⁰⁻³¹

The present study investigated the non-covalent modification of silk fibroin surfaces with adsorbed avidin and the subsequent functionalization with biotinylated molecules for biomedical applications. Avidin was chosen for this work because it carries a net positive charge at neutral pH (isoelectric point of 10-10.5) making it possible to take advantage of non-specific interactions with the negatively charged silk surface. Utilizing biotinylated-horseradish peroxidase (B-HRP) as an indicator of avidin adsorption, identification of the maximum B-HRP adsorption by varying avidin and B-HRP concentrations was investigated. Subsequent desorption of HRP molecules was investigated for application to drug delivery. Utilizing optimal adsorption immobilization conditions, electrostatic immobilization of avidin was compared with the standard protein coupling technique, carbodiimide coupling chemistry. Furthermore, the silk surfaces were functionalized with B-RGD to provide cell attachment domains. These B-RGD-modified surfaces were evaluated for the ability to enhance short-term cell attachment and growth on the modified silk surfaces.

EXPERIMENTAL SECTION

Silk Extraction.

Aqueous silk solutions were prepared as previously described.³² In brief, *B. mori* silkworm cocoons were boiled in 0.02 M sodium carbonate (Sigma-Aldrich, St. Louis, Missouri) for 30 min to remove the sericin protein and then washed with ultrapure water (Milli-Q, Millipore, Burlington, Massachusetts). The extracted silk fibers were dissolved in 9.3 M lithium bromide (Sigma-Aldrich) for 3 h at 60°C. The resultant silk solution was dialyzed using 3,500 Da molecular weight cutoff tubing (Fisher Scientific, Hampton, New Hampshire) for 3 days against ultrapure water with at least six water changes to remove the lithium bromide. Non-soluble particulates present during the extraction process were removed through centrifugation (2×20 min at 9,000 rpm). A silk solution of 6% (w/v) was obtained as a final product and was diluted with ultrapure water to the desired final silk concentration.

Silk Film Fabrication in Well Plates.

Silk films were utilized as a model system for rapid analysis of the adsorption properties of avidin to silk fibroin surfaces. To form the films, 50 or 150 μ L of 2% (w/v) silk solution was pipetted into 96- or 48- well plates, respectively, (Cellstar, Greiner Bioone, Monroe, North Carolina) and allowed to dry overnight. The dried silk films were treated with methanol for 1 h to render the silk films insoluble. Following the methanol treatment, the films were incubated with PBS for 30 min to remove soluble silk fragments. Films for cell culture were UV-sterilized for 30 min on both sides after the methanol treatment, then washed with sterile PBS.

Silk Functionalization.

The silk fibroin surface was functionalized through the adsorption of avidin via electrostatic interactions, followed by immobilization of biotinylated molecules (B-HRP and B-RGD) utilizing avidin-biotin interactions. Covalent coupling of B-HRP was also performed as a comparison to standard coupling techniques. Avidin adsorption or EDC/NHS coupling was individually performed in each well. The general principles of attachment for both the electrostatic and covalent modification techniques are shown in Figure 1. B-HRP (14 atom spacer, Thermo Scientific, Waltham, Massachusetts) was utilized for detection and quantification.

Characterization of Avidin Adsorption to Silk Films Indicated by HRP Activity.

Silk films were incubated with 100 μ L of 10^{-4} - 10^3 μ g/mL avidin (Invitrogen, Carlsbad, California) overnight at room temperature. Also, a set of wells were treated in parallel with 1 mg/mL avidin-horseradish peroxidase (A-HRP, Invitrogen) to confirm adequate washing between steps. Following overnight incubation, excess avidin and A-HRP were removed through rapid PBS washing. During the wash procedure, the PBS was periodically removed and evaluated for the presence of soluble A-HRP using 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB solution as a colorimetric indicator of enzymatic activity. A lack of color change (conversion of the clear TMB solution to blue) in the wash PBS after 15 min defined complete washing/extraction. The avidin-modified silk films were then incubated with 100 μ L of 5×10^{-5} - 5×10^2 μ g/mL B-HRP for 2 h. Excess protein was removed through PBS washing and lack of enzymatic conversion of TMB was used to confirm complete washing. To determine the level of A-HRP and B-HRP on the silk films, the TMB solution was added directly to the wells. Images of the wells were taken periodically prior to reading the absorbance of the converted TMB substrate. Additionally, one image was taken after the addition of 2 M sulfuric acid to stop the enzymatic reaction (Figure S1).

Covalent Coupling of HRP Variants to Silk Films.

Silk films were incubated in 4-morpholinoethanesulfonic acid (MES) buffer (50 mM, 150 mM NaCl, pH 6) for 30 min at room temperature. Subsequently, a solution of 0.5 mg/mL EDC (Thermo Scientific, Waltham, Massachusetts) and 0.7 mg/mL NHS (Thermo Scientific) dissolved in MES buffer was applied to the silk films for 15 min to form a stable NHS ester capable of conjugating primary amines. Excess EDC/NHS was removed via washing with MES buffer with a final wash step using PBS. The silk films were then incubated with 5 μ g/mL horseradish peroxidase (HRP, Sigma) or B-HRP for two h at room temperature. Excess HRP or B-HRP were removed with PBS washing.

Detection of HRP Variants.

The HRP substrate TMB (for the initial avidin and B-HRP concentration range experiment: 1-Step™ Ultra TMB-ELISA Substrate Solution, ThermoFisher Scientific, Waltham, MA; for all other experiments: Extended Range One Component, BioRx Laboratories, Owings Mills, Maryland) was used to detect HRP activity. TMB was applied directly to the silk films for adsorption and functionality experiments or at a 1:1 ratio with PBS for the confirmations during the wash-steps. For the initial experiment, TMB conversion was monitored detected

using a Spectramax M2 Microplate Reader (Marshall Scientific, Hampton, New Hampshire) at 650 nm. For all other experiments, TMB conversion was monitored every 15 s for 20 min on a Spectramax 250 Microplate Reader (Marshall Scientific, Hampton, New Hampshire) at 650 nm. Data were generated in SOFTmax® Pro software (Molecular Devices, Sunnyvale, California) and analyzed using Microsoft Excel. Data were normalized by translocation to the origin by subtracting the first recorded value of each well from all subsequent data points, allowing for easier visual comparisons of the slopes. The level of enzymatic activity was determined from the slope of the linear region of the data; subtracting the initial data point does not affect the slope.

Avidin Release.

Since avidin-biotin interaction is the strongest non-covalent interaction known, it was expected that loss of this interaction via desorption would not be observed; thus, A-HRP was used for simplicity. To investigate the release or desorption of avidin from silk fibroin surfaces, the silk films were incubated in 0.1 mg/mL A-HRP for one hour or overnight, followed by PBS washing to remove unbound A-HRP. Following washing, 50 µL of PBS was added to each well and the plate was placed in a 37°C incubator. The full volume of PBS was collected with a time delta of 0.25, 0.5, 1, 2, 4, 12, 24, 48, and 96 h corresponding to samples at 0-0.25, 0.25-0.75, 0.75-1.75, 1.75-3.75, 3.75-7.75, 7.75-19.75, 19.75-43.75, 43.75-91.75, 91.75-187.75 h range, respectively. A-HRP release was evaluated using the TMB colorimetric assay. New PBS was added to the well after each collection time point. To develop a release curve, absorbance values from one-time point (405 s) within the linear region of the A-HRP kinetics curves for all time deltas were compared.

Cell Culture.

Human foreskin fibroblasts (hFFs, P6-P12, kindly provided by Dr. George D. Pins) were cultured in Dulbecco's Modified Eagle Medium with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. Human mesenchymal stem cells (hMSCs, P1-P4, kindly provided by Dr. Tanja Dominko) were cultured in the hFF media, supplemented with 5 ng/mL Fibroblast Growth Factor (FGF-2, PeproTech, Rocky Hill, New Jersey). Medium was changed every 2 d until confluence was achieved, and cells were passaged after treatment with trypsin. A seeding density of 5,000 cells/well in a 48 well plate was used for all cell experiments.

The effect of avidin adsorption to silk films on cell growth was examined. Sterile silk films were incubated with 1 mg/mL avidin overnight and washed three times with sterile PBS. hFFs were cultured on the films for 7 d and evaluated by a resazurin metabolic assay on days 1, 3, and 7. Unmodified silk films, tissue culture plate (TCP), and TCP with adsorbed 1 mg/mL avidin served as controls.

The effect of RGD functionalization of silk films on cell growth was also examined. Sterile silk films were incubated with 1 mg/mL avidin overnight or EDC/NHS for 15 min, then washed three times with sterile PBS. High (1×10^{-7} M) or low (1×10^{-8} M) concentrations of Biotin-GRGDS (B-GRGDS) or GRGDS (Biomatik, Willmington, Delaware) were applied to the silk films for two h. The "high" concentrations of B-GRGDS and GRGDS

used were molar equivalents of the identified B-HRP concentration. Unmodified silk films, unmodified silk films with peptide applied, and TCP served as controls. Excess peptide was removed through sterile PBS washing. Immediately following washing, hFFs or hMSCs were seeded on the silk films.

Resazurin Metabolic Assay.

Resazurin (0.15 mg/mL, Acros Organics, Pittsburgh, Pennsylvania) was added in a 1:5 ratio with the cell medium. Cells were incubated with resazurin for 3 h at 37°C. Following incubation, resazurin fluorescence was read at an excitation of 544 nm and an emission at 590 nm on a plate reader (Victor³, Perkin Elmer, Waltham, Massachusetts).

Statistics.

TMB colorimetric absorbance values for terminal functionality data are presented as mean $\pm$ standard error of the mean (SEM) and are representative of multiple experiments. Kinetic activity was analyzed by comparing the slopes with one-way ANOVA followed by Tukey post hoc analysis ($p < 0.05$). Statistical differences in A-HRP release values were determined by student t-test ($p < 0.05$). Resazurin fluorescence data is expressed as mean $\pm$ SEM unless otherwise indicated. The statistical significance between resazurin fluorescence values from cells cultured in the different well formats was determined by one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). Statistical analysis was performed in SPSS 22.0 software (IBM, Armonk, NY).

RESULTS AND DISCUSSION

Characterization of B-HRP Immobilization to Silk Films by Varying Avidin and B-HRP Concentration.

To determine if avidin was passively adsorbed to silk, an initial screening experiment was conducted. Through the use of an array, where each well was a different combination of avidin and B-HRP, an optimal concentration for avidin and B-HRP, within the design space of the experiment, was determined while confirming that the detection was not due to B-HRP adsorbing to silk directly (Figure 2, Figure S1). A darker blue color or a higher absorbance value indicated greater amounts of B-HRP (i.e. HRP activity). The highest avidin concentration tested, 1,000 $\mu\text{g/mL}$, resulted in the maximum HRP activity. However, intermediate B-HRP concentrations (0.5 – 5 $\mu\text{g/mL}$) were optimal. The optimal concentrations at intermediate levels may be due to steric hindrance preventing access to binding sites within the avidin molecules. At the higher concentrations tested, the avidin may be tightly packed on the silk surface where adjacent avidin molecules interfere with the proper conformation for biotin recognition of the active binding site. Alternatively, even if all the active binding sites of avidin are accessible, biotin-HRP molecules bound to the avidin may inhibit additional biotin groups from binding at adjacent sites. Previous work suggested steric hindrance reduced the binding of high densities of avidin to biotinylated surfaces.³³⁻³⁵ The absorbance values of some wells with low amounts of avidin were higher than expected (e.g. 50 $\mu\text{g/mL}$ B-HRP, 0.01 $\mu\text{g/mL}$ avidin). This was likely due to imperfect silk film formation (wrinkles, lifting) causing a gap between the film and the well plate making adequate washing difficult. Additionally, random sampling was employed to verify

complete washing between steps, making it plausible that some wells were washed better than others were. However, the few wells with unexpected absorbance values did not affect data interpretation, as they were nearly an order of magnitude lower than the maximum absorbance values.

Comparison of Terminal Molecule Functionality on Avidin-Modified Silk Surfaces.

There are many methods to immobilize proteins to biomaterial surfaces. One common method utilizes EDC/NHS activation of exposed carboxylic acids for subsequent reaction with primary amines. For comparison, side-by-side experiments were performed to determine how the non-covalent modification method developed here compared to the covalent coupling through EDC/NHS to immobilize B-HRP. The enzymatic activity of B-HRP conjugated to avidin-modified silk films was significantly higher than the B-HRP conjugated through EDC/NHS coupling (Figure 3, Figure S2) as evidenced by the faster rate of absorbance change. Quantification of the slope for avidin/B-HRP demonstrated significantly higher enzymatic activity than all groups, including EDC/NHS conjugated B-HRP (Table 1). This suggests avidin modification better preserves terminal molecule functionality, allows more terminal molecules to be immobilized, or a combination of both.

The downward slope of the avidin/B-HRP reaction after 135 s was not due to loss of functionality of the B-HRP over time, but rather due to high amounts of B-HRP exhausting the TMB substrate, which results in a precipitate and lowers the absorbance value (Figure 3). In contrast, the EDC/NHS wells did not form a precipitate, but still displayed lower absorbance values compared to other methods, including the absorbance values of the background binding controls. This suggests that the EDC/NHS coupling method decreases the activity of the terminally conjugated protein. Two distal heme pocket residues, Arg38 and His42, have been shown to play an important role in the ability of HRP to oxidize substrates.³⁶ It is possible that the lower activity of the EDC/NHS conjugated HRP could be a result of the non-specific aminization performed by the NHS ester affecting one of these amino acids during the conjugation reaction.

One avidin molecule is capable of binding four biotin molecules.³⁷ Therefore, increased immobilization of B-HRP by avidin is not surprising since each NHS ester formed will conjugate one amine group (or one molecule) to the silk surface compared to the ability of the adsorbed avidin to bind up to four biotinylated molecules. As suggested above, the random nature of the covalent amide bond formation may result in an inactive HRP molecule. If the bond formation between avidin and biotin is similarly random, then the higher number of available binding sites increases the likelihood of some B-HRP docking in a favorable conformation. Another possibility is that bond lengths and steric hindrance played a role in the accessibility of the molecules.³⁸ The B-HRP spacer arm is 14 atoms long, which may allow the extension of the HRP away from the surface of the avidin molecule and reduce steric hindrance for additional B-HRP molecule binding. In contrast, EDC/NHS coupling is a zero-length crosslinker, which could result in the bound HRP blocking adjacent activated groups. Overall, the results of this experiment demonstrate the possibility for avidin-biotin conjugation to better preserve terminal protein functionality and thus, create a more functional biomaterial surface than EDC/NHS activation.

A low amount of B-HRP and HRP was observed to bind non-specifically to the silk, possibly due to hydrophobic or electrostatic interactions, as previously reported.⁹ These background amounts of B-HRP and HRP exhibited significantly lower enzymatic activity than avidin-immobilized B-HRP, indicating that the non-specific binding was negligible (Table 1). Adsorbing bovine serum albumin (BSA) to silk fibroin prior to functionalization may prevent the non-specific binding, likely through hydrophobic interactions, of B-HRP to the silk films and could reduce background effects. Since BSA is also negatively charged at neutral pH, it would not be expected to interfere with avidin adsorption through electrostatic interactions to the silk surface.^{25, 39}

Desorption of Avidin from Silk Surfaces.

After confirming adsorption and functionality of this avidin-based system, avidin desorption from silk films was investigated. Avidin desorption is of interest as it would allow for long-range effects in tissue engineering applications and potential sustained release of bioactive compounds.^{26, 40} Sustained release and tunable release are both favorable characteristics of drug delivery systems. The effect of avidin adsorption time was also evaluated as increased adsorption time may increase mass adsorption and alter the desorption kinetics. The data are presented as the rate of A-HRP released per min during the specified time interval (Figure 4) extracted from A-HRP kinetic curves (Figure S3). The release profiles of the A-HRP adsorbed to the silk overnight or for one-hour were similar through the first six-time intervals, or through 19.75 h, of release (Figure 4). However, the amount of A-HRP released from the overnight adsorption groups between 19.75 h and 187.75 h (or ~8 d) was significantly higher than the amount released from the one-hour adsorption group. Release of A-HRP would be expected to continue if the experiment were continued due to the presence of sink conditions.

Additionally, A-HRP was present on the surface of the silk films at the end of the release experiments (Figure S4). Significantly higher amounts of A-HRP remained on the overnight adsorption surfaces than on the 1-hour adsorption surfaces, determined by comparing the slope of the enzymatic activity curves (Table S1). Together, the release data and the surface bound data suggest that an increased amount of A-HRP was immobilized on the silk surface with the increased incubation time. This finding can be used to manipulate the release of molecules from silk surfaces. Further manipulation of avidin release time could be achieved through changes to the silk film crystallinity, as suggested in previous work.^{5, 10}

In addition to providing a functionalized scaffold for tissue engineering applications, the release of avidin from the silk surface could lead to therapeutic applications. For example, biotinylated growth factors could be released from a silk surface to promote tissue regeneration in areas not immediately adjacent to the material surface. Additionally, biotinylated therapeutics could be released for disease treatments. A similar approach has been explored with the incorporation of doxorubicin, a chemotherapy medication, into a hyaluronic acid micro-hydrogel through neutravidin-biotin interactions for cancer treatment.⁴¹ The *in vitro* release of the doxorubicin was initiated by the addition of excess biotin to the solution surrounding the microhydrogel. The additional biotin caused disassembly of the hydrogel and resulted in a rapid release of doxorubicin. The use of electrostatic interactions

in the work presented here eliminates the need for an additional biotin injection step to release the therapeutic, as the avidin will desorb in the presence of ions within the body. Additionally, the silk-avidin modification scheme allows for sustained release of the therapeutic from the silk surface rather than full material disassembly.

Since electrostatics have previously been shown to be the driving force behind the initial interaction between silk and cationic small molecules,⁵ charge interactions are believed to be the initial driver for silk-avidin interactions. However, hydrophobic interactions and changes in protein conformation may also contribute to the adsorption and desorption of proteins to silk.⁴²⁻⁴³ As avidin interacts with the silk surface it likely changes conformation driven by thermodynamic processes, exposing different regions of the protein to the surface resulting in multiple types of interactions.⁴⁴⁻⁴⁵ Previous work has shown that the release of drugs from silk is similar *in vitro* and *in vivo*.⁴⁶ However, the presence of other proteins within the body may result in competitive interactions and avidin desorption.⁴² Additionally, the presence of proteases *in vivo* may shorten the release duration through degradation of avidin or silk. Potential applications of this system for drug delivery may be for tumors, liver, and the kidneys as avidin and biotin have been shown to accumulate in these locations.^{26, 47}

hFF and hMSC Growth on RGD Functionalized Silk Surfaces.

To determine the utility of this modification scheme for potential tissue engineering applications, we evaluated hFF and hMSC growth on avidin adsorbed-silk films. The presence of adsorbed avidin on silk films did not significantly affect the growth of hFFs (Figure S5A), while avidin applied to TCP significantly lowered cell growth on days 3 and 7 when compared to unmodified TCP (Figure S5B). Based on these results, avidin adsorption to silk films did not significantly affect hFF growth.

Next, RGD was used as the terminal functional molecule to alter the cell-material interaction. RGD is an integrin binding domain found in many extracellular matrix proteins that has been shown to increase cell attachment and proliferation on many biomaterial surfaces.^{19, 32, 48-51} hFFs grown on all avidin-modified silk films with either B-GRGDS or GRGDS exhibited significantly higher cell growth, as compared to the silk control, on days 3 and 7 as indicated by higher metabolic activity of these groups (Figure 5). In contrast, only one EDC/NHS group (high GRGDS) had significantly higher cell growth than the silk control on days 3 and 7. Increased metabolic activity was not seen with films with GRGDS or B-GRGDS adsorption alone, demonstrating the effect observed is due to the silk functionalization method immobilizing the GRGDS or B-GRGDS molecule. It is likely that GRGDS, with its net negative charge, may have interacted with avidin via electrostatic interactions, supporting enhanced cell growth without avidin-biotin docking. Therefore, it is shown that the interaction between the RGD peptides and avidin significantly increased cell growth on the silk surface.

Previous work has demonstrated that biotinylation of cells also increases cell attachment and growth to avidin modified surfaces.^{33-34, 52-53} However, while biotinylated cells have demonstrated initial adhesion, this process decreases over time.^{33, 52-53} Loss of biotin moieties on cells has been observed within 1-3 d of culture, due to endocytosis by the cells.^{52, 54} Additionally, daughter cells produced during proliferation will not be biotinylated and

may not attach to the surface as readily as the original biotinylated cells, negatively impacting the long term efficacy of biotinylated cells. Loss of adhesion was not observed in our system as cells continued to grow and remain attached to the silk surface through the seven-day experiment.

To examine if the modification technique could promote growth of more than one type of cell, an initial study was performed to determine the feasibility of using these avidin-modified silk films for increased adhesion of hMSCs through 7 d of culture. hMSCs were chosen because they offer potential for regenerative therapies and are relevant for many tissue engineering applications.⁵⁵⁻⁵⁶ On day 7, the cell growth of hMSCs in the avidin-modified silk groups with GRGDS was significantly greater than growth of hMSCs in the silk control group (Figure 6). Increased cell growth was also observed on days 1 and 3 for avidin-modified silk with a high GRGDS concentration, but not for avidin-modified silk with a low GRGDS concentration suggesting a delayed effect for lower RGD concentrations (Figure S6). It is possible that GRGDS was better able to interact with avidin because it carries a negative charge, and without the addition of biotin, it may have reduced steric hindrance for interaction with the surface. However, significantly greater growth of hFFs was observed in both B-GRGDS and GRGDS wells. It is possible that avidin contains cell binding domains recognized by hMSCs that rendered the inclusion of B-GRGDS unnecessary or these sites were covered or altered by the docking of B-GRGDS.⁵⁷⁻⁵⁸ This agrees with previous findings that hMSCs will non-specifically bind to avidin, perhaps due to the surface charge of the cells.²⁵ The non-specific binding can be reduced by pre-blocking with BSA.²⁵ Our results suggest that, for certain cell types, avidin-modification of the surface without additional biotinylated molecules may be sufficient to induce greater cell attachment and proliferation on silk surfaces.

Cell adhesion is dependent upon the bioactive molecules selected for attachment.²⁷ While avidin-modification alone may be sufficient to promote the attachment of hMSCs, additional modification was required to promote the attachment of hFFs to silk. The interchangeability of the terminal biotinylated molecule in this work creates flexibility for this system. Different biotinylated molecules could be immobilized to promote attachment of more than one cell type at a time or allow spatial control over cell attachment. Additionally, growth factors may be immobilized to direct differentiation of stem cells or maintain the differentiation of cells.⁵⁹⁻⁶¹

CONCLUSION

Silk is an abundant, biocompatible, and mechanically tunable biomaterial that can be used in a variety of applications including tissue engineering and drug delivery. A method for noncovalent functionalization of silk films with avidin is presented. The method is based on the physical adsorption of positively charged avidin onto the negatively charged silk surface. Avidin adsorbed to silk surfaces at a physiologically relevant pH, eliminating the need for harsh buffers or reagents to activate chemical groups on the silk surface. When compared with chemical conjugation of avidin, physical adsorption resulted in higher HRP activity, suggesting that this method promoted the retention of macromolecule activity or increased adsorption. Physical adsorption also allowed for macromolecule desorption under

physiological conditions, showing the utility of this approach as a sustained release system, in addition to a surface modification approach. Furthermore, it was found that adsorption time altered the release kinetics and the remaining adsorbed A-HRP level, suggesting a system for tunable release kinetics. Cell responses to the avidin-adsorbed silk films were also evaluated with and without coupling of B-RGD as a cell binding domain. This approach supported or enhanced cell attachment and growth, suggesting a method for easy modification of silk surfaces for tissue engineering applications. Overall, this is a promising silk modification method that relies exclusively on physical adsorption of avidin under mild conditions and provides a facile linker for functionalization with biotinylated compounds with potential applications in drug delivery, tissue engineering, and biological sensing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic of conjugation and detection.

(A) The conjugation and detection method for modifying silk films with avidin, which then interacts with biotinylated molecules, and (B) the conjugation and detection method for modifying silk films with EDC/NHS carbodiimide coupling. pI indicates the isoelectric point of the molecule. Representative biotin structure used. Linker length of commercially provided product is 14 atoms. PDB file for the avidin structure used is 1VYO (DOI: [10.2210/pdb1VYO/pdb](https://doi.org/10.2210/pdb1VYO/pdb)).

Figure 2. Colorimetric characterization of avidin and B-HRP concentrations.

Avidin and B-HRP concentrations were varied to determine the optimal combination of concentrations. HRP was detected via the addition of a clear substrate (TMB) that is cleaved by HRP, forming a blue color. (A) Image of the array study where the triangles indicated the concentration gradients for each compound. A darker blue color indicates higher HRP activity correlating to increased avidin adsorption. (B) Quantification of the visible light absorbance indicating optimal avidin concentration at 1,000 $\mu\text{g/mL}$ and optimal B-HRP concentration at 0.5 – 5 $\mu\text{g/mL}$.

Figure 3. Kinetic curves of adsorbed or covalently coupled HRP moiety functionalized silk films. Biotin-HRP or HRP (5 $\mu\text{g/mL}$) were adsorbed onto silk films modified with avidin or carboxylate activation. HRP activity was monitored via TMB cleavage forming a blue chromophore detected at 650 nm (15 s between each measurement). Data were normalized to first recorded value for each well. A faster rate of change (higher slope) indicates greater HRP activity. (A) All kinetic curves are displayed with the same y-axis for direct comparison. To more readily make comparisons (B) AB-H and E/NB-H are compared, (C) AH and E/NH are compared, and (D) B-H and H are compared. AB-H modified silk films had the highest HRP activity. Data are presented as mean $\pm$ SEM of three independent experiments with 3-6 samples each. Key: **AB-H**: Avidin with Biotin-HRP; **AH**: Avidin with HRP; **E/NB-H**: EDC/NHS with Biotin-HRP; **E/NH**: EDC/NHS with HRP; **B-H**: Biotin-HRP background control; **H**: HRP background control well.

Figure 4. A-HRP release from silk films over time.

Rate of release of A-HRP from silk film for different time intervals sampled. Rates were determined from time points in the linear region of A-HRP activity curve using TMB as the substrate. Detectable quantities of desorbed A-HRP were observed for both group studies. When A-HRP was adsorbed overnight (black bars) versus for 1-hour (white bars), increased levels of HRP were detected after 19.75 h of release. Data were normalized to first recorded value for each well and are representative of three independent experiments with 8 samples per time point (** $p < 0.01$, *** $p < 0.001$ when comparing overnight to 1-hour samples).

Figure 6. Metabolic activity of hMSCs on modified silk films.

Normalized fluorescence of resazurin incubated with hMSCs after 7 d of culture. Data shown as average with standard deviation from a single experiment with four samples. All values are normalized to the average fluorescence for hMSCs cultured for 1 day on silk only wells (* $p < 0.05$; ** $p < 0.01$ as compared to silk only controls). H indicates 10^{-7} M. L indicates 10^{-8} M. + indicates inclusion, – indicates exclusion. TCP is tissue culture-treated plastic.

Table 1.

Slope of linear region of HRP activity curves from covalently coupled HRP moiety functionalized silk films

Group	B-HRP Slope (AU/s)	HRP Slope (AU/s)
Avidin	$3.38 \times 10^{-3} \pm 1.88 \times 10^{-4}$ ****	$9.79 \times 10^{-5} \pm 1.13 \times 10^{-5}$
EDC/NHS	$7.17 \times 10^{-5} \pm 2.37 \times 10^{-4}$	$3.56 \times 10^{-5} \pm 1.28 \times 10^{-5}$
Binding control	$2.02 \times 10^{-4} \pm 1.32 \times 10^{-4}$	$5.13 \times 10^{-5} \pm 1.62 \times 10^{-5}$

Data represented as mean $\pm$ SEM of three independent experiments with 3-6 samples. Slope of Avidin/B-HRP group is significantly higher than all other groups

 $p < 0.0001$.