Self-assembling of Chimeric Mussel-inspired Bioadhesives Originated from *Mytilus Californianus* and *Anabaena Flos-aquae*: A New Approach to Develop Underwater Adhesion

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Research Article

Keywords: Mussel foot protein, Gas vesicle Protein A, Self-assemble, Underwater adhesive

Posted Date: March 17th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-294097/v1>

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Mytilus Californianus and *Anabaena flos-aquae*: A New Approach to Develop
Underwater Adhesion

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Running Title: Novel Mussel-inspired Adhesive with amyloid features

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Abstract:

Bio-adhesives play a pivotal role in a wide range of medical applications. However, there are some problems about their application in varied pH values and low adhesion force under wet conditions. Here, we report new recombinant fusion protein achieved by mussel foot proteins (Mfps) of *Mytilus Californianus* and gas vesicle protein A (GvpA) of *Anabaena flos-aquae* by genetic engineering methods. These chimeric proteins self-assembled into β -sheet rich fibres because of GvpA amyloid structure. Also, their adhesion forces were significantly increased especially in alkaline environment based on Mfp-3 and Mfp-5. This study illustrates that copolymer of Mfp-5-GvpA:GvpA-Mfp-3 can be used as an underwater sturdy adhesive with tolerance to auto-oxidation, especially at basic conditions.

Keywords: Mussel foot protein, Gas vesicle Protein A, Self-assemble, Underwater adhesive

Introduction:

Naturally, bio-inspired adhesives originated from mussel, barnacles, Notaden frogs and gecko have had extensive interest among scientists due to being water-based adhesive¹⁻⁵. On the one hand, the presence of large quantities of Dihydroxyphenylalanine (DOPA) known to encourage adhesion on wet surfaces⁶ is a special characteristic of Mussel Foot Proteins⁷. Tremendous features in Mussel Foot Proteins (Mfp-3 and Mfp-5) such as resistance in heavy water flow just as changes in temperature and saltiness are so impressive for designing a superglue. Two cited proteins contain highest DOPA (in byssal plaque of *Mytilus californianus*) in their protein structures⁸.

On the other hand, amyloids as bio-inspired adhesives can make novel opening for surface/interface functionalization⁹. The versatility of amyloids have attracted the attention of scientists¹⁰. In a significant number of pathologies, including neurodegenerative diseases and systemic amyloidosis, amyloid fibrils have been identified¹¹. A Polypeptide molecules capable of self-assembly into β -sheet rich linear aggregates are amyloidogenic proteins¹². A β -hairpin that forms an antiparallel β -sheet is found in amyloid monomers. Amyloids are versatile proteins for interfacial underwater adhesion. For interfacial underwater adhesion, such fibrillar structures have incredible advantages¹³. A study was conducted regarding the presence of amyloid fibers in Gas vesicle protein A (GvpA) of *Anabaena flos-aquae*. This protein has some advantages such as tolerance to proteolysis, providing the strength of protein structure and amphiphilic properties¹⁴.

In our study, we have logically planned for a novel chimeric bioadhesive involving mussel-inspired adhesive and amyloidogenic protein. For having superglue, Mfp-3 and Mfp-5 have been selected with highest Tyrosine (DOPA-based mussel foot protein) among other Mfps and GvpA single protein as amyloidogenic protein has been chosen from Gas Vesicle wall of *Anabaena flos-aquae*. Also, Mfp-5-GvpA and GvpA-Mfp-3 were built. Due to having self-assembly of GvpA as

amyloidogenic protein, it is expected that both Mfp-5-CsgA and CsgA-Mfp-3 could self-assemble together into amyloid fibrils with adhesive features by showing DOPA residue on the outside of amyloid scaffold to create copolymers according to previous research work (Figure 1) ⁴. They can also build more strength, resistant to proteolysis and protein flexibility and elasticity, achieved by GvpA in chimeric protein¹⁴. moreover, we have measured force adhesion between these functional chimeric proteins and mica under wet conditions. The impact of varied pH values on adhesion has been investigated too. We have reached maximum force adhesion at neutral pH and found that copolymers at alkaline pH had the most force adhesion. Nonetheless, other approaches have failed to attain underwater adhesion at neutral pH^{15,16}.

Results and discussion:

Expression of recombinant Mfp-5- GvpA and recombinant GvpA-Mfp-3 hybrids:

The results illustrated in this research work match the state-of-the-art chimeric proteins. Formerly, *Mfp-5- GvpA* and *GvpA-Mfp-3* cDNAs were specifically optimized for *E. coli* (Mfp-3 and Mfp-5 genes from *Mytilus californianus* and GvpA gene from *Anabaena flos-aquae*), synthesized (by Biomatik Co., Canada) and inserted into a pET-11a expression vector, and then were transformed into *E. coli* BL21 (DE3). Induction of Mfp-5-GvpA and GvpA-Mfp-3 recombinant hybrids were performed by IPTG 1mM but there are not expressed proteins in SDS-PAGE and Western Blotting, which can be due to toxicity of produced recombinant proteins^{17,18}. Hence, *E. coli* BL21-AI have been used as alternative for resolving this problem^{19,20}. After the induction of aforementioned recombinant mussel-inspired amyloid hybrids with L-arabinose 0.2% and IPTG 1mM, cells were grown for 3 hours. Most of the recombinant proteins were expressed as inclusion body (IB) in *E. coli*. Total expressed proteins were analyzed with SDS-PAGE 10% (Coomassie Brilliant Blue G-250 staining) (Figure 3) and Western Blotting (Anti-histidine antibody) (Figure 4).

Recombinant protein purification:

Protein purification was performed by nickel-containing affinity resin. Recombinant proteins were solubilized by 8M urea, and they were not bound to nickel resin due to aggregated proteins. Consequently, we have used fresh 6M guanidine HCl (GuHCl) (stronger chaotrope than 8M urea) as denaturant for enhancing the solubility of recombinant proteins, where eventually recombinant proteins could be bound to nickel resin. After purification with nickel-IDA agarose 6% under denatured conditions (Figure 5), concentration of purified proteins was obtained at 0.7439 (mg/mL) and 0.8937 (mg/ml) for Mfp-5-GvpA and Mfp-3-GvpA proteins through the BCA Protein Assay, respectively.

Modification and confirmation of recombinant protein with tyrosinase treatment qualitatively and quantitatively:

In order to obtain adhesive features, tyrosinase was added to purified proteins (unmodified Mfp-5-GvpA and unmodified Mfp-3-GvpA). After the filtration of mussel-inspired adhesives, they have been dialyzed in 5% acetic acid (to prevent auto-oxidation) at 4 °C. Conversion Tyrosine to DOPA in recombinant GvpA-Mfp-5 and recombinant Mfp-3-GvpA proteins have been detected by redox-cycling nitroblue tetrazolium (NBT) staining, where modified Mfp-5-GvpA and modified Mfp-3-GvpA were stained with a blue-purple band (Figure 6) (Unmodified and modified proteins referred to before and after tyrosinase treatment). For measuring the quantity of this conversion (% Tyr to DOPA), acid-borate difference spectrum (ABDS) method was utilized (Table 1). According to the protein concentration gained from BCA method, molarity of tyrosine and DOPA were calculated for both Mfp-5-GvpA and Mfp-3-GvpA: M(Tyr) for GvpA-Mfp-3: 0.77252635(mM); M(Tyr) for Mfp-5-GvpA: 0.7944(mM); M(DOPA) for GvpA-Mfp-3: 0.09538(mM); M (DOPA) for GvpA-Mfp-5: 0.08973(mM) (supposedly Mfp-5-GvpA and Mfp-3-

GvpA have 24 and 18 tyrosines, respectively). Then, the percentage of tyrosine converted into dopa were achieved (Table 1).

Congo red staining:

This assay verified amyloidogenic characteristics of GvpA-Mfp-3 and Mfp-5-GvpA (Figure 7).

3-5-Force Adhesion Measurement:

Atomic force spectroscopy (AFS) was utilized to measure adhesion force of GvpA-Mfp-3, Mfp-5-GvpA and copolymer (in both unmodified and modified states) with silica tip on mica surface in a wet environment. Comparative tests with BSA (F/R=3.1 mN/m) and Cell-Tak™ (F/R=24.7 mN/m) were conducted as negative and positive control. Modified copolymer (F/R=157.1938 mN/m) and unmodified Mfp-3-GvpA (F/R=17.2744 mN/m) have had the most and least adhesion respectively (at pH=5) (Figure 8). In previous studies, it has been shown that recombinant Mfps had adhesive features without tyrosinase treatment, but adhesion force was less than the modified protein with tyrosinase treatment²¹. Nonetheless, unmodified hybrids show more force adhesion than the BSA as a negative control (Unmodified GvpA-Mfp-3=17.2744, Unmodified Mfp-5-GvpA=34.0444, and Unmodified GvpA-Mfp3:Mfp-5-GvpA=15.6575). This indicated that, in addition to DOPA, some residues such as lysine and arginine in Mfp-5 have a key role in binding to wet surfaces²²⁻²⁴. The adhesion forces of Gvp-A-Mfp-3 and Mfp-5-Gvpa were respectively 2.5 and 2 times more than the cited adhesion force for Mgf-3A and Mgf-5²¹. Additionally, force adhesion of Mfp-5-GvpA was almost two times higher than that for mfp-151 measured under identical conditions²¹. Furthermore, adhesion force of modified copolymer (GvpA-Mfp3:Mfp-5-GvpA) is 1.5 and 3 times higher than that for modified Mfp-5-GvpA (99.9368 mN/m, $P \leq 0.001$) and modified GvpA-Mfp-3 (53.6650 mN/m, $P \leq 0.0001$)(Figure 8). It was supposed that GvpA-

Mfp3:Mfp-5-GvpA copolymer widens surface-area-to-volume ratio for DOPA residues to fulfil robust underwater adhesion. This issue was confirmed by previous studies that the self-assembly of GvpA into gas vesicles appears to be regulated in a similar way to that of CsgA and silk spidroin (MaSp1) self-assembly^{4,14,25}.

Also, the force adhesion was evaluated on Mfp-5-CsgA:CsgA-Mfp-3 fibres under wet environment with different pH values. The order of adhesion strength from highest to lowest is as follows: pH= 5, 7, 2.5, 12. In a previous study, AFS was applied for measuring adhesion force of Mfp-5-CsgA-CsgA-Mfp-3 at different pH values⁴. Recent study reported that Mefp5 had one-third of the lower adhesion energy at pH 5 than that at pH 2.6. Moreover, other studies reveal that the force adhesion of Mfp3 and Mfp5 in basic pH is virtually eliminated²⁶. Oxidation of Dopa residues in alkaline solutions resulted in a reduction in force adhesion (9). For this reason, Gas vesicle protein A (GvpA) was selected as a tolerant agent against auto-oxidation¹⁴. Notably, we worked on modified copolymer as an strong underwater adhesive at pH values: 5 and 2.5, and our modified copolymer reveals a force of adhesion of ~158 and 122.4850 mN/m; the values that are higher than those in a previous study for under water superglue Mfp-5⁸. A similar pattern of chimeric protein was attained in another research. Mfp3-MaSp1 was chosen as a chimeric mussle-inspired adhesive with amyloid features. Adhesion force of this bioadhesive was 55 mN/m, which was lower than our study²⁷. In line with previous studies, modified Mfp-5⁽³⁾ (Mfp5⁽²⁾-Intein^N + Intein^C-Mfp5⁽¹⁾) had the force of adhesion ~ 201 mN/m at pH=5, the strongest mussel-inspired adhesive among other studies under the same condition. Nevertheless, the force of adhesion was not measured at other pH values and probably did not have tolerance to auto-oxidation¹⁶. Overall, our adhesive hybrid mainly showed tolerance to auto-oxidation in comparison with previous studies on another Mfps. In comparison to our study, the adhesion force of our bioadhesive has

been reinforced at pH:7(152.4125 mN/m) (Figure 9). To explain, GvpA is strongly hydrophobic and negatively charged at pH:7²⁸ making it an excellent choice for medical application in physiologic and basic conditions.

Self-assembly of GvpA-Mfp3 and Mfp-5-GvpA (Copolymerization of two recombinant amyloid proteins): phosphate solutions were used to dissolve purified proteins (pH = 7.2) and were altered to pH =5.0. Copolymer fibers (Mfp-5-GvpA:GvpA-Mfp3:) with 5:5 molar ratios have had more adhesion rather than 3:7 and 7:3. According to our study (Figure 10), the (GvpA-Mfp3)-co-(Mfp5-GvpA) copolymer hybrid demonstrated the sturdiest underwater adhesion at pH:7 among all former studies on mussel-inspired bioadhesive under the same condition^{4,27,29-31}.

All in all, we showed a novel mussel-inspired bioadhesive with amyloidogenic properties including Mfp-5-GvpA and GvpA-Mfp-3. These structures demonstrated strong wet adhesion especially in neutral and alkaline pH. Adhesion force was measured by atomic force spectroscopy with colloidal probe and evaluated in different pH values. As mentioned, at pH=12, there was the strongest adhesion. For the first time, our mussel-inspired adhesive based on amyloid features of GvpA was utilized as a new chimeric underwater bioadhesive, and had the ability to adhere in variety of pH values which can be used for a wide range of biomedical applications as a medical device.

Materials and Methods:

Gene design: Having recombinant MFP-3-GvpA and recombinant MFP-5-GvpA, the optimized genes were synthesized artificially by Biomatik corporation (Canada, QC SG180626). Polyhistidine tags were introduced in C-terminal of these gene structures. GS linker was inserted between Mfps and GvpA in recombinant forms. pET-11a was selected as an expression vector.

Initially, pET11a was cut by two restriction enzymes (BamHI and NdeI), and then gene constructs were separately inserted between BamHI and NdeI restriction site. Inserted sequences were verified by colony PCR and enzymatic method (BamHI and NdeI enzymes). Colony PCRs were performed with T7 forward and reverse primers. Sequencing was performed by Biomatik corporation. Plasmid construction are mapped into Figure 2.

Protein Expression: Generally, our procedure is based on methods used in protein expression. It includes the induction of recombinant protein, confirmation of expressed protein, refolding and purification. The recombinant constructs, pET-11a-GvpA-Mfp3, and pET-11a-Mfp5-GvpA, have been transformed in *E. coli* BL21 AI. Then, these bacteria have been grown to OD₆₀₀~0.4 in Terrific broth medium consisting of 50 mg/mL ampicillin at 37°C. After that, recombinant protein was expressed as inclusion bodies by 0.2% Arabinose and 1mM IPTG at 30°C for 3 h.

Cell extract: One liter of the cell culture of *E. coli* is centrifuged at 5000g at 4°C. The supernatant was removed and the weight of the pellet is determined. Then, Cell lysis buffer (guanidine hydrochloride (GdnHCl, 8M), 50 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0) and 100 mM NaCl) were added. Pellets are resuspended afterwards. A total of 100 mM PMSF and then 10 mg/ml lysozyme (Sigma) were added. The whole suspension was stirred. After that deoxycholic acid were subjoined. The suspension was stored at 37°C while stirring sporadically. When Lysate became viscous, 1 mg/ml DNase was added. Lysates have been incubated at 4°C overnight.

SDS-PAGE and Western Blotting

Initially, samples were mixed with 1x SDS loading buffer (50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% bromophenol blue, and 10% (v/v) glycerol), and they were then boiled. SDS-PAGE was prepared with 15% separating gel and 5% stacking gel. After running

the SDS-PAGE, the gels were stained with Coomassie Brilliant Blue. Having samples run on 15% SDS-polyacrylamide gels, they were transferred onto Nitrocellulose membrane. Chimeric proteins were marked by Monoclonal Anti-polyHistidine-Peroxidase (Sigma, A7058) at a dilution of 1:2000. The expressed proteins were confirmed using H₂O₂, and diaminobenzidine generated a brown precipitate.

Purification with 6% Nickel-IDA Agarose

Ten bed volumes of Binding buffer (50mM NaH₂PO₄, 300mM NaCl, 10 mM imidazole, Urea 8M, pH:7) were added and mixed thoroughly at 4°C overnight on a rotator. Having removed supernatant, E. coli lysate was added to gel and incubated at 4°C overnight. Binding buffer was added 10 bed volumes and mixed gently on a rotatory shaker. The washing step (50mM NaH₂PO₄, 300mM NaCl, 20 mM imidazole, Urea 8M, pH:7) was repeated 4 times and 1.5 % Tween 20 was added to further reduce contamination. Suspensions were centrifuged at 500g for 5 minutes to sediment agarose. One bed volume of elution buffer (50mM NaH₂PO₄, 300mM NaCl, 250 mM imidazole, Urea 8M, pH=7) was added and mixed gently on a rotatory shaker. This suspension was centrifuged at 500g for 5 minutes and his-tagged protein containing supernatant was transferred into new tube and stored on ice. After that, imidazole was removed by dialysis.

Post-translational modification was performed by Tyrosinase (from mushroom). To study adhesion, purified recombinant unmodified proteins of Mgfp-5-GvpA, GvpA-Mfp-3 and copolymer have been resolved in 5% acetic acid to halt auto-oxidation of DOPA residues and 25 mM ascorbic acid-containing buffer and have been modified with 10-unit mushroom tyrosinase to create DOPA at room temperature for 6h at pH=6.5(17). BSA (18) and Cell-Tak were selected for negative and positive control respectively.

Self-assembly of Mfp-5-GvpA and GvpA-Mfp-3:

After purification of Mfp-5-GvpA and GvpA-Mfp-3, protein concentration was measured by the bicinchoninic acid (BCA) assay. In phosphate solutions (pH=7), these proteins (45 μ M) were dissolved in. Then, pH was changed to pH=5. Following that, these proteins were stored overnight at 4°C. Two chimeric proteins were incubated with a variety of molar ratios.

Nitroblue tetrazolium (NBT) used for staining Dopa-containing proteins:

Acid-urea PAGE was performed to validate the presence of DOPA residues with redox cycling staining involving nitroblue tetrazolium (NBT) and glycinate of modified purified recombinant Mfp-5-GvpA, GvpA-Mfp-3 and (Mfp-5-GvpA:GvpA-Mfp-3)(19).

Acid-borate Difference Spectrum: a quantitative test to confirm DOPA

Evaluation of the amount of dopa in modified mussel-inspired adhesives was performed by Acid-borate difference spectrum(20). Absorbance of DOPA in acid conditions is 280nm. Wavelengths were changed by the creation of diol-borate at pH=7-12 (287nm).

Molarity of tyrosine in unmodified adhesives was measured: (n: the number of Tyrosine in unmodified adhesive proteins)

$n \times (\text{protein concentration in BCA assay}) / (\text{Molecular weight of unmodified adhesive proteins})$

M(Tyr) for GvpA-Mfp-3: 0.77252635(mM), M(Tyr) for Mfp-5-GvpA:0.7944(mM)

DOPA concentration was measured by Acid-borate difference spectrum

M DOPA for GvpA-Mfp-3: 0.09538(mM), M DOPA for GvpA-Mfp-5: 0.08973(mM)

Congo Red Assay

The nitrocellulose membranes were used for spotting 50 µg/mL bio-inspired adhesive samples. After that, membranes with associated proteins were saturated in 0.0025 (m/v%) Congo red solution for 1 hour at RT. After washing with water, these membranes were incubated overnight.

Adhesion Force Measurement:

Adhesion force between our chimeric adhesion proteins on mica surface and colloidal probe (Silica) was measured by Atomic Force Spectroscopy (AFS)(21). AFM force measurements were conducted at room temperature with varied pH values in buffered solutions (pH = 2.5, 5.0, 7.0 and 12.0) using an AFM5100N (HITACHI). Adhesion force measurements were performed at a rate of 0.2 to 2.3 Hz, using Silica cantilever (coated with glass sphere). Afterwards, the force curves were analyzed by Hitachi Navigation system and Origin 8.1 software. Then, 45 µM bio-inspired adhesive solution was covered on a mica surface under wet environment with different pH values (pH = 2.5, 5.0, 7.0 or 12.0) for 1h to completely cover mica surfaces. A total of 64 measurements were conducted for each adhesive fibre type. In the end, adhesion forces have been measured as reported by the Johnson-Kendall-Roberts(22):

$$F_{ad} = 3\pi R E_{ad} \quad R: \text{Touch Radius} \quad E_{ad} = \text{Adhesion Energy per area}$$

Authors' Contribution:

HI, SH, and SNH writing paper and data analysis. HHF and SZ data collecting. SB and MAS conceptualization, Investigation, Formal analysis, Writing-review & editing. All co-authors commented on the manuscript. All authors read and approved the final manuscript.

Acknowledgments:

This research work was supported by Pasteur Institute of Iran and Iran National Science Foundation by grant No: 96016699. Also, it was financially supported by grant No: 961207 from the Biotechnology Development Council of the Islamic Republic of Iran. We acknowledged Mr. Saadi Hosseini and Mrs. Neda Moazzezy for providing proof reading the article.

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Figure 1: Genetic engineering of fusion proteins, expression and self-assembling of Mfp-5-GvpA and GvpA-Mfp-3 conducted successfully (Created with BioRender.com).

2-1-Gene constructs:

Figure 2: Pet11a-Mfp-5-GvpA and Mfp-3-GvpA plasmid constructions. Gene sequences were located between BamHI and NdeI restriction sites.

Figure 3: Over-expression of two bio-inspired amyloid adhesives were analyzed by SDS-PAGE gel with G250 staining: 1) Protein marker, 2,8) Induction of *E. coli* BL21 AI with Plasmid without gene 3,7) Induction of *E. coli* BL21 AI without plasmid 4) Expressed Mfp-3-GvpA (Green arrow) with M.W: 18279.44 Da 6) Expressed GvpA-Mfp-5 (Orange arrow) with M.W=20371.22 Da 5) Protein marker.

Figure 4: Protein expression confirmed by Western Blotting method through anti-histag antibody. Line 1 and 2: Protein marker, Line 3: GvpA-Mfp-5 (Orange arrow), Line 4: Mfp-3-GvpA (Green arrow).

Figure 5: SDS-PAGE analysis: Protein purification by Immunoaffinity purification; Lane 1 and 4 protein markers; Lane 5 and 6: GvpA-Mfp-5(Orange arrow); Lane 2, 3: Mfp-3-GvpA(Green arrow) respectively.

	GvpA-Mfp3	Mfp5-GvpA
DOPA quantity (%)	63.7%±2.4	74.3%±3.8

Table 1: ABDS analysis: measurement of DOPA content in GvpA-Mfp-3 and Mfp-5-GvpA.

Figure 8: Adhesion force of bioadhesives with silica tip: This figure demonstrates comparison of adhesion force (mNm⁻¹) between mussel-inspire adhesives: Unmodified and modified GvpA-mfp-3, mfp-5-GvpA and (Gvp-A-Mfp3)-co-(Mfp-5-GvpA) copolymer. ***P ≤ 0.001, ****P ≤ 0.0001

Figure 9: Adhesion force of (Gvp-A-Mfp3)-co-(Mfp-5-GvpA) copolymer at different pH values: As shown in this figure, in pH=5(Yellow Colour) and pH=7(Green Colour) copolymers have the most force adhesion respectively.

Figure 10: Comparison of adhesion force between copolymer hybrids (GvpA-Mfp3:GvpA-Mfp-5) with 3 ratios. molar ratio, 5:5(157.1937 mN/m):3:7(47.8312 mN/m): 7:3(43.5175mN/m).

Figures

Figure 1

Genetic engineering of fusion proteins, expression and self-assembling of Mfp-5- GvpA and GvpA-Mfp-3 conducted successfully (Created with BioRender.com).

coli BL21 AI without plasmid 4) Expressed Mfp-3-GvpA (Green arrow) with M.W: 18279.44 Da 6)
Expressed GvpA-Mfp-5 (Orange arrow) with M.W=20371.22 Da 5) Protein marker.

Figure 4

Protein expression confirmed by Western Blotting method through anti-histag antibody. Line 1 and 2: Protein marker, Line 3: GvpA-Mfp-5 (Orange arrow), Line 4: Mfp-3-GvpA (Green arrow).

Figure 5

SDS-PAGE analysis: Protein purification by Immunoaffinity purification; Lane 1 and 4 protein markers; Lane 5 and 6: GvpA-Mfp-5(Orange arrow); Lane 2, 3: Mfp-3-GvpA(Green arrow) respectively.

Figure 6

NBT assay. Lane 6 and 13: protein marker; Lane 9 and Lane 1: Purified Mfp-3-GvpA (Green arrow) and Purified GvpA-Mfp-5 (Orange arrow) after modification respectively. Lane 5 and Lane 12: total protein after over-expression without tyrosinase treatment; Lane 2, 3, 7 and 8: Negative control; L-tyrosine; 4, 10 and 11

Figure 7

Congo red staining: 1) GvpA; 2) Modified Mfp-3- GvpA; 3) Modified GvpA-Mfp-5; 4) GvpA-Mfp3:Mfp-5-GvpA

Figure 8

Adhesion force of bioadhesives with silica tip: This figure demonstrates comparison of adhesion force (mNm⁻¹) between mussel-inspired adhesives: Unmodified and modified GvpA-mfp-3, mfp-5-GvpA and (GvpA-Mfp3)-co-(Mfp-5-GvpA) copolymer. *** $P \leq 0.001$, **** $P \leq 0.0001$

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Adhesion force of (Gvp-A-Mfp3)-co-(Mfp-5-GvpA) copolymer at different pH values: As shown in this figure, in pH=5(Yellow Colour) and pH=7(Green Colour) copolymers have the most force adhesion respectively.

Figure 10

Comparison of adhesion force between copolymer hybrids (GvpA-Mfp3:GvpA-Mfp-5) with 3 ratios. molar ratio, 5:5(157.1937 mN/m);3:7(47.8312 mN/m); 7:3(43.5175mN/m)

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