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Dual-Functioning Antifouling Silk Surfaces via Covalent Grafting of a Calcium-Chelating Agent

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by

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ABSTRACT OF THE THESIS

Dual Functioning Antifouling Silk Surfaces via Covalent Grafting of a Calcium-Chelating Agent

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University of California, Riverside, June 2026
Dr. Younjin Min, Chairperson

Bacterial contamination and biofilm formation on material surfaces remain major challenges in USDA-relevant food and agricultural environments, where durable coatings are needed to reduce microbial attachment, persistence, and cross-contamination, including from multidrug-resistant pathogens. Silk fibroin is an attractive coating platform because it is a biocompatible structural protein whose surface chemistry and interfacial behavior can be tailored through chemical modification. In this study, silk fibroin was functionalized with 1-hydroxyethylidene-1,1-diphosphonic acid (HEDP) using EDC/NHS coupling to create a phosphonate-modified surface with enhanced ion-binding capability and altered wettability. The resulting silk-HEDP materials were characterized by Fourier transform infrared (FTIR) spectroscopy, water contact angle measurements, and calcium depletion experiments. FTIR confirmed successful incorporation of phosphonate functionality while preserving the characteristic amide features of silk. Secondary-structure analysis further indicated changes in the relative β-sheet and α-helix contents after modification and post-treatment, showing that chemical grafting influenced both surface composition and protein organization. Contact-angle

measurements showed that silk-HEDP exhibited a lower water contact angle than unmodified silk, consistent with increased surface hydrophilicity due to the introduction of polar phosphonate groups. Calcium depletion assays demonstrated substantially greater ion-binding interactions for silk-HEDP than for unmodified silk and glass controls. Overall, covalent HEDP grafting provides an effective route to engineer silk-based coatings with tunable structure, wettability, and calcium-chelating functionality, establishing a promising platform for future antimicrobial and antifouling surfaces aimed at limiting bacterial attachment and biofilm development.

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1 Introduction

Microbial biofilms formation remains a persistent challenge in food processing, biomedical, and industrial environments. Once bacteria adhere to a surface, they begin producing a sticky protective layer called an extracellular polymeric substance (EPS). This matrix holds the cell together and shields them from cleaning agents and physical removal. Biofilms contribute to contamination, reduced system efficiency, and increased maintenance costs. Because biofilms are highly structured and protective, they are much harder to remove free-floating (planktonic) bacteria. This makes it important to design surfaces that prevent biofilm formation in the first place, rather than relying only on antimicrobial treatments after they develop.

Traditional antifouling approaches often rely on the incorporation or release of biocidal agents such as silver nanoparticles or quaternary ammonium compounds, which inhibit bacterial growth or kill organisms upon contact to prevent surface colonization and biofilm formation. [1,2] Although effective, these strategies may raise concerns regarding cytotoxicity, environmental persistence, leaching, and the development of antimicrobial resistance. Consequently, there is growing interest in non-biocidal approaches that interfere with biofilm formation mechanisms without directly inducing bacterial death.

To provide a basis for comparison, quercetin was used as a model compound in bacterial growth assays. This enabled comparison between conventional growth inhibition and the proposed ion-sequestration mechanism. This enabled comparison between conventional growth inhibition and the proposed ion-sequestration mechanism.

Dialect metal ions, particularly calcium (Ca^{2+}), play a critical role in biofilm stability. Calcium ions contribute to bacterial adhesion and crosslink the negatively charged components, enhancing mechanical strength and structural integrity. Local depletion of Ca^{2+} at the material interface represents a promising strategy to disrupt early biofilm development by destabilizing matrix formation and weakening bacterial attachment.

2 Background

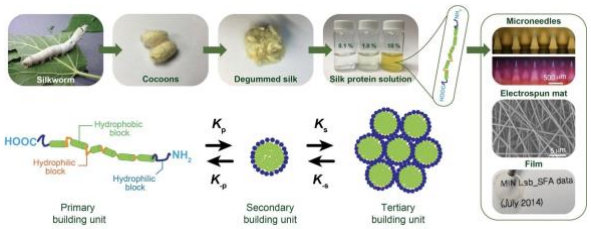

Figure 1: Overview of silk fibroin processing from *Bombyx mori* cocoons and representative hierarchical structures and material formats derived from silk fibroin [3]

Silk Fibroin (SF) is a naturally derived protein polymer obtained from *Bombyx mori* cocoons and is widely used due to its biocompatibility, biodegradability, and adjustable mechanical properties. Its structure consists of a combination of crystalline β-sheet domains and amorphous regions such as random coils and α-helices, which can be modulated through processing treatments such as solvent exposure. This structural adjustability allows SF to exhibit a wide range of mechanical and surface properties, making it an attractive candidate for surface modification applications.

Surface fouling and biofilm formation remain critical challenges in food processing and industrial systems. Biofilm development begins with bacterial adhesion, followed by the formation of an extracellular polymeric substance (EPS) matrix that enhances structural stability and resistance to removal. [4] Traditional antifouling

strategies often rely on biocidal agents; however, these approaches may introduce toxicity concerns and promote antimicrobial resistance.

Divalent metal ions, particularly calcium ions (Ca^{2+}), play a key role in biofilm stability the EPS matrix and enhancing bacterial adhesion to surfaces. Calcium ions interact with negatively charged functional group within the matrix. Therefore, controlling calcium availability at the surface represents a promising strategy for disrupting biofilm formation at an early stage.

In this context, surface modification method that remove or bind calcium ions have become promising non-biocidal antifouling strategies. Compounds containing phosphonate groups, such as HEDP, strongly interact with calcium ions and can help reduce Ca^{2+} levels near the material surface. Adding these functional groups onto surfaces may help reduce mineral buildup, interfere with biofilm formation, and decrease bacterial attachment.

3 Methods

3.1 Preparation of Silk Solution

SF was prepared from *Bombyx Mori* cocoons following a standard degumming and dissolution protocol. Raw silk cocoons were first cut into small pieces and boiled in 0.02 M sodium carbonate (Na_2CO_3) solution for 30 minutes to remove sericin. The fibers were then thoroughly rinsed with MilliQ water multiple times to eliminate residual salts. The degummed silk fibroin was allowed to dry overnight under vacuum inside of the desiccator. Dried silk was subsequently dissolved in a 9.3 M lithium bromide (LiBr)

solution at 60°C for 4 hours to obtain a homogeneous silk solution. The resulting solution was transferred into dialysis tubing (MWCO = 3.5 kDa) and dialyzed against MilliQ water for roughly 48 hours to remove LiBr. After dialysis, the silk fibroin solution was centrifuged to remove insoluble particulates. [5]

3.2 Preparation of Silk-HEDP

SF is a structurally delicate protein that is highly sensitive to pH conditions. Near its isoelectric point (pI = 4.2), reduced electrostatic repulsion between SF chains promotes aggregation and can lead to gelation. [6] To maintain solution stability throughout the reaction, 2-(N-morpholino)ethanesulfonic acid (MES) buffer was used, with sodium hydroxide (NaOH) added as needed to adjust the pH. The initial MES buffer pH (~3.5) was adjusted to approximately 5.05 using NaOH. The starting SF concentration was 2 mg/mL in the MES/NaOH buffer, with an initial pH of 5.09. Based on literature, approximately 3% of the SF primary structure consists of carboxylic acid groups [7]. The molar ratio of SF carboxylic groups to EDC and NHS was maintained at 1:1 and 1:2, respectively. HEDP was added five times the molar amount of SF carboxylic groups to ensure sufficient availability and improve the likelihood of successful conjugation. In this procedure, the SF solution in the MES buffer was first mixed with EDC and NHS and allowed to react and stir for 15 minutes. Prior to the addition of HEDP, the pH of the solution was adjusted to approximately 6.10 to prevent the system from approaching the isoelectric point of SF to minimize the risk of aggregation and gelation. HEDP was then added, and the reaction mixture was stirred for approximately 24 hours to ensure complete modification. Following the reaction, the solution was

dialyzed against the MES buffer (pH = 6.3) to maintain a stable pH environment during purification. Incomplete reaction or improper pH control during dialysis can induce aggregation and lead to the gelation of silk fibroin.

3.3 Mechanism

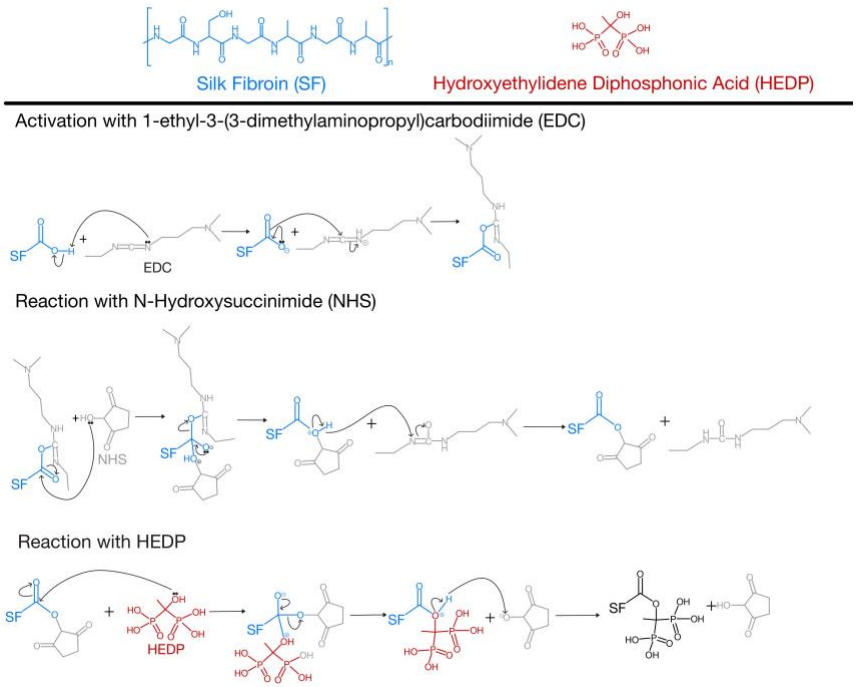

Figure 2: Schematic illustration of EDC/NHS-mediated conjugation between silk fibroin and HEDP

EDC-mediated coupling proceeds through activation of carboxylic acid groups on silk fibroin under mild acidic conditions. At this pH, EDC efficiently reacts with carboxyl groups to form a highly reactive O-acrylisourea intermediate. This intermediate is unstable and susceptible to hydrolysis, which can reduce coupling efficiency if not further stabilized. To address this, NHS is introduced to convert the unstable O-acylisourea intermediate into a more stable NHS ester. The formation of this intermediate enhances the stability of the activated carboxyl group and improves the overall efficiency

of the coupling reaction. The NHS ester subsequently reacts with nucleophilic groups present on HEDP, forming a covalent linkage between silk fibroin and HEDP, which produces silk-HEDP. Reaction conditions are carefully controlled, as higher pH reduces EDC reactivity while increasing nucleophilicity, whereas overly acidic conditions can promote side reactions or instability. Thus, a mildly acidic to near-neutral pH is optimal to balance activation and nucleophilic attack. [8]

3.4 Fourier-Transform Infrared Spectroscopy

Fourier-transform infrared (FTIR) spectra were collected in the range of 500 – 4000 cm^{-1}. Samples were analyzed using transmission mode and spectra were used to identify functional group changes following modification.

3.5 Contact Angle Goniometer

Water contact angle measurements were performed using a contact angle goniometer to evaluate the surface wettability of silk fibroin and modified silk (silk-HEDP) films. A droplet of MilliQ water was deposited onto the sample surface, and the contact angle was measured at room temperature. Measurements were taken at multiple locations on each sample to ensure accuracy. Changes in contact angle were used to assess variations in surface hydrophilicity after chemical modification.

4 Results and Discussion

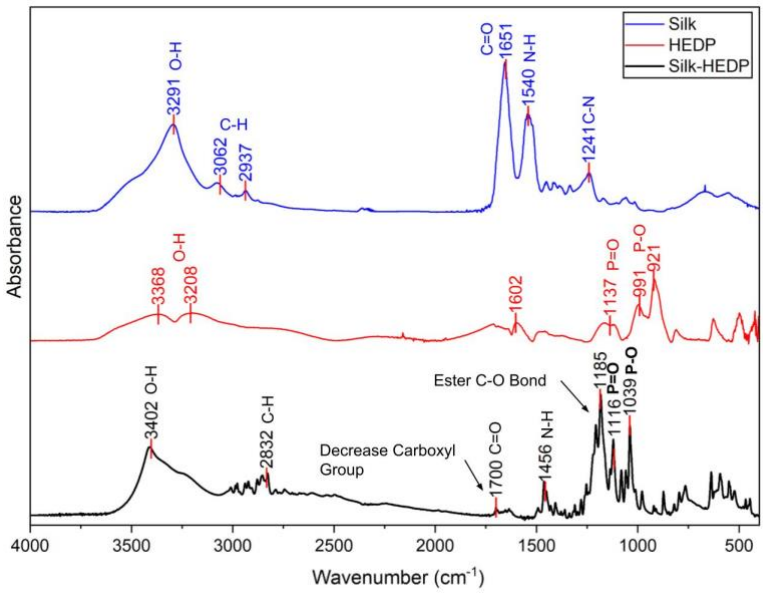

Figure 3: Comparative FTIR analysis of silk fibroin, HEDP, and silk-HEDP demonstrating spectral changes following phosphonate functional groups.

The FTIR spectra of SF, HEDP, and silk-HEDP are shown in Figure X. Pure silk fibroin exhibits characteristics of amide I and amide II peaks at approximately 1651 cm^{-1} and 1540 cm^{-1}, corresponding to C=O stretching and N-H bending. In contrast, HEDP displays prominent peaks in the 1000 - 1200 cm^{-1} region, which are attributed to P-O and P=O stretching associated with phosphonate groups. After modification, the silk-HEDP spectrum retains the characteristic amide peaks of silk, indicating that the overall protein structure is preserved. Additional peaks and increased intensity in the 1000 - 1200 cm^{-1} region are observed, which is consistent with the presence of phosphonate functional groups from HEDP. These spectral changes suggest successful incorporation of HEDP

onto the silk fibroin. The presence of both silk amide bands and phosphonate-related peaks indicates successful modification of silk fibroin with HEDP.

The characteristic amide I and amide II peaks were still present after modification, suggesting that the overall silk fibroin structure remained intact during the coupling reaction. This is important because the silk structure remained stable after modification while still gaining new surface properties. The appearance of phosphonate-related peaks in the silk-HEDP spectrum further supports successful incorporation of HEDP onto the silk surfaces. These phosphonate groups are expected to increase surface polarity and calcium-binding capability, which may contribute to the enhanced wettability and antifouling behavior, which was observed in later analysis.

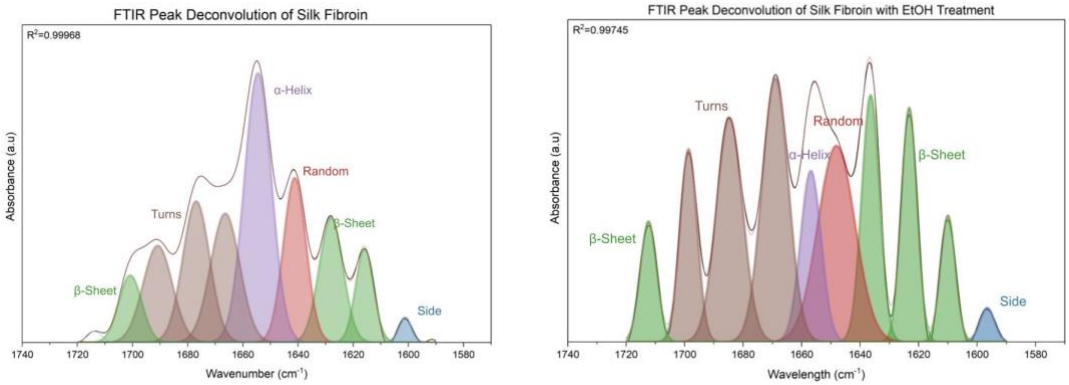

Figure 4: FTIR amide I peak deconvolution of silk fibroin before and after ethanol treatment showing changes in secondary structure composition.

FTIR peak deconvolution of the amide I region revealed significant changes in the secondary structure of silk fibroin following ethanol treatment. Untreated silk exhibited β-sheet and α-helix contents of approximately 22.1% and 26.0%, respectively. After ethanol treatment, β-sheet content increased to 30.3%, while α-helix content decreased to 9.0%, suggesting a transition toward a more ordered and crystalline structure. Ethanol treatment promotes intermolecular hydrogen bonding, which stabilizes β-sheet domains

and enhances molecular packing within the silk fibroin structure. [9] Increased β-sheet content also reduces the exposure of polar functional groups at the surface, resulting in lower surface polarity and reduced wettability. This behavior is consistent with the higher water contact angle observed after ethanol treatment, indicating increased hydrophilicity. Overall, these results suggest that ethanol treatment induces structural rearrangement from less ordered α-helix conformations into more stable β-sheet structures.

The increase in β-sheet content after ethanol treatment suggests improved structural stability and tighter molecular packing within the silk fibroin matrix. Increased crystallinity is commonly associated with reduced chain mobility and lower exposure of hydrophilic functional groups at the surface. This structural rearrangement likely contributed to the water contact angle observed after ethanol treatment. These findings demonstrate that ethanol treatment not only alters the secondary structure of silk fibroin but also influences surface behavior that may affect material performance in antifouling applications.

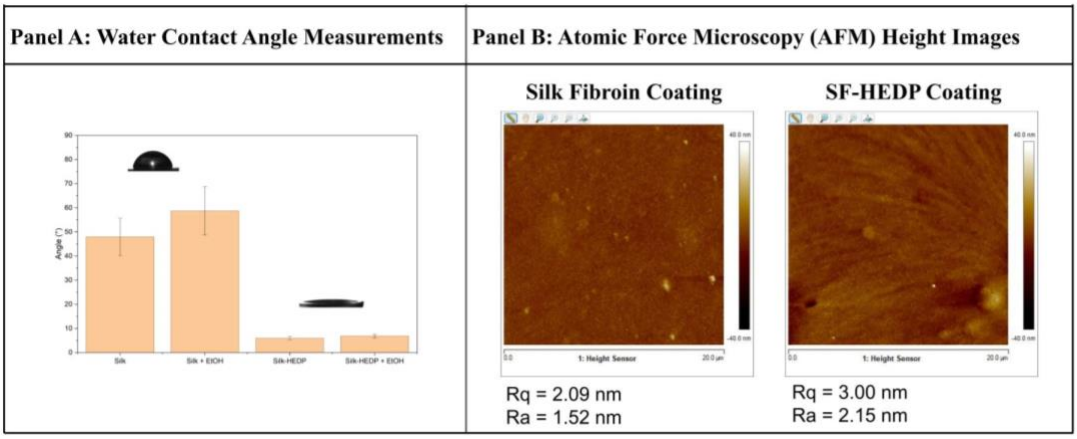

Figure 5: Surface wettability comparison of silk fibroin films before and after ethanol treatment and HEDP modification

The water contact angle measurements demonstrate clear changes in surface wettability following ethanol treatment and HEDP modification. Ethanol-treated silk exhibited an increase in contact angle compared to untreated silk, indicating a more hydrophobic surface. This behavior is attributed to the induction of β-sheet structures during ethanol treatment, which promotes tighter molecular packing and reduces the availability of polar functional groups at the surface. As a result, surface energy decreases, leading to reduced wettability. In contrast, HEDP-modified silk showed a significant decrease in water contact angle, indicating enhanced hydrophilicity. This is attributed to the introduction of phosphonate and hydroxyl groups from HEDP, which increases the surface polarity and promotes stronger interactions with water molecules. The increased presence of hydrophilic functional groups enhances surface wettability and contributes to improved antifouling potential. Increased surface hydrophilicity is also associated with reduced fouling, as hydrophilic surfaces minimize bacterial adhesion. Therefore, the decrease in water contact angle after HEDP modification suggests improved antifouling performance.

Overall, the wettability results support the successful modification of silk fibroin surfaces through both ethanol treatment and HEDP functionalization. The increase in water contact angle after ethanol exposure suggests a more ordered and compact surface structure, while the lower contact angle observed for silk-HEDP indicates increased surface polarity. These findings agree with the FTIR and deconvolution results, which showed structural rearrangement after ethanol treatment and incorporation of phosphonate groups after HEDP modification. Together, the results suggest that surface

chemistry and secondary structure both contribute to the observed changes in wettability and may influence the antifouling behavior of the material.

AFM height images further demonstrate morphological differences between untreated silk surfaces that appeared relatively smooth and uniform, whereas the SF-HEDP surface exhibited increased surface features and visible patterned regions. These features may be associated with the incorporation and distribution of HEDP within the silk coating. The changes in surface morphology after HEDP modification may help explain the increased hydrophilicity behavior observed in the water contact angle. Changes in surface structure and chemical properties can affect how the material interacts with water and calcium ions, which may influence antifouling behavior.

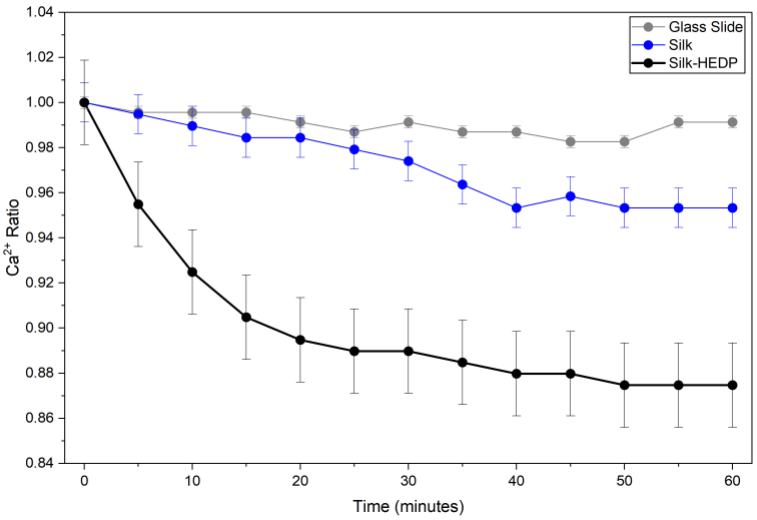

Figure 6: Calcium depletion profiles comparison ion-binding behavior of glass, silk fibroin, and HEDP-functionalized silk surfaces over time.

The calcium depletion results demonstrate the enhanced ion-binding capability of HEDP-modified silk surfaces. Over time, the Ca^{2+} concentration decreased more significantly in the presence of silk-HEDP compared to untreated silk and the glass

control. In Figure X, it is shown that the Ca^{2+} ratio for silk-HEDP decreased to approximately 0.88 after 60 minutes, compared to 0.96 for untreated silk and around 1.00 for the glass slide. The rapid initial decrease followed by a gradual plateau indicates that calcium ions quickly bind to the surface at first, then reach a stable equilibrium over time. Notably, the Ca^{2+} ratio for silk-HEDP remains consistently low over time without significant calcium recovery, unlike pure silk and the glass slide, which show slight increase after a certain point. This behavior suggests stronger and more stable calcium binding on the HEDP-modified surface, with reduced desorption and enhanced binding affinity. The enhanced ion-binding behavior is attributed to the introduction of phosphonate groups from HEDP, which have a high affinity for calcium ions. In contrast, pure silk shows only a slight decrease in Ca^{2+} concentration, indicating limited interaction with calcium ions. These results confirm that HEDP modification improves the calcium-chelating ability of silk surfaces and contributes to improved antifouling performance.

Overall, the calcium depletion results demonstrate that surface modification significantly altered the interaction between silk fibroin, and calcium ions. Compared to untreated silk, the HEDP-functionalized surface maintained a lower Ca^{2+} concentration over time, suggesting more stable ion interactions at the material interface. This behavior is important because calcium ion contribute to biofilm stability and bacterial attachment. The observed depletion trend therefore supports the potential use of silk-HEDP coatings as non-biocidal antifouling materials. In addition, the calcium depletion behavior agrees with the FTIR and wettability results, indicating that the introduced phosphonate groups successfully changed the surface chemistry and interfacial properties.

5 Summary and Conclusions

This study demonstrated the successful modification of silk fibroin through covalent grafting of HEDP using EDC/NHS coupling chemistry. FTIR analysis confirmed the incorporation of phosphonate-related functional groups while retaining the characteristic amide bands of silk fibroin, indicating that the overall protein structure was preserved after modification. Secondary structure analysis showed that ethanol treatment increased β-sheet content and promoted a more ordered silk structure, while water contact angle measurements revealed that HEDP modification significantly increased surface hydrophilicity. AFM imaging further demonstrated changes in surface morphology following modification, suggesting that HEDP incorporation altered the surface patterning of the silk coating. Calcium depletion experiments showed substantially enhanced calcium ion binding for silk-HEDP compared to unmodified silk and glass controls, confirming the effectiveness of the introduced phosphonate groups. Collectively, these results demonstrate that HEDP-functionalized silk combines enhanced hydrophilicity and calcium-chelating capability, supporting its potential as a non-biocidal antifouling material for applications in food processing, biomedical devices, and other environments where biofilm formation is a concern. Future work will focus on evaluating bacterial adhesion and biofilm formation on silk-HEDP surfaces to directly assess antifouling performance. Additional studies will investigate protein adsorption behavior, phosphonate leaching, cytocompatibility, and coating reusability to further establish long-term stability and practical applicability of the modified silk coatings.

References

1. Franci G, Falanga A, Galdiero S, Palomba L, Rai M, Morelli G, Galdiero M. Silver nanoparticles as potential antibacterial agents. Molecules. 2015 May 18;20(5):8856-74. doi: 10.3390/molecules20058856. PMID: 25993417; PMCID: PMC6272636.

2. Jiao Y, Niu LN, Ma S, Li J, Tay FR, Chen JH. Quaternary ammonium-based biomedical materials: State-of-the-art, toxicological aspects and antimicrobial resistance. Prog PolymSci. 2017 Aug;71:53-90. doi: 10.1016/j.progpolymsci.2017.03.001. Epub 2017 Mar 12. PMID: 32287485; PMCID: PMC7111226.

3. Y. Zhang, Y. Zuo, S. Wen, Y. Hu, and Y. Min, "Distinctive Stress-Stiffening Responses of Regenerated Silk Fibroin Protein Polymers under Nanoscale Gap Geometries: Effect of Shear on Silk Fibroin-Based Materials," *Biomacromolecules*, vol. 19, no. 4, pp. 1363–1374, 2018.

4. Flemming, HC., Wingender, J., Szewzyk, U. *et al.* Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* 14, 563–575 (2016). https://doi.org/10.1038/nrmicro.2016.94

5. D. N. Rockwood, R. C. Preda, T. Yücel, X. Wang, M. L. Lovett, and D. L. Kaplan, "Materials fabrication from *Bombyx mori* silk fibroin," *Nature Protocols*, vol. 6, no. 10, pp. 1612–1631, Oct. 2011, doi: 10.1038/nprot.2011.379.

6. V. Catto, S. Farè, I. Cattaneo, M. Figliuzzi, A. Alessandrino, G. Freddi, A. Remuzzi, and M. C. Tanzi, "Small diameter electrospun silk fibroin vascular grafts: Mechanical properties, in vitro biodegradability, and in vivo biocompatibility," *Materials Science and Engineering: C*, vol. 54, pp. 101–111, 2015.

7. Hongshi Zhao, Eva Heusler, Gabriel Jones, Linhao Li, Vera Werner, Oliver Germershaus, Jennifer Ritzer, Tessa Luehmann, Lorenz Meinel, Decoration of silk fibroin by click chemistry for biomedical application, Journal of Structural Biology, Volume 186, Issue 3, 2014, Pages 420-430, ISSN 1047-8477, https://doi.org/10.1016/j.jsb.2014.02.009.

8. R. Liu, J. Ming, H. Zhang, and B. Zuo, "EDC/NHS crosslinked electrospun regenerated tussah silk fibroin nanofiber mats," *Fibers and Polymers*, vol. 13, no. 5, pp. 613–617, 2012, doi: 10.1007/s12221-012-0613-y.

9. Kaewpirom S, Boonsang S. Influence of alcohol treatments on properties of silk-fibroin-based films for highly optically transparent coating applications. RSC

Adv. 2020 Apr 21;10(27):15913-15923. doi: 10.1039/d0ra02634d. PMID: 35493649; PMCID: PMC9052366.