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Mussel-Inspired Polymerization of Peptides: The Chemical Activation Route as Key to Broaden the Sequential Space of Artificial Mussel-Glue Proteins

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A previously introduced tyrosinase-activated polymerization of Tyr- and Cys-bearing peptides yielding artificial mussel-glue proteins is realized without the need of the specific enzyme by a chemical activation route. This decouples the sequence of polymerizable peptides (unimers) from the constraints of tyrosinase substrates and enables the polymerization of minimal motifs such as Dopa-Lys-Cys (U_{mini}^{*KC}) or Dopa-Gly-Cys (U_{mini}^{*GC}). In the polymerization procedure, sodium periodate is used to oxidize Dopa residues of the unimers to Dopaquinones to which the thiol of a Cys residue is added in a Michael-type reaction. The resulting $polyU_{mini}^{*KC}$ and $polyU_{mini}^{*GC}$ exhibit a thiol–catechol connectivity as a potent adhesive functionality at each repeat unit. QCM-D experiments show the excellent substrate adsorption properties of the products from the chemically activated polymerization. On aluminum oxide surfaces, $polyU_{mini}^{*KC}$ rapidly forms a coating, even under seawater model conditions and the coating resists rinsing with hypersaline solution of 4.2 M salt mixtures. While the sodium periodate oxidation is less specific than the tyrosinase reaction and requires the implementation of Dopa instead of Tyr residues into the polymerizable unimers, the chemical route makes scale-up more easily accessible.

Water-based adhesives for underwater or water-resistant gluing applications are still technologically challenging, since adhesion to the target surfaces might be weakened or even prevented by the surface-bound water layers.[1] In evolution, several marine animals, such as the blue mussel, adapted to this difficulty by providing an adhesion apparatus that offers universal adhesion capabilities and modulus matching.[2,3] Mussels can adhere to virtually any solid surface, including glass, plastics, steel, wood, cement, or Teflon, by using a concerted secretion deposition of a set of mussel foot proteins (mfp).[4] In these proteins, L-3,4-dihydroxyphenylalanine (Dopa) was identified as one of the most important amino acid derivatives,[5] that contributes significantly to both adhesion and cohesion (**Figure 1**a).[6] Dopa presents a catechol side chain functionality that proves remarkable interaction strengths with a broad range of different surfaces.[7] Besides that, the Dopa residues were responsible to set up and modulate cohesion in the mussel-glue, for example, by intermolecular iron complex formation[8,9] as well as covalent quinone-quinone couplings[10,11] or Michael-addition of nucleophiles to quinones.[12,13]

Advances in understanding of the functions of Dopa residues in mfps[14–16] triggered a rich set of mussel-glue inspired polymers, which present catechol functionalities on various synthetic polymers.[17–24] These mussel-glue inspired polymers proved their potentials in applications reaching from adhesives and coatings, to functionality anchors as well as to gelators for hydrogels.[25–33] Recently, the mussel-inspired polymerization (MIP) of defined oligopeptides (unimers) was described[19] to bridge the gap between mussel-glue inspired polymers, where function relies often exclusively on the implementation of catechol functionalities, and mfps with their complex functions resulting from a distinct sequence. The peptide AKPS**S**PPT**Y**KGGGC (U_1^C), which represents a derivative of the consensus sequence from mfp-1, was enzymatically activated with A*b*PPO4 tyrosinase,[34] which selectively transformed Tyr9 to Dopa-quinone by a two-step oxidation.[19] This allowed an intermolecular Michael-addition of the β-thiol functionality from the Cys14 residue to form the artificial mussel-glue protein ($polyU_1^C$). The polyaddition reaction led to high-molecular weight macromolecules with thiol-catechol-connectivities (TCCs) at each repeat unit. The TCCs were held responsible for a fast and robust adsorption onto different surfaces and superior adhesive energies have been revealed compared to the isolated mfp-1 that acted as source of inspiration. On the one hand, the use of an enzyme-triggered reaction allows highly specific oxidation of Tyr residues and on-demand formation of Dopa-functionalities from easy-to-store, stable precursors.[5,19,27,28,35] On the other hand, batch-to-batch variations of the tyrosinase enzyme and large-scale demands for materials science applications impose potential bottlenecks that make scale-up of this

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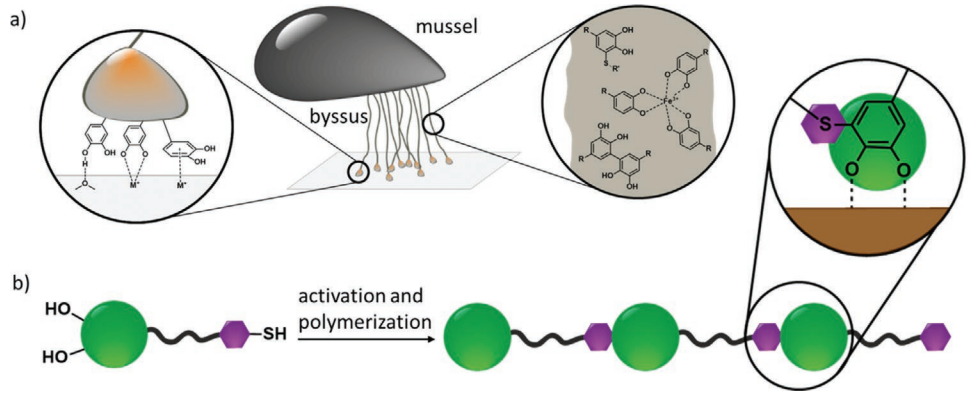

Figure 1. Illustration of the chemically activated MIP. a) The mussel adhesive process as a source of inspiration shows the relevance of Dopa residues for adhesion and cohesion. The schematic structure of the artificial mussel foot proteins (b, right) obtained by chemically activated polymerization of a peptide-based unimer with Dopa and Cys residues (b, left).

method difficult. Though combinatorial approaches have been described recently to select tyrosinase substrates for distinct tyrosinase batches,[35] the sequence selectivity of tyrosinases limits possibilities to expand the enzyme-activated MIP toward peptide sequences more suitable for technical adhesives.

Here, we present our work on expanding the MIP of Tyr- and Cys-bearing oligopeptide (polymerizable unimers) from the enzymatic strategy to a chemical activation route. The chemical oxidation requires implementing Dopa residues into the peptide unimers, which increases the efforts during unimer synthesis, but enables ease of activation by common oxidation reagents (Figure 1b). This decouples the applicable peptide unimers from the constrains of tyrosinase substrates, thus enabling to broaden the sequence space and overcoming the availability bottleneck of tyrosinase for potential scale-up of artificial mussel-glue proteins.

The peptide AKPS**S**PPT**Y**KGGG**C** (U_1^C) can be enzymatically activated with A*b*PPO4, oxidizing Tyr9 selectively to Dopaquinone, which reacted in an intermolecular Michael-type addition with thiols from Cys14 to form polyU_1^C.[19] For the chemical activation route, the peptide AKPS**S**PPT**Y***KGGG**C** (U_1^{*C}) containing Dopa9 (Y*) was synthesized by solid-phase peptide synthesis (SPPS), using the building block Fmoc-Dopa(acetonide)-OH.[36] After liberation from the support, U_1^{*C} was isolated and UPLC-ESI-MS confirmed the chemical identity (Section 4.5, Supporting Information).

Dopa residues can be oxidized effectively to the corresponding Dopa quinones by sodium periodate.[37] This was confirmed in a control experiment by oxidizing the non-polymerizable peptide Y*GG with $NaIO_4$ (Figure S8, Supporting Information). UV/Vis-spectroscopy exhibited the appearance of an absorption band at 380 nm after $NaIO_4$ addition, which confirmed the formation of quinone moieties.[38] The maximum absorption value was reached practically instantly and the intensity was not affected by 1.3 equiv. of $NaIO_4$ compared to the catechol moiety.

The chemical activation of U_1^{*C} with $NaIO_4$ leads to the formation of polymeric products (polyU_1^{*C}) under aqueous conditions. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) confirmed the polymerization by showing a broad polymer band with apparent molecular weights of 10–25 kDa ($DP_{app.}$ ≈7–18) (Figure S7, Supporting Information). The chemical structure of polyU_1^{*C} was confirmed by matrix-assisted laser desorption/ionization-time of flight MS, exhibiting a clean homologous row of the desorbable fraction with signal spacings corresponding to the repeat unit mass (Figure S6, Supporting Information). Interestingly, the polymerization product of the chemically-activated unimer shows high similarities to the products of the tyrosinase-activated process. In both cases, polymer growth is highly rapid. As given by SDS-PAGE, the product bands are not shifting further after 5–10 min and the primary polymer fraction is apparently limited to 20–25 kDa. In the enzymatic activation process, this growth limitation was explained by a lack of availability of reactive end groups for enzymatic activation and/or polymerization.[19] The results of the chemical route support the hypothesis of regulated growth by end-group availability, but suggest a reduction of the polyaddition rate as no enzyme is present in the reaction mixture.

The chemical route decouples the sequence of the polymerizable peptide-based unimers from constrains of tyrosinase substrate properties and provides high flexibility for exploiting the sequential space. For instance, peptide sequences might be used that show material-specific adhesion, biological signaling, or guide biomimetic crystallization.[39–42] Besides those complex functions, minimal adhesion motifs are of interest, because from mfps and synthetic Dopa-presenting polymers, an optimum of adhesiveness was found at about 30 mol% of Dopa.[43] To meet this criterion, a minimal unimer of the type of **Y*KC** (U_{mini}^{*KC}) tripeptide (**Figure 2**a) was synthesized by SPPS (Figure S1, Supporting Information). Polymerization of U_{mini}^{*KC} was initiated by different equivalents of $NaIO_4$ with respect to the catechol moieties (Figure 2b). The SDS–PAGE results suggested that both a substoichiometric deficit (<1 equiv.) and high excess (>3 equiv.) of $NaIO_4$ oxidation reagent hindered the desired polymerization reaction. While the former was due to incomplete activation, the latter is probably due to oxidative side reactions getting dominant.[44] Hence, further polymerizations of U_{mini}^{*KC} were performed using an excess of 1.5 equivalents $NaIO_4$ in buffered solution at room temperature. A slight excess of oxidizing agent has been chosen to avoid potential occurrence of aryloxy radical

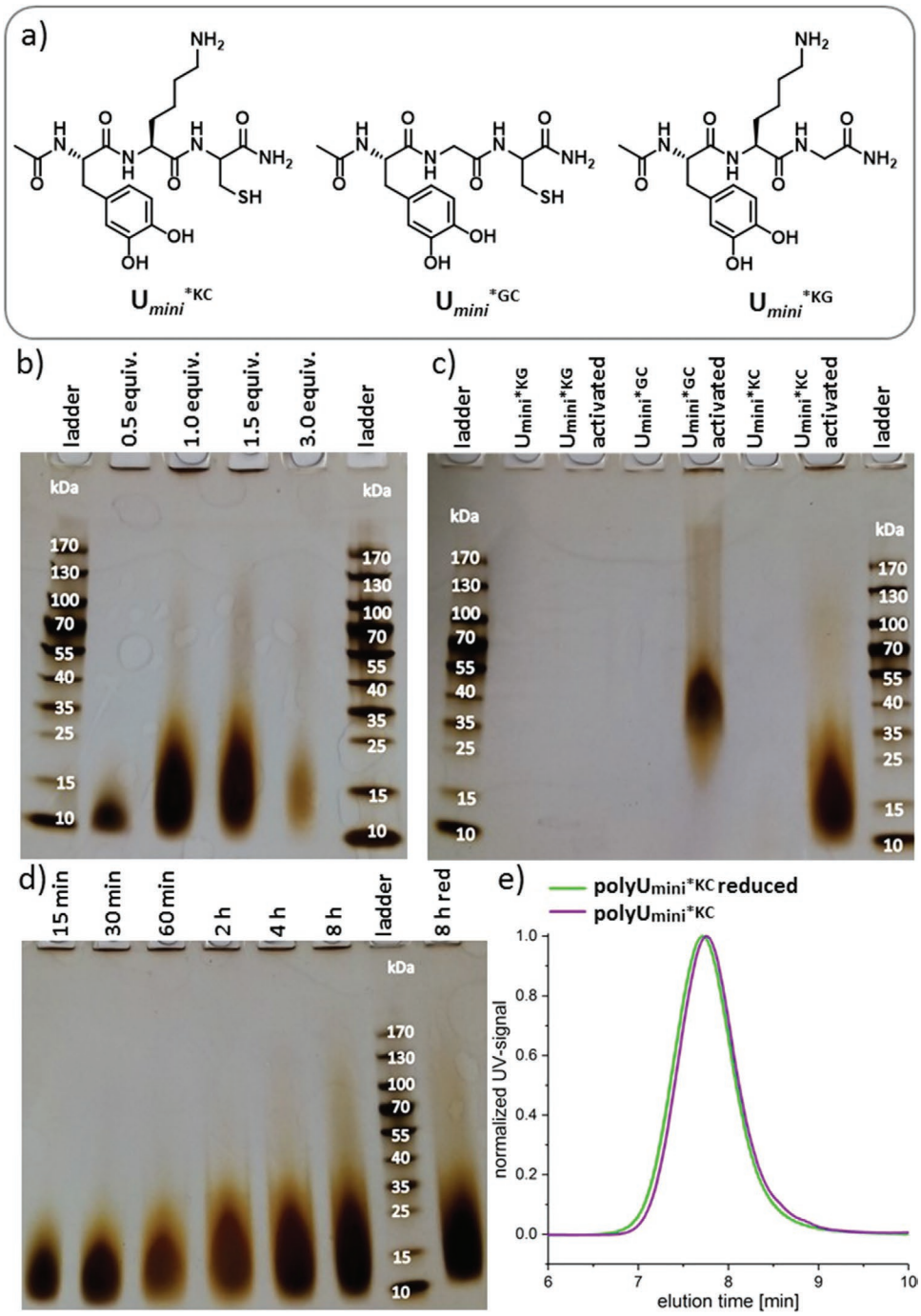

Figure 2. Investigation of the chemically activated polymerization of U_{mini}^{*KC}. a) Chemical structures of the minimal unimer U_{mini}^{*KC} as well as respective control unimers U_{mini}^{*GC} and U_{mini}^{*KG}. b) SDS–PAGE analysis of the chemical activation of U_{mini}^{*KC} using different equivalents of sodium periodate in MIP process and c) respective control polymerizations of U_{mini}^{*KC}, U_{mini}^{*GC}, and U_{mini}^{*KG} (w and w/o activation). d) Kinetic SDS–PAGE analysis of the U_{mini}^{*KC} polymerization and e) the GPC traces of polyU$_{mini}^{*KC}$ product after 4 h prior and after reduction of potential disulfide connectivities (conditions: 3.3 mM U_{mini} in ammonium acetate buffer [20 mM, pH 7]; [DOPA]:[$NaIO_4$] = 1:1.5).

coupling reactions that were described as dimerization pathways of quinones with catechols being simultaneously present in the reaction mixture.[45]

MALDI-TOF MS verified the formation of polyU$_{mini}^{*KC}$ product by showing equidistant mass signals up to the 11*mer* species with differences in m/z signals accurately corresponding to those of the U_{mini}^{*KC} repeat unit mass (Figure S14, Supporting Information). MALDI-TOF MS/MS fragmentation analysis proved the growth mechanism by showing the required y and b fragment ions, indicative of the TCC (Figure S16 and Table S1, Supporting Information). Additionally, SDS–PAGE confirmed the rapid formation of high molecular weight

polyU$_{mini}$*KC products with up to $M_{app.}$ = 25 kDa already present after 15 min reaction time (Figure 2d). The observed mass distribution increased further with time, reaching $M_{app.}$ of up to 40 kDa and 130 kDa after 2 h and 8 h reaction time, respectively. SDS–PAGE shows a second, high molecular weight fraction that remained in the injection pocket. Apparently, the molecular weight of this fraction is beyond the size exclusion limit of the PAGE gels. The occurrence of this fraction shows notable analogies to the polyU$_1^C$ product, obtained in the tyrosinase-induced peptide polymerization.[19] Probably it can be explained by the formation of branched or cross-linked structures due to secondary reaction pathways, which potentially lead to lysinyl-Dopa connectivities,[46] diDopa connectivities,[47] or multi thiol substitutions at the same catechol center.[48,49]

An alternative growth mechanism that might be considered for the chemical activation process would be the oxidative disulfide formation of β-thiols from Cys residues, which is accompanied by, for example, diDopa or lysinyl-Dopa formation to provide linear or branched polymer growth. To exclude this alternative route, the polyU$_{mini}$*KC reaction mixture was incubated with 5 equiv. of sodium dithionite with respect to [U$_{mini}$*KC] to cleave disulfide bridges.[50] SDS–PAGE showed practically no changes prior and after reduction, which proved the absence of noticeable amounts of disulfide connectivities in the backbone of polyU$_{mini}$*KC (Figure 2d).

Aqueous gel permeation chromatography (GPC) analysis was consistent with molecular weight analysis by SDS–PAGE. The GPC traces of polyU$_{mini}$*KC exhibit an apparent molecular weight of $M_{n,\,app.}$ = 14.000 ($DP_{n,app.}$ = 30), a dispersity of Đ = 1.71, and reach a maximum of about $M_{app.}$ = 150.000 ($DP_{app.}$ = 320) after 4 h polymerization (Figure 2e, and Figure S12, Supporting Information). The GPC traces of the polyU$_{mini}$*KC are showing only a negligible shift within the error of the method prior and after sodium dithionite reduction, confirming the absence of significant amounts of disulfide bridges in the backbone of polyU$_{mini}$*KC. In contrast to the SDS–PAGE results, the GPC traces of polyU$_{mini}$*KC were monomodal. The absence of a high molecular weight fraction peak suggested that the respective fraction, which could only be observed in SDS–PAGE, was most likely cross-linked and not molecularly soluble.

From MALDI-TOF MS/MS analysis of the polyU$_{mini}$*KC, it was evident that the formation of TCC linkages is the dominating polymerization pathway (Table S3, Supporting Information). To elucidate the polymerization in more detail and exclude other potentially relevant mechanisms of secondary polymer growth, for example, via lysinyl-Dopa or diDopa formation, model polymerization reactions have been performed. For that purpose, two control unimers **Y*KG** and **Y*GC** (U$_{mini}$*KG and U$_{mini}$*GC) were synthesized (Figures S2 and S4, Supporting Information and Figure 2a) and the products of different test reactions were investigated by SDS–PAGE analysis (Figure 2c). In the absence of $NaIO_4$, none of the unimers U$_{mini}$*KG, U$_{mini}$*GC, or U$_{mini}$*KC showed the formation of higher molecular weight products within 4 h. Thus, oxidation of the Dopa residues in those unimers by dissolved oxygen could be excluded as a pathway that could trigger polymerization or crosslinking. As expected, $NaIO_4$ activation leads to high molecular weight materials of both U$_{mini}$*GC and U$_{mini}$*KC, exhibiting Cys residues. It seems to be noteworthy, that no indication of a polymerization product was found for the reaction of U$_{mini}$*KG with $NaIO_4$. This allows for the exclusion of both lysinyl-Dopa and diDopa reaction pathways. Apparently, under the given conditions, these pathways are not dominating side reactions in the polymerization, even if they occur prominently in the formation of poly(dopamine) from dopamine monomers.[51]

An adsorption study of the resulting TCC-containing, artificial mussel-glue protein (polyU$_{mini}$*KC) was carried out, using quartz crystal microbalance with dissipation monitoring (QCM-D). Adsorption and desorption kinetics on aluminum oxide-coated sensors as well as the stability of resulting coatings against rinsing with buffer, saline sea water model, and hypersaline dead sea water model solutions were analyzed. The polyU$_{mini}$*KC shows a remarkably rapid adsorption from aqueous solutions onto the alumina surface, resulting in a frequency shift of Δf = –17 Hz and reaching after 5 min about 90% of the final material deposition (**Figure 3**a) . In the following 3.5 h, the frequency shift converged very slowly to about Δf = –19 Hz. The dissipation does not increase and shows no overtone dispersion, indicating the absence of viscoelastic behavior of the coating. This requires to apply the Sauerbrey model to estimate mass deposition and film thickness.[52] Taking the model into account, 340 ng cm^{-2} of polyU$_{mini}$*KC was deposited during incubation, leading to a coating thickness of approximately 2.9 nm (Equations S1 and S2, Supporting Information). Interestingly, the subsequent rinsing step with buffer resulted in further decrease of the frequency to reach –22 Hz after 2 h. This effect is not expected, but also not uncommonly observed for strong, high molecular weight binders, where coatings are formed by kinetically controlled deposition and film equilibration proceeds slower by taking up solvents/salts. The slow reswelling of the coating is accompanied by a slight increment in dispersion of the dissipation overtones, indicative of a minor increase in viscoelasticity (Figure S20, Supporting Information). At this point, it should be emphasized that despite the change in dissipation, the coating can be considered as a rather rigid film. The resulting polyU$_{mini}$*KC coating proved remarkable stability in washing experiments under intensified conditions with sea water model solution (599 mM NaCl)[53] or even 4.2 M dead sea water model solution.[54] As provided in Figure 3b and 3c, the coating resists even those rigorous washing steps with different saline solutions. Maximum mass losses of only 3% were observed, which is within the error of the method. It has to be mentioned, that the thiol/quinone-Michael addition yields polymers with TCC functionalities in the catechol oxidation state. Slight excess of $NaIO_4$ or oxygen from air might lead to a partial oxidation of the TCC groups to quinones, which have a reduced adhesion capability.[7] In principle, the addition of ascorbic acid would be a straightforward manner to reverse quinone formation and maximize the number of reduced TCC functionalities to optimize adhesiveness. However, the QCM analysis results confirmed the excellent performance of the TCC-bearing polymers obtained directly from the polymerization mixture and thus proving that a treatment with further additives is not required. This was impressively

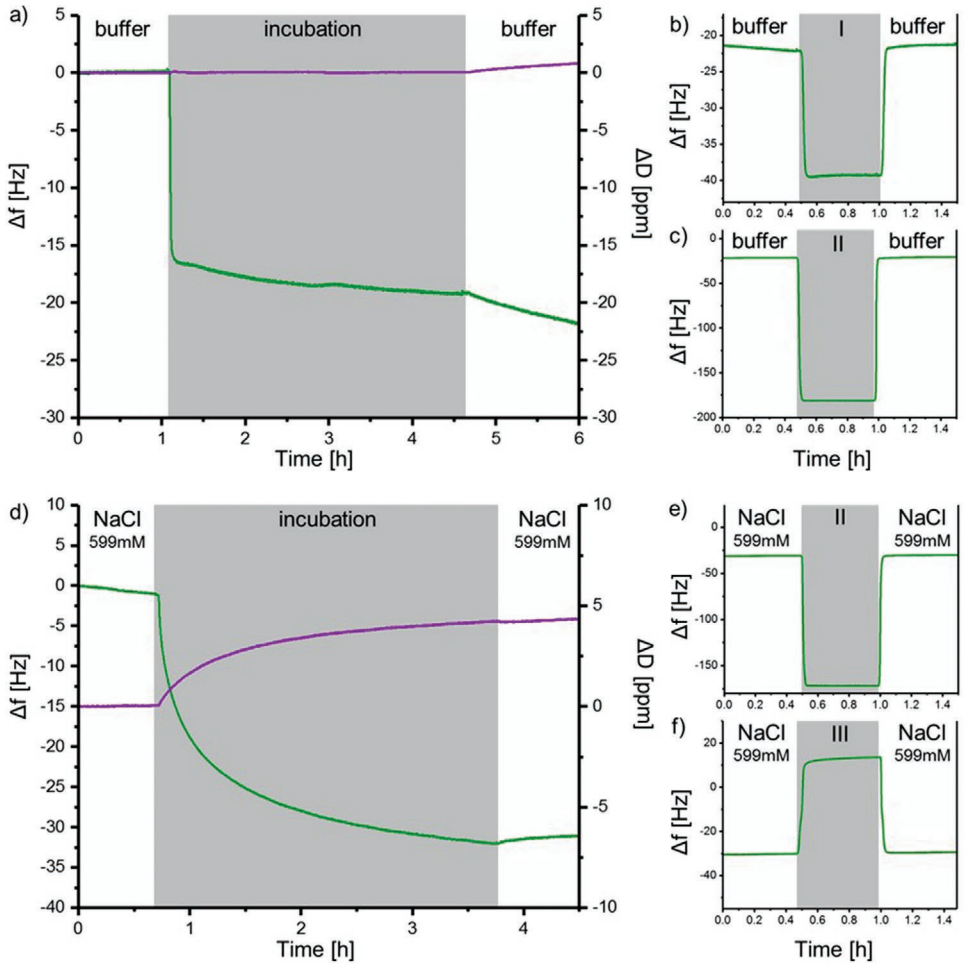

Figure 3. QCM-D adsorption and desorption kinetics of polyU$_{mini}$^{*KC} on alumina substrates. Incubation and coating deposition from a) aqueous polyU$_{mini}$^{*KC} solution and d) seawater model solution (NaCl 599 mM). Examination of film stability against washing with b) 559 mM NaCl (I), c,e) 4.2 M hypersaline salt mixtures (II), or f) with Milli-Q water (III).

indicated by adsorption of polyU$_{mini}$^{*KC} onto aluminum oxide surfaces under seawater model condition. QCM analysis confirmed the capability of polyU$_{mini}$^{*KC} to form coatings on aluminum oxide-coated sensors from 599 mM NaCl solutions (Figure 3d). As expected the polymer deposition occurs slower compared to deposition from buffer solutions, but leads to coatings with similar resistance against washing even with hypersaline solutions of 4.2 mol L^{-1} salt mixtures. In contrast to films deposited from buffer solutions, the coating shows slight viscoelastic properties as indicated by overtone dispersion of frequency and dissipation shifts (Figure S20, Supporting Information).

It seems to be an interesting advantage with respect to the commonly established poly(dopamine) coatings that polyU$_{mini}$^{*KC} is a soluble, well processable polymer, which not only leads to non-covalently cross-linked coatings, but also provides the film cohesion required to withstand these harsh washing conditions. One detail appears to be notable, as the film deposition is highly rapid, the material coatings are seemingly not equilibrated with the aqueous surrounding (cf. reswelling while buffer rinsing). Potentially, this might indicate a "drying mechanism" where water is expelled from hydrated substrate surfaces, thereby reproducing one of the extraordinary effects found in the mussel adhesion process.[55]

In summary, the synthetic strategy to produce artificial mussel-glue proteins by MIP of peptides was expanded from enzymatic means toward chemical activation. $NaIO_4$ was applied as a highly reactive, chemical oxidant instead of a selective but expensive tyrosinase. This requires implementing chemically synthesized Dopa moieties instead of Tyr residues into the polymerizable peptide unimers in addition to Cys residues. As the chemical route decouples, the applicable unimer sequence from constrains of tyrosinase substrates, a straightforward polymerization of minimal peptide unimers such as Dopa-Lys-Cys or Dopa-Gly-Cys is possible. $NaIO_4$ oxidized the Dopa residues to Dopa quinone, which were reacting with thiols of the Cys residues in a Michael-type addition reaction. High molecular weight adhesive polymers with TCCs at each repeat unit of the polymer backbone were obtained. The properties were shown by QCM-D experiments, revealing fast adsorption onto alumina substrates,

leading to coatings that resist even harsh rinsing steps with 4.2 M dead sea water model solution. The polyU_{mini}^{*KC} coatings not only show high stability against dilution and effectively defy extreme ionic strength conditions, but also they deposited remarkable well under seawater model conditions. The chemical route overcomes the potential difficulties of enzyme availability and batch-to-batch variations. Considering the strongly reduced costs of peptide synthesis within the last decades, the chemically activated MIP route paves the way to reaction scale up. This might provide sufficient amounts of TCC-presenting polymers for addressing adhesive applications. Additionally, the decoupling from sequence constrains allows to envision the use of functional sequences, for example, material-specific adsorption domains or bioactive segments as selected from phage display biopanning to advent next-generation bio-based, material-specific adhesives.

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.

Keywords

Dopa polymerization, mussel mimetic adhesion, seawater stable coatings, sequence polymerization, water-based peptide glues

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