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Supramolecular β -Sheet Suckerin Based Underwater Adhesives

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Abstract

Nature has evolved several molecular strategies to ensure adhesion in aqueous environments, where artificial adhesives typically fail. One recently unveiled molecular design for wet-resistant adhesion is the cohesive cross- β structure characteristic of amyloids, complementing

the well-established surface-binding strategy of mussel adhesive proteins based on 3,4-L-Dihydroxyphenylalanine (Dopa). A family of structural proteins that self-assemble into cross- β -sheet networks are the suckerins discovered in the sucker ring teeth of squids. Here, we shed light on the wet adhesion of cross- β motifs by producing recombinant suckerin-12, naturally lacking Dopa, and investigating its wet adhesion properties. Surprisingly, the adhesion forces measured on mica are as high as *ca.* 70 mN m⁻¹, exceeding those measured for all mussel adhesive proteins to date. The pressure-sensitive adhesion of artificial suckerins is largely governed by their cross- β motif, as evidenced using control experiments with disrupted cross- β domains that result in almost complete loss of adhesion. We also incorporate Dopa in suckerin-12 using a residue-specific incorporation strategy that replaces Tyrosine with Dopa during expression in *Escherichia coli*. Although the replacement does not increase the long-term adhesion, it contributes to the initial rapid contact and enhances the adsorption onto model oxide substrates. Our findings suggest that suckerins with supramolecular cross- β motifs are promising biopolymers for wet-resistant adhesion.

1. Introduction

Wet-resistant synthetic bioadhesives have attracted growing interest in the past decade, with a main objective to create adhesives that combine biocompatibility, processability, and ability to retain adhesive functionality in wet environments.^[1] The development of such bioadhesives is particularly relevant for restorative applications and tissue engineering, where there is a strong need for adhesive materials that can efficiently attach to both hard and soft tissues.^[2] Because nature abounds of organisms that have mastered the ability to attach to immersed or hydrated surfaces,^[3] efforts are increasingly aiming at mimicking the molecular strategies unveiled in the adhesive systems of organisms such as mussels,^[1, 4] sandcastle worms,^[5] barnacles,^[6] or slugs.^[7]

Several synthetic adhesives such as cyanoacrylates, resorcinol and formaldehyde have been considered for tissue adhesion due to their strong adhesiveness, high mechanical strength, and rapid curing. However, their cytotoxicity makes them unsuitable for medical applications.^[8] In contrast, protein-based adhesives such as fibrin and gelatin have good biocompatibility but their application is limited by their relatively low adhesion strength, in particular because they are often processed in the form of cross-linked hydrogels containing a large amount of water that hinders adhesion.^[9] Polyethylene glycol-based adhesives provide strong adhesion but their usage is limited by their hypersensitivity reactions when applied topically, including urticaria and delayed allergic reactions.^[10]

Among protein-based bioadhesives, the non-canonical amino acid 3,4-Dihydroxyphenylalanine (Dopa) first discovered in mussel adhesive proteins has attracted a strong interest, and multiple approaches are being explored to introduce the Dopa moiety in synthetic adhesives.^[10d, 11] In recent developments, the complex biochemistry of mussel adhesive proteins has highlighted that relying on Dopa alone is not sufficient to engineer effective wet-resistant adhesives, because underwater adhesion is facilitated by synergistic

effects of multiple molecular mechanisms.^[4b, 12] For instance, Dopa undergoes auto-oxidation to the less adhesive quinone moiety at neutral pH^[12c] and Dopa-based biomimetic adhesives should be designed to reduce the resulting loss of surface-binding ability. Moreover, it was recently highlighted that Dopa and other non-catecholic aromatic residues (Phenylalanine, Phe and Tyrosine, Tyr) can form strong protein-protein cross-links with positively charged amino acids via cation- π interactions.^[12b, 13] An adhesive protein film at the interface between two surfaces typically comprises a random network of polymer-like segments connected to each other (*e.g.*, via cation- π cross-links) or to the surfaces (*e.g.*, via adsorption of Dopa). Surface adhesion ultimately depends on the density and strength of the bonds that must be broken to separate the surfaces, generating failure planes at the surfaces^[12b] and fractures within the protein layer.^[14] The interplay between surface bonds and crosslinks provides additional opportunities to engineer wet adhesives at the molecular level, *e.g.*, to direct micro-fracture within the protein layer where cohesive molecular domains can provide additional mechanical dissipation.^[7b, 15]

One type of nanostructures that has been recognized to yield adhesive properties are functional amyloids, which are implicated in the adhesion of many marine bacteria, as well as in fungi, invertebrates, algae, or barnacles.^[16] Amyloids are proteinaceous materials consisting of ordered cross β -sheet structures.^[17] The cross- β molecular design, comprised of a large number of repeating intermolecular β -strands, is sought to provide cohesive strength, and amyloid-based adhesives have thus opened up new avenues to produce wet-resistant bioadhesives, with recent studies highlighting the importance of self-assembly in enhancing adhesion strength. For example, self-assembled barnacle cement protein 19k enriched in β -sheets were recently shown to exhibit stronger adhesion than the same protein in the unassembled state.^[18]

Figure 1. Schematic illustration of supramolecular self-assembled β -sheets. **a)** Suckerin-12 and **b)** suckerin-12-Dopa with a residue-specific incorporation of Dopa. Red stars depict the Dopa residues in suckerin-12-Dopa. **c)** Molecular sequence design of Suckerin-12. Ala, Ser, Thr, Val, and His-rich modules are highlighted in red, Gly and Tyr-rich modules are in blue.

In one study, Zhong *et al.* combined Dopa chemistry with the adhesive properties displayed by amyloidogenic curli fibers secreted by *Escherichia coli* (*E. coli*), and produced an adhesive by recombinant expression of a fusion protein containing both mussel adhesive proteins and curli domains.^[19] The latter assembled into cross- β amyloid fibrils hypothesized to favor underwater adhesion due to the increase in the interfacial contact area of the fibrils, the high cohesive strength associated with the cross- β supramolecular structure, as well as the hydrophobicity that may protect Dopa against auto-oxidation. Combining both Dopa and curli adhesive motifs allowed the authors to enhance underwater molecular-scale adhesion as measured using Atomic Force Microscopy (AFM). In this design, Dopa functionality was

1 obtained post-expression by subjecting the fusion protein to tyrosinase enzyme that converted
2 Tyr to Dopa residues. Furthermore, the two domains of the fusion proteins were spatially
3 segregated.

4 Since Dopa side-chains and cross- β secondary structure both appear to enhance wet
5 adhesion, the first objective of the present work was to produce a strong underwater adhesion
6 by designing a protein polymer whereby the two adhesive motifs were integrated in a modular
7 fashion and not separated in two large, segregated blocks. The second objective was to directly
8 incorporate the post-translated Dopa instead of Tyr in the protein during the translation process
9 inside *E. coli* cells, thereby avoiding the complexity associated with the multi-step procedure
10 required to convert Tyr to Dopa enzymatically *in vitro*, *i.e.*, including tyrosinase
11 immobilization and treatment, and purification of the modified protein. To reach these
12 objectives, we selected the recently discovered suckerin proteins as the foundation for an
13 adhesive for the following reasons. Suckerins, which constitute the sole building blocks of the
14 hard sucker ring teeth (SRT) lining the arms and tentacles of squids, exhibit a block co-polymer
15 molecular architecture that self-assembles into a semi-crystalline network made of Tyr-rich
16 amorphous domains intervened by nano-sized, amyloid-like β -sheets.^[20] Because the
17 amorphous domains are enriched with Tyr, we reasoned that Dopa could substitute Tyr during
18 expression by a residue-specific incorporation strategy^[21] based on an *E. coli* auxotrophic
19 strain,^[22] during which Tyr is depleted from the media and substituted by Dopa (**Figure 1**).

20 Together with the β -sheet containing domains, we adopted a two-pronged approach in which
21 the adhesive propensity of both Dopa and cross- β amyloids would be exploited. The suckerin
22 (suckerin without Dopa) and Dopa-containing suckerin (suckerin-Dopa) were successfully
23 produced and their adhesive properties investigated using complementary surface adsorption
24 and nanomechanical measurements, including Quartz Crystal Microbalance (QCM),
25 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), Atomic

Force Microscopy (AFM), and Surface Forces Apparatus (SFA). We found that suckerin without Dopa could mediate strong underwater adhesion ($W_{\text{ad}} \sim 15 \text{ mJ m}^{-2}$), and cohesion ($W_{\text{co}} \sim 9 \text{ mJ m}^{-2}$), which exceeds those of the strongest underwater adhesion of the mussel adhesive protein (mfp-5) measured with the SFA.^[11i, 23] Although Dopa did not significantly increase further the strong adhesion of suckerin-12, it did enhance irreversible adsorption to model oxide substrates. Our results suggest that the cross- β sheet structural motif is the main contributor to the high adhesion of recombinant suckerin. Given the established non-cytotoxicity of suckerins,^[24] this work establishes the potential of recombinant suckerins as a bioadhesive for hard and soft tissue repair.

2. Results and Discussion

2.1. Production and characterization of suckerin-12 and Dopa-incorporated suckerin-12

Suckerin-12 has a block co-polymer primary structure that self-assembles into a semi-crystalline network made of Gly- and Tyr-rich amorphous domains intervened by nano-sized, amyloid-like β -sheets (see Supporting Information for the full-length sequence of suckerin-12). Thirty-five Tyr residues in suckerin-12 were intended to be replaced with the non-canonical Dopa through the *in vivo* residue specific incorporation. Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) results of total cell extracts after expression of suckerin-12 and suckerin-12-Dopa are shown in **Figure 2a**. Both proteins were mostly expressed in the inclusion body fractions with similar yields. Following purification, the yield of suckerin-12 and suckerin-12-Dopa were calculated as 20 mg L^{-1} and 25 mg L^{-1} , respectively. Amino acid composition analysis of purified suckerin-12s closely matched the predicted composition of the suckerin-12s primary sequences (**Table S1 and S2**, Supporting Information). The purity of the suckerins was above 95% based on the amino acid composition analysis and SDS-PAGE gel image analysis. To verify that Dopa was incorporated into suckerin-12, we employed nitroblue tetrazolium (NBT) and Arnow stain assays, which detect redox-active residues in

proteins such as Dopa (**Figure 2b and S1**, Supporting Information). Suckerin-12-Dopa was NBT-positive on the SDS-PAGE gel, whereas suckerin 12 was NBT-negative. Furthermore, quantitative amino acid analysis confirmed the depletion of Tyr and the apparition of a Dopa peak in suckerin-12-Dopa (**Figure S2, Table S1 and S2** Supporting Information). The molecular weights obtained by Matrix Assisted Laser Desorption-Time of Flight mass spectrometry (Maldi-ToF) for suckerin-12 and suckerin-12-Dopa were 24,534 Da and 25,071 Da, respectively (**Figure 2c**). Therefore, Dopa incorporation yield in suckerin-12-Dopa was *ca.* 98% based on Maldi-ToF spectra, but lower at *ca.* 88% based on the ratio of Dopa/Tyr measured by amino acid analysis, which is attributed to partial oxidation of Dopa during acid hydrolysis prior to amino acid analysis and to trace Tyr in the tyrosine auxotroph.^[25] For subsequent underwater adhesion measurements, suckerin-12 and suckerin-12-Dopa were always kept under argon atmosphere and dark conditions prior to measurements in order to minimize Dopa oxidation.^[26]

Circular dichroism (CD) spectra were used to assess the secondary structure of suckerin-12 and suckerin-12-Dopa (1 and 6 mg mL⁻¹ in 5% acetic acid) in solution. At 1 mg mL⁻¹, the spectra exhibited random-coil structure with a minimum at 195–205 nm, and at 6 mg mL⁻¹ the spectra exhibited a minimum at 220 nm and a maximum at 210 nm, which are the spectral signatures of β -sheet structures (**Figure 3**). These data indicate that there was a conformational switch observed at higher protein concentration (6 mg mL⁻¹), which corroborates previous results obtained with recombinant suckerin-19.^[27] In addition, the overall secondary structure of suckerin-12 remained similar after the incorporation of Dopa. These results confirm that both suckerin-12 and suckerin-12-Dopa refolded into β -sheet enriched structures in 5% acetic acid at 6 mg mL⁻¹, which was further confirmed by ATR-FTIR measurements presented in Section 2.2.

Figure 2. Protein expression profiles of recombinant suckerin-12 and suckerin-12-Dopa obtained by residue-specific incorporation of Dopa into suckerin-12. **a)** SDS-PAGE gel stained with Coomassie blue and **b)** redox-cycling NBT staining, confirming the presence of L-Dopa in suckerin-12-Dopa. Maldi-ToF MS spectra of **c)** suckerin-12 (24,314 Da) and **d)** suckerin-12-Dopa (24,874 Da), purified from recombinant expression. See also Figures S1 and S2 for further confirmation of Dopa incorporation by Arnow staining and amino acid analysis.

Figure 3. Secondary structures of suckerin-12 and suckerin-12-Dopa as determined by circular dichroism spectroscopy in different concentrations in 5% acetic acid.

2.2. Adsorption of suckerin proteins on oxide surfaces

In order to study the adsorption behavior of suckerin-12 and suckerin-12-Dopa on oxide surfaces, we performed adsorption measurements with silicon dioxide (SiO₂) and titanium dioxide (TiO₂) surfaces by QCM and ATR-FTIR. Resonance frequency change during QCM experiments is directly correlated to mass adsorption changes. Suckerin-12-Dopa displayed greater adsorption on both TiO₂ ($\Delta f_{\text{ads}} = 11.4$ Hz) and SiO₂ surfaces ($\Delta f_{\text{ads}} = 9.2$ Hz) than suckerin-12 ($\Delta f_{\text{ads}} = 5.2$ Hz and 5.9 Hz on TiO₂ and SiO₂ surfaces, respectively) (**Figure 4a, b**). After washing, both proteins showed negligible desorption (notably, lower than mussel adhesive proteins^[28]) indicating strong binding to oxide surfaces for both proteins.

ATR-FTIR spectra were obtained from 0.1 mg mL⁻¹ solutions of both proteins in 5% acetic acid on a ~100 nm thick TiO₂ nanoparticle film (**Figure 4c,d**). For both proteins, spectra were characterized by strong absorbance at ~1623–1641 cm⁻¹ assigned to the amide I region, as well as peaks at ~1520 cm⁻¹ corresponding to the amide II region (**Figure S3**, Supporting Information). The semi-quantitative evaluation of proteins secondary structure was determined by deconvolution of the amide I band, which is very sensitive to proteins' conformation.^[29] The ATR-FTIR spectrum showed bands at 1652 and 1626 cm⁻¹ that originate from random coils and β -sheets, respectively. The changes in amide I band can be associated to structural rearrangement of adsorbed suckerin-12 and suckerin-12-Dopa. Furthermore, a band at 1638 cm⁻¹ assigned to β -sheets appeared and its intensity increased over time. Concurrently the intensity of the band at 1652 cm⁻¹ decreased, indicating that Dopa incorporation in suckerin-12-Dopa did not disrupt the formation of cross- β amyloids upon surface adsorption. Together, these trends indicate an increase in β -sheets and concomitant decrease in random coil upon adsorption of both suckerin proteins.

Figure 4. Adsorption of suckerin-12 and suckerin-12-Dopa assessed by QCM and ATR-FTIR. QCM plots for the adsorption of suckerin-12 and suckerin-12-Dopa on **a)** SiO₂ and **b)** TiO₂ surfaces. ATR-FTIR spectra of adsorbed **c)** suckerin-12 and **d)** suckerin-12-Dopa on TiO₂. Spectra were obtained at 2 min intervals from 0.1 mg mL⁻¹ solution adsorbed on a TiO₂-coated diamond prism. The 3750–3300 cm⁻¹ and 3300–2900 cm⁻¹ wavenumber regions correspond to liquid-like and surface-bound water, respectively.

ATR-IR spectra of adsorbed suckerin-12-Dopa on TiO₂ also revealed spectral features of OH groups (νOH stretching mode in the ~3800–2800 cm⁻¹ region) that parallel those previously observed for Dopa-containing adhesive proteins from the green mussel *Perna viridis*.^[28] In particular, we observed that negative bands progressively developed in the ~3800–3300 cm⁻¹

and $\sim 3300\text{--}2950\text{ cm}^{-1}$ regions, which can be attributed due to dislodgment of liquid-like and surface-bound water molecules, respectively (**Figure 4d**). For suckerin-12, the band associated with liquid-like (bulk) water also decreased but the removal of surface-bound water was not observed (**Figure 4c**). These data therefore show that suckerin-12-Dopa is more efficient than suckerin-12 to displace interfacial water molecules from a wet hydrophilic surface (TiO_2), probably due to the bidentate coordinative complex formation of Dopa and TiO_2 .^[30]

2.3. Wet adhesion of oxide surface coated with suckerin-12 proteins

Next, we assessed the adhesion and cohesion behavior of suckerin-12 variants with both SFA and AFM measurements. Two types of SFA experiments were conducted, namely in the symmetric mode for cohesion, whereby both mica surfaces were coated with an adsorbed layer of suckerins, and the asymmetric mode for adhesion when only one mica surface was coated (see insets of **Figure 5**). For all measurements, the surfaces were brought into contact in acidic buffer (0.1 M acetic acid, pH 3.0), contacted from 2 min to 1 h, and then separated at a rate of $\sim 5\text{ nm s}^{-1}$. The adsorption of suckerin-12 and suckerin-12-Dopa to mica was confirmed by the increase in the steric wall distance (D_{sw} , defined as the confined protein film thickness under the normal load or pressure, $F/R \sim 20\text{ mN m}^{-1}$). The thickness of suckerin films, as measured by D_{sw} , was *ca.* 4.3 nm, which is similar to values of the β -sheets domains of native suckerins ($\sim 2.5 \times 3.3\text{ nm}$) reported in a previous study.^[20c] Since the film thickness did not depend on the amount of Dopa in the protein, adsorption appeared to be driven by protein-surface interactions that did not involve Dopa, most likely electrostatic interactions between positively charged suckerins at pH 3.0^[20c] and the negatively charged mica surfaces.

In the asymmetric mode, strong adhesion between mica and suckerin was initially measured at contact time, $t_c = 2\text{ min}$. Suckerin-12-Dopa exhibited higher adhesion to mica ($F_{\text{ad}}/R = -35.25 \pm 0.99\text{ mN m}^{-1}$) than that of suckerin-12 ($F_{\text{ad}}/R = -22.14 \pm 4.30\text{ mN m}^{-1}$).

However, as t_c increased from 2 min to 1 h, the maximum adhesion was measured for suckerin-12 ($F_{ad}/R = -67.73 \pm 9.47 \text{ mN m}^{-1}$), whereas for suckerin-12-Dopa, $F_{ad}/R = -57.77 \pm 1.17 \text{ mN m}^{-1}$ (**Figure 5e**). These results suggest that the introduction of Dopa enhances the initial adsorption of suckerin-12 to oxide surfaces; however, it does not increase its long-term adhesive properties.

Cohesive forces were slightly lower in the symmetric mode compared with the asymmetric mode, with maximum cohesion forces for suckerin-12 of $F_{co}/R = -41.19 \pm 1.10 \text{ mN m}^{-1}$, whereas for suckerin-12-Dopa we measured $F_{co}/R = -46.80 \pm 0.89 \text{ mN m}^{-1}$ (**Figure 5f**). There were no significant differences in adhesive forces measured between suckerin-12 and suckerin-12-Dopa in both modes. We highlight that the mean highest adhesive force for suckerin-12 in the asymmetric mode after 1 h of contact time ($F_{ad}/R = -67.73 \pm 9.47 \text{ mN m}^{-1}$) is similar to that of the most adhesive Dopa-rich mussel adhesive protein in the same experimental setup, namely mfp-5 for which maximum forces of $F_{ad}/R = -64.9 \text{ mN m}^{-1}$ have been reported.^[23] However in the symmetric mode, suckerin-12 exhibits adhesive forces roughly 4-fold higher than mfp-5 in the same experimental conditions.^[23] We also measured higher maximum adhesive forces as the contact time increased from 2 min to 1 h in all cases (**Figure 5**), indicating that both suckerin-12 and suckerin-12-Dopa favorably developed molecular reorientation or rearrangement, which are typical signatures of pressure-sensitive adhesives.^[11h, 31] Furthermore, the higher adhesive forces of suckerin-12 at longer contact times correlated with decreased steric wall thickness D_{sw} , from 13.7 nm at $t_c = 2 \text{ min}$ to 6.9 nm at $t_c = 1 \text{ h}$, which corroborates the molecular rearrangement of pressure-sensitive adhesives.^[32]

In both asymmetric and symmetric modes, suckerin-12 exhibited adhesive forces that were equivalent to those from suckerin-12-Dopa, suggesting that adhesion is mediated by molecular mechanisms beyond those uncovered in Dopa-containing adhesives.^[1, 11k] Recent SFA studies have revealed that cation- π interactions based on Dopa, Tyr, or Phe residues can enhance wet

adhesion.^[11j, 12b, 13] Since our suckerin constructs are enriched in aromatic residues, a similar interfacial adhesion enabled by π interactions (e.g., π - π stacking, cation- π interaction) is also plausible and would be efficient with both Dopa and Tyr-based suckerins. However, we surmise that a different mechanism is predominant here. Since suckerins are amyloidogenic with a self-assembly behaviour mediated by β -sheet formation, this supramolecular network may lead to a cohesive type of failure associated with the unraveling of intermolecular β -sheets domains, which would also be consistent with the higher forces measured in the symmetric case. To verify this hypothesis, we measured the adhesion under 2 M urea, a well-known chaotropic agent that can unfold the nanoconfined β -sheets domains in aqueous buffers.^[20c] Under these conditions, adhesion was indeed completely lost for suckerin-12 in both modes. Adhesion measurements after β -sheet disruption in the symmetric mode also suggests a cohesive type of failure mediated by β -sheets. Additionally, we introduced 1 nmol of sodium periodate (NaIO_4) in the buffer solution to assess the effect of Dopa oxidation. In the case of asymmetric mode of suckerin-12-Dopa, the adhesive force decreased from $F_{\text{ad}}/R = -57.77 \pm 1.17 \text{ mN m}^{-1}$ to $-16.11 \pm 1.45 \text{ mN m}^{-1}$ when it was treated with NaIO_4 . Furthermore, both repulsive interactions and D_{sw} increased after the NaIO_4 treatment during the surface approach. The repulsive force for cohesion generated from a distance of $\sim 120 \text{ nm}$ and D_{sw} was almost 1.8-fold higher than before Dopa oxidation at 20 mN m^{-1} compression (**Figure S5**, Supporting Information), which can be attributed to a reduced flexibility of the peptide backbone upon oxidation of Dopa to Dopa-quinone.^[33] Furthermore, we performed ATR-FTIR measurements of suckerin-12 and suckerin-12-Dopa (6 mg mL^{-1}) in the absence or presence of 2 M urea to verify unfolding of β -sheets. Without urea, the Amide I band for both suckerin-12 and suckerin-12-Dopa exhibited peaks at 1630 and 1641 cm^{-1} characteristic of β -sheets. Upon treatment with urea, the intensity of these peaks strongly decreased whereas a peak at 1652 cm^{-1}

¹ appeared, confirming the secondary structural change towards random coil (Figure S4, Supporting Information).

Figure 5. Force vs. distance profiles at different contact times (t_c) measured using SFA as a function of surface separation distance. **a)** Suckerin-12 vs. mica. **b)** Suckerin-12 vs. suckerin-12. **c)** Suckerin-12-Dopa vs. mica and **d)** Suckerin-12-Dopa vs. suckerin-12-Dopa. Normalized adhesion force and energy as a function of t_c in the **e)** asymmetric and **f)** symmetric modes for suckerin-12 and suckerin-12-Dopa. The addition of 2 M urea for suckerin-12 and 1 nmol NaIO₄ for suckerin-12-Dopa was conducted by

injecting the solution into the gap between asymmetric or symmetric surfaces. The error bars represent the s.e.m. (standard error of mean), where $n = 3$ in each group.

A previous study that combined Dopa chemistry with amyloidogenic curli fibers^[19] showed that this protein exhibited strong adhesion exceeding that measured here for suckerin-12, though the values are not directly comparable since they were measured with two different techniques, namely colloidal probe AFM for curli fibers and SFA in our study. The difference might be due to the fibrillar structure of curli-based fibers that may provide a larger surface area for interfacial adhesion. While investigations of the curli-based adhesive suggested that the amyloid motif may play a role in adhesion, there were no direct experimental evidences linking the cross- β sheet structure with adhesion. In contrast, the present study unambiguously establishes the important contribution of cross- β sheet structure to wet-resistant adhesion.

In AFM based force measurements, the uncoated silicon nitride (Si_3N_4) probe was pushed into an adsorbed suckerin layer initially, eventually reaching the underlying TiO_2 substrate (*i.e.*, at probe-substrate separation distance, $D = 0$, **Figure S6a**, Supporting Information). Upon retraction of the probe from the hard-wall contact, a typical AFM force *vs.* distance curve showed multiple adhesive peaks, *i.e.*, negative force minima followed by a sudden decrease of force magnitude and a jump in separation distance (**Figure S6a**, Supporting Information). The peaks were irregularly spaced over a distance range reaching $D = 250$ nm and had different force magnitudes. Assuming a distance of 0.38 nm between consecutive α -carbons along the amino acid sequence (see Supporting Information), the extended (contour) length of a suckerin protein is $l_c \approx 90$ nm. Considering that much of this length is packed into β -sheet domains with nanometer size (**Figure 1a**), force minima observed at $D > l_c$ reveal a significant level of protein aggregation within the adsorbed layer. Adhesion is thus attributed due to the progressive stretching of flexible polymer-like segments in a random network of proteins,

1 eventually leading to protein bond rupture.^[14-15] The network is both cross-linked (*e.g.*, via
 2 cation- π bonds or amyloidogenic interactions inducing β -sheet formation) and attached to the
 3 Si_3N_4 probe and TiO_2 substrate (*e.g.*, Dopa-surface bonds). Adhesive peaks were due to the
 4 rupture of protein bonds, either a protein-protein cross-link or protein-surface bond. The
 5 maximum adhesive force F_0 on the order of 0.1–1.0 nN is consistent with the rupture of single
 6 bonds,^[11e, 14] whereas the adhesive energy $W = -\int FdD$ characterizes the adhesive properties
 7 of the protein network, cumulating the effect of breaking multiple bonds of different types.
 8 Both F_0 and W showed a large variation among different measurements, even when all
 9 experimental parameters such as AFM tip velocity, or dwell time and maximum load at contact
 10 were kept constant (**Figure S6c,d**, Supporting Information). In comparison, the force curves
 11 obtained for uncoated TiO_2 showed a single adhesive peak at a short distance $D < 1$ nm due to
 12 Van der Waals attraction between the probe and TiO_2 substrate (**Figure S6b**, Supporting
 13 Information), and the values of F_0 and W were distributed over narrower ranges (**Figure S6c,d**,
 14 Supporting Information). Analysis of the values obtained before and after absorbing the protein
 15 layer on the TiO_2 substrate showed that suckerin-12-Dopa significantly increased both F_0 and
 16 W relative to uncoated substrates, whereas suckerin-12 produced a smaller increase of F_0 and
 17 did not significantly increase W (**Figure 6** and **Figure S6c,d**, Supporting Information).
 18 Analysis of the values obtained before and after absorbing the protein layer on the TiO_2
 19 substrate showed that suckerin-12 and suckerin-12-Dopa increased both F_0 and W relative to
 20 uncoated substrates (**Figure 6** and **Figure S6c,d**, Supporting Information). However the
 21 increase was significantly smaller for suckerin-12, namely $\Delta F_0 = (83.8 \pm 3.0)$ pN and $\Delta W =$
 22 $(6.9 \pm 1.6) \times 10^{-20}$ J, than for suckerin-12-Dopa, namely $\Delta F_0 = (185.4 \pm 7.0)$ pN and $\Delta W = (53.7$
 23 $\pm 3.5) \times 10^{-20}$ J. The presence of Tyr instead of Dopa is expected to weaken surface-protein
 24 bonds, particularly on the TiO_2 substrate,^[30] whereas it marginally increases cation-
 25 π interactions^[12b] and does not affect β -sheets domains. Therefore suckerin-12-Dopa produced

1 a larger adhesion than suckerin-12 in AFM experiments most likely because AFM-based force
2 measurements involved the breaking of Dopa titania bonds that were absent for Tyr-containing
3 suckerin-12. In comparison, Dopa did not increase the average adhesion measured in SFA
4 experiments, either because protein-surface bonds did not involve Dopa (*e.g.*, proteins were
5 adsorbed via electrostatic attractions) or because Dopa-surface bonds were not broken during
6 the measurement, at the expense of other and possibly weaker bond types.

7 Marine mussels have been the archetype model living organisms for Dopa-mediated wet
8 adhesion.^[1] Because most mussel adhesive proteins are viewed as flexible polyelectrolytes with
9 a high fraction of intrinsically disordered domains,^[34] the role of Dopa in mussel underwater
10 adhesion has mostly focused on its biochemical aspect.^[11] Dopa is a post-translationally
11 modified amino acid which is chemically unstable: it quickly oxidizes into reactive
12 dopaquinone in the presence of oxygen or at basic pH. As the result, two additional steps,
13 namely enzymatic reaction for Dopa conversion and thiol-rich reducing environments for Dopa
14 protection, are mandatory for mimicking Dopa mediated mussel adhesion via protein
15 engineering.^[12c] Compared to adhesion of Dopa-containing mussel proteins, the most
16 distinctive feature of suckerins is their ordered cross β -sheet structures, an aspect which proved
17 central for the wet adhesion of suckerins in this study. Adhesion of the cross β -sheet-enriched
18 structure of suckerin-12 exceeds that of the stickiest protein in mussel adhesion, mfp-5.^[1, 23]
19 Considering the instability of Dopa in the presence of oxygen or at basic pH,^[13] the cross β -
20 sheet structure of suckerins could be utilized as a complementary protein engineering strategy
21 for mimicking biological adhesion with enhanced chemical stability.

Figure 6. AFM measurements. **a)** Maximum adhesive force, F_0 and **b)** adhesion energy, W , for suckerin-12-Dopa and suckerin-12. The values are compared to control measurements on bare TiO₂ substrates to account for the variability of AFM tip shape, affecting the number of molecules and adhesive interactions probed in an AFM measurement. Error bars show the s.e.m. of the samples. Differences between average values indicated with asterisks were statistically significant, with probability $p < 0.0001$ for the null-hypothesis given by the t -test.

3. Conclusion

In summary, suckerins and Dopa-containing suckerins were produced in *E. coli*, whereby Dopa was incorporated via a residue-specific incorporation strategy during expression, with the goal to obtain an underwater adhesive that synergistically exploits both the cross- β amyloid structural motif of suckerins as well as the Dopa chemistry of mussel adhesive proteins. Our results show that regular suckerin-12 (free of Dopa) exhibits an underwater adhesive

performance on par with the strongest mussel adhesive protein on mica in the asymmetric mode and exceeds it in the symmetric mode, whereas Dopa incorporation to suckerin enhances instantaneous adhesion and adsorption to wet substrates. The cross- β amyloid motif of suckerins provides strong, reversible, pressure-sensitive and self-healing properties. Reversibility is inferred from repetitive adhesion observed in cyclic loading/unloading runs at the same contact point, whereas the pressure-sensitive and self-healing properties are deduced from the increase in adhesive forces with contact time attributed to molecular re-arrangement.^[32] Despite not significantly enhancing long-term adhesive forces, Dopa in suckerin-12-Dopa can serve as a cross-linker during maturation of the underwater adhesive. The approach opens up interesting opportunities in biomolecular materials design through direct incorporation of Dopa into recombinant adhesive proteins, and could be applied towards protein materials used in biomedical applications, such as elastin-like peptides or resilin.

4. Experimental Section

Cloning, Expression and Purification of Suckerin Proteins: Unmodified recombinant suckerin-12 (suckerin without Dopa) was produced in *E. coli* as previously described.^[27] Suckerin-12 was cloned into pQE-80L using *Bam*HI and *Hind*III restriction enzymes. For the residue-specific incorporation of Dopa the tyrosine auxotroph stem *E. coli* JW2581 (purchased from *E. coli* Genetic Stock Centre, Yale University, New Haven, USA) was transformed with the generated plasmid. The above-mentioned construct was grown in M9 minimal medium supplemented with 0.4% (w/v) of glucose, 0.1 mM of calcium chloride, 1 mM of magnesium sulfate, 35 $\mu\text{g mL}^{-1}$ of thiamine, 20 amino acids (40 mg L⁻¹), 100 $\mu\text{g mL}^{-1}$ ampicillin and 100 $\mu\text{g mL}^{-1}$ kanamycin. When the optical density of the culture reached 0.8 to 1.0 at 600 nm, cells were centrifuged for 10 min (5,000 rpm) at 4 °C. The cell pellets were washed twice with 0.9% (w/v) of sodium chloride (NaCl) solution. The cells were then re-suspended in M9 minimal medium supplemented with 19 amino acids (40 mg L⁻¹) lacking tyrosine. The resuspended cells

1 were added to different test tubes containing M9 minimal medium containing L-tyrosine (40
2 mg L⁻¹) as a positive control, Dopa (1 mM) as a test. Reducing agents such as 100 µM
3 Dithiothreitol (DTT) and 1 mM ascorbic acid were added to the medium supplemented with
4 Dopa for minimizing dopa auto-oxidation during fermentation. After incubation at 37 °C for
5 10 min to deplete intracellular tyrosine pools, 1 mM of isopropyl β-d-1-thiogalactopyranoside
6 (IPTG) was added to induce the protein expression and allowed to proceed for 7 h. The cells
7 were harvested by centrifugation at 4,000 rpm for 30 min and stored at -80 °C until further
8 use.^[22a, 22b] Afterwards, the cells were resuspended in 10 mL of lysis buffer (50 mM Tris at
9 pH 7.4, 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 100 mM Borate) per
10 1 g of wet cell pellet. Subsequently, the cells were disrupted by sonication in a properly fitted
11 ice bath for 10 min (pulse 2 s on and 5 s off, 0.75 amplitude) and transferred to a centrifuge
12 tube. The disrupted cells were centrifuged for 30 min at 16,000 rpm and 4 °C. The supernatant
13 was separated by decanting and the pellet was washed with 30 mL of wash buffer I (2% Triton
14 X-100, 100 mM Tris at pH 7.4, 5 mM ethylenediaminetetraacetic acid (EDTA), 2 M urea,
15 5 mM Dithiothreitol (DTT) and 100 mM Borate). Therefore, the initially sticky pellet was
16 resuspended by pipetting up and down. After centrifugation at 6,450 rpm for 10 min at 4 °C,
17 the remaining pellet was washed further two times with wash buffer I by pouring 20 mL into
18 the centrifuge tube, vortexing for 2 min, placing the whole tube for 5 min at -80 °C to destroy
19 the foam, adding the remaining 10 mL wash buffer I to the centrifuge tube and shaking for an
20 additional 2 min before centrifugation for 6,450 rpm and 10 min at 4 °C. Subsequently, this
21 procedure was applied in the same manner for three washing steps with wash buffer II (100
22 mM Tris pH 7.4, 5 mM EDTA, 5 mM DTT and 100 mM Borate). Again, the supernatant was
23 removed by decanting and the remaining pellet was extracted two times with 20 mL of 5%
24 acetic acid. Therefore, 3 min of sonication (pulse 2 s on and 5 s off, 0.75 amplitude) and 30 min
25 of centrifugation (19,000 rpm, 4 °C) were applied. The supernatants were transferred to dialysis

bags (Spectra/Por 6 Dialysis Tubing, molecular weight cut off 15,000 Da) and dialyzed against 5 L of 5% acetic acid for 48 h. Afterwards, the purified protein solution was lyophilized and stored at -80 °C under argon atmosphere. The molecular weight of the purified proteins was analyzed by Maldi-ToF. During the whole protein purification, the samples were covered with an aluminum foil and kept on ice in order to prevent oxidation of Dopa.

Staining Assays: Nitroblue tetrazolium (NBT) and sodium glycinate solutions can specifically stain for Dopa-containing proteins by catalyzing redox-cycling reactions at an alkaline pH 10. The NBT assay was thus used to confirm the successful incorporation of Dopa in place of Tyr into suckerin-12. 100 μL of 0.1 mg mL^{-1} of suckerin-12 and suckerin-12-Dopa were spotted onto Protran BA83 nitrocellulose membranes (Whatman) with a dot blot manifold (Schleicher & Schuell Minifold-I Dot-Blot System). The membranes spotted with protein solutions were incubated in 20 mL of freshly made 0.6 mg mL^{-1} NBT solution in 2 M potassium glycinate buffer (pH = 10.0) at room temperature in the dark for 45 min. The membranes were washed with 10 mL 0.16 M sodium borate solution twice and soaked in another 20 mL sodium borate solution overnight.^[19, 35] For the Arnow stain assay, the membranes spotted with protein solutions were immersed in 0.5 N hydrochloric acid, followed by 1.45 M sodium nitrite/0.41 M sodium molybdate, and finally in 1 N sodium hydroxide for 5 min under dark conditions.^[36]

Circular Dichroism: The secondary structures of suckerin-12 and suckerin-12-Dopa were characterized by CD using a Chirascan spectropolarimeter (Model 420, AVIV Biomedical Inc.). Suckerin-12 and suckerin-12-Dopa were prepared at concentrations of 1 and 6 mg mL^{-1} in 5% acetic acid. Measurements were conducted in triplicates at wavelengths ranging from 190 to 260 nm, with 1 nm step size and 1 nm bandwidth at room temperature. The spectra were smoothed by the Savitzky–Golay method with a second-order polynomial.^[28]

Amino Acid Analysis: In order to determine the ratio of Tyr to Dopa and thereby to quantify the incorporation of Dopa during protein expression, a Ninhydrin-based amino acid analyzer was used (Sykam, Eresing, Germany). For sample preparation, 2 mg of purified suckerin-12 and suckerin-12-Dopa were transferred to a glass tube containing 200 μ L of 6 M HCl solution and 10 μ L 100% phenol. The tube was sealed *in vacuo* and placed in a heating block at 110 °C protected from light for 24 h. The hydrolysate was transferred to a separate glass tube and into a speed vacuum system (ScanVac, City Country, etc) and washed in two times with Milli-Q water (Milli-Q, Millipore) and one time with methanol (30 min; 2,000 rpm; 51 °C). Afterwards, the washed hydrolysate was re-solubilized in 250 μ L sample dilution buffer, transferred to a 1.5 mL tube and spun for 2 min at 13,300 rpm. The supernatant was transferred to a glass vial and stored at 4 °C until use.^[28] Since Dopa is not present in the standard solution, Dopa was independently run on the analyzer to obtain its elution time. In addition to the operating conditions recommended by the manufacturer, acidic buffer was used to separate the hydrolyzed amino acids (Buffer A, pH = 3.45 led to a poor separation between the Leu and Dopa peaks during amino acid analysis; a lower pH of 3.05 for Buffer A was used and found to enhance the separation of Leu from Dopa).

Quartz Crystal Microbalance (QCM): 5-MHz piezoelectric quartz crystals coated with SiO₂ and TiO₂ were purchased from Q-Sense (Frölunda, Sweden). The crystals were ozone treated for 10 min prior and after immersion in 2% SDS in Milli-Q water for 30 min. The cleaned surfaces were then washed thoroughly with Milli-Q water, dried with nitrogen gas and used immediately. Real-time QCM measurements were performed using a Q-Sense E4 QCM (Q-Sense, Sweden) instrument with four-channel IPC pump (Ismatec SA, Switzerland). Three cleaned crystals were mounted in the micro-fluidic chambers of the QCM. On attaining stable

resonant frequencies, proteins were injected in the four different channels, respectively, at a constant flow rate of 50 mL min^{-1} . Suckerin-12 and suckerin-12-Dopa were prepared in degassed 5% acetic acid at a concentration of 0.1 mg mL^{-1} .^[22b, 28] In these experiments, $250 \text{ }\mu\text{L}$ of 0.1 mg mL^{-1} of suckerin-12 and suckerin-12-Dopa were introduced for 2 min (adsorption step) before washing with buffer to desorb weakly bound proteins (desorption step).

Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Spectroscopy: ATR-FTIR spectra were recorded with a MIRacle accessory equipped with a three-reflection diamond prism (Pike Technologies) in an infrared spectrometer (Vertex 70, Bruker) equipped with a KBr beamsplitter and a liquid nitrogen cooled mercury cadmium telluride detector. The optical bench was continuously purged with nitrogen gas. The diamond ATR prism was coated with a $\sim 100 \text{ nm}$ thin film of TiO_2 (anatase; $< 25 \text{ nm}$ in diameter) (Sigma-Aldrich) nanoparticles by drying $50 \text{ }\mu\text{L}$ of 10 mM sonicated aqueous TiO_2 suspensions overnight in air. A volume of $40 \text{ }\mu\text{L}$ of suckerin-12 and suckerin-12-Dopa at 0.1 mg mL^{-1} were dissolved in 5% acetic acid and it were placed on the TiO_2 nanoparticle film-coated diamond prism, and ATR-FTIR spectra were collected from 128 co-added scans at 4 cm^{-1} resolution with Opus software (version 6.5). Background was from 5% acetic acid on the TiO_2 film. The spectra were corrected for the frequency dependence of the penetration depth with the ATR correction programme available in the Opus 6.5 software.^[28, 37]

Surface Forces Apparatus (SFA): A surface forces apparatus 2000 (Surface LLC, Santa Barbara, CA, USA) system was used to measure the adhesion and cohesion force of suckerin-12 and suckerin-12-Dopa proteins.^[38] To prepare protein coated-surfaces, freshly cleaved back-silvered muscovite mica (Grade #1, S&J Trading, Floral Park, NY, USA) was glued on to the cylindrical glass disk. Then the mica surface was dipped into each protein solution (0.5 mg mL^{-1}

¹ in 5% acetic acid) for 1 h and rinsed thoroughly with 5% acetic acid in order to remove unbound proteins. For adhesion force (asymmetric), one protein coated surface and one bare mica surface were transferred into the SFA chamber, whereas for cohesion force (symmetric), two protein coated surfaces were transferred into the SFA chamber with a crossed-cylinder geometry that corresponds to a sphere of radius $R \sim 2$ cm approaching a flat surface, respectively. A droplet of buffer (50 μ L, 0.1 M acetic acid, pH = 3.0) was injected into the two opposing surfaces. The SFA chamber was saturated with water vapor to prevent the buffer from evaporation during the experiments. After sealing the SFA chamber, the system was equilibrated for 1 h to adjust with room temperature. During the force measurements, the two opposing surfaces were approached with a constant speed (~ 5 nm s⁻¹ in this study) to reach a steric wall distance (D_{sw}) by a motor connected to the lower surface and kept in contact for 2, 30, 60 min to investigate the contact time (t_c) effect which can cause the molecular rearrangement at a longer contact. Then, the surfaces were separated. To investigate the effect of urea on suckerin-12 protein, 20 μ L of 2 M urea solution was injected into the gap between asymmetric or symmetric surfaces, whereas to investigate the effect of NaIO₄ on suckerin-12-Dopa, 20 μ L of 1 nmol NaIO₄ solution was injected into the gap between asymmetric or symmetric surfaces. Both solutions were prepared using 0.1 M acetic acid. We note that mfp-5 (~ 9.5 kDa) and mfp-3 (~ 5.3 kDa) contain 30% and 20% Dopa in their amino acid composition and that treatments with 1 nmol and 0.6 nmol NaIO₄ completely abolished adhesion forces for both proteins, respectively.^[23] Considering that suckerin-12-Dopa (~ 25 kDa) consists of 8.8% of Dopa, 1 nmol of NaIO₄ is in excess amount for the full oxidation of Dopa in suckerin-12-Dopa. The force and distance profile were determined through the fringes of equal chromatic order (FECO) using a multiple beam interferometry (MBI).^[39] The interaction forces (F) were measured as a function of the absolute distance (D) of surface separation and the deflection of double cantilever spring ($k = 2,304$ N m⁻¹) connected to the lower surface.^[39-40] The measured

F was converted into the interaction energies per unit area ($W = 2F/3\pi R$) between two surfaces using the Johnson-Kendall-Roberts (JKR) model for soft and deformable materials.^[39-41] All force versus distance profiles were repeated at least three times and at two different contact points for each condition to verify their reproducibility.

Atomic Force Microscopy (AFM): Lyophilized frozen suckerin-12 and suckerin-12-Dopa proteins were thawed at room temperature and dissolved at a concentration of 0.1 mg mL⁻¹ in the Milli-Q water containing acetic acid (0.02 mg mL⁻¹, pH 2–3). Flat TiO₂ substrates with an approximate area of 1 cm² were completely coated with the protein solution and sealed in a Petri dish saturated with water vapor to prevent evaporation. Protein adsorption was allowed for 20 min, after which the surfaces were abundantly rinsed with (protein-free) acetic acid solution. During the AFM experiments, the surface was completely covered with a drop of rinsing solution and the AFM probe was completely immersed in the drop. The adhesive forces generated by the adsorbed protein layers were measured using an AFM (Nanoscope III, Digital Instrument, Bruker) in force spectroscopy mode.^[42] The AFM probe had a triangular cantilever with 0.06 N m⁻¹ nominal elastic constant ending with a sharpened pyramidal Si₃N₄ tip (DNP-S10, Bruker). For each TiO₂ substrate, at least three different areas were tested and a minimum of 60 measurements were carried out in each area, before and after adsorption of the protein. The result of an AFM measurement was a force curve relating the force F to the separation distance D between the AFM probe and the TiO₂ substrate. From the curve obtained upon retracting the probe from the surface, we extracted the maximum adhesion force F_0 (i.e., the deepest minimum in the curve) and the adhesion energy $W = -\int FdD$ (**Figure S6a,b**, Supplementary Information for representative AFM force curves and definition of F_0 and W). The integral was calculated from the distance where the force first became attractive ($F < 0$) upon retraction to a large distance where the force was zero.

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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Suckerins, the building blocks of squid sucker ring teeth, self-assemble into supramolecular cross- β motifs. In this study, we demonstrate that suckerins can serve as strong wet-resistant adhesives, with an adhesive strength exceeding that of mussel adhesive proteins. The study shows that the cross- β motif is the major contributor to the strong wet-adhesion, offering new opportunities in bioinspired wet adhesives.

Keyword: underwater adhesive, suckerin, Dopa, residue specific incorporation, SFA

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Supramolecular β -Sheet Suckerin Based Underwater Adhesives

TOC illustration