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Mussel-Inspired New Approach for Polymerization and Cross-Linking of Peptides and Proteins Containing Tyrosines by Fremy's Salt Oxidation

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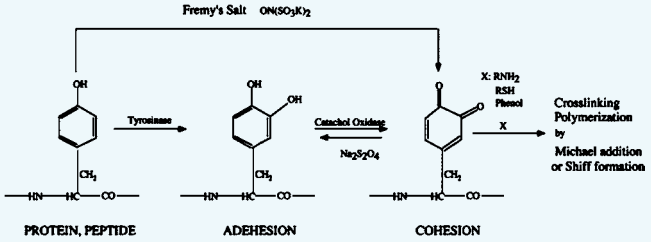
ABSTRACT: Our objective was to develop a method mimicking the natural process of coherence in marine mollusks, by direct chemical conversion of protein tyrosine residues to DOPA-o-quinones, which consequently generates polymerization and cross-linking. Fremy's salt, $(ON(SO_3K)_2$, was used to convert tyrosine residues in peptides and proteins to reactive *o*-quinones. The conversion of tyrosines to DOPA-o-quinones, and their ability to polymerize or cross-link, was tested on tyramine, peptides, and proteins. The peptides tested were as follows: biotin-PEG_4-tyramine (PEG-BT), and two decapeptides (identical to the repeating units comprising the mussel's adhesive protein). The proteins tested were as follows: bovine pancreatic ribonuclease A (RNase), lysozyme, IgG, avidin, and streptavidin. The oxidized peptides and proteins were all shown to incorporate oxygen atoms and undergo polymerization and cross-linking, depending on the availability of nucleophiles, mostly lysine amino groups of proteins. All the peptides and the noninteracting proteins such as RNase and lysozyme underwent homopolymerization upon Fremy's salt oxidation. When Fremy's salt oxidaized PEG-BT was mixed with the above proteins, it did not react with any of these proteins because PEG-BT underwent fast self-polymerization. Conversely, streptavidin or avidin cross-linked with PEG-BT after preincubation, thus showing that biorecognition is a prerequisite for cross-linking. Polymerization and cross-linking also occurred, following Fremy's salt oxidation of interacting proteins such as avidin and strepavidin with biotinylated lysozyme or biotinylated RNase. This indicates that only proteins in very close proximity readily cross-link and polymerize via tyrosine residues. Attempts to convert DOPA-quinone to DOPA by reduction with sodium dithionite $(Na_2S_2O_4)$, was successful as far as small peptides were used. Fremy's salt oxidation can serve as an easy and useful tool to polymerize and cross-link proteins, for fabrication of biomaterials and to study protein−protein interactions.



INTRODUCTION

DOPA (3,4-dihydroxyl-L-phenylalanine) is a post translational modification of tyrosine and is present in most adhesive proteins of marine mollusks, e.g., mussels and barnacles.[1] Mussels, for example, adhere to their underwater substrates by synthesizing mussel's adhesive protein (MAP), a polyphenolic protein of about 130 kDa, consisting of 80 repeats of various peptides. MAP tyrosines are converted to DOPA derivatives by tyrosinase. The catechol side chains of DOPA are known to be metal-liganding agents, which explains how marine creatures adhere to ship hulls.[2] Part of the DOPA is converted to DOPA-o-quinone by catechol oxidase, which is present in high amounts in adhesive protein producing organisms, thus augmenting their adhesive/cohesive properties. *ortho*-Quinones are very unstable and readily react with nucleophiles such as lysines, cysteines, or tyrosines via mechanisms such as the Michael addition reaction or Schiff base formation, leading to protein polymerization and cross-linking.[1] Previous studies suggest that cross-linking between different proteins also occurs via this mechanism and plays an important role in the high level of adhesive strength observed in mussel proteins.[3−5]

It has become increasingly important to prepare DOPA and dopamine containing polymers that are MAP mimetic and can be used for surface modification, for nanomaterials, and for clinical applications.[6]

Direct chemical conversion of the phenol group of tyrosine proteins to DOPA has been impossible until now; therefore, DOPA has been site specifically incorporated by genetic engineering into proteins, using E-coli or yeast, and the amber nonsense codon TAG.[7]

Several decades ago, we[8] showed that tyrosine derivatives and peptide tyrosines are converted by Fremy's salt directly to DOPA-o-quinones, which can consequently be reduced with sodium dithionite to DOPA. Upon short incubation times with the oxidant, followed by immediate reduction, tyrosines were converted to DOPA. After longer incubation periods, precipitation was observed, indicating polymerization. The nature of the polymers was not studied at that time.[8]

Fremy's salt produces DOPA-o-quinone directly from tyrosine similar to the reaction of catechol oxidase with DOPA as shown in Scheme 1.

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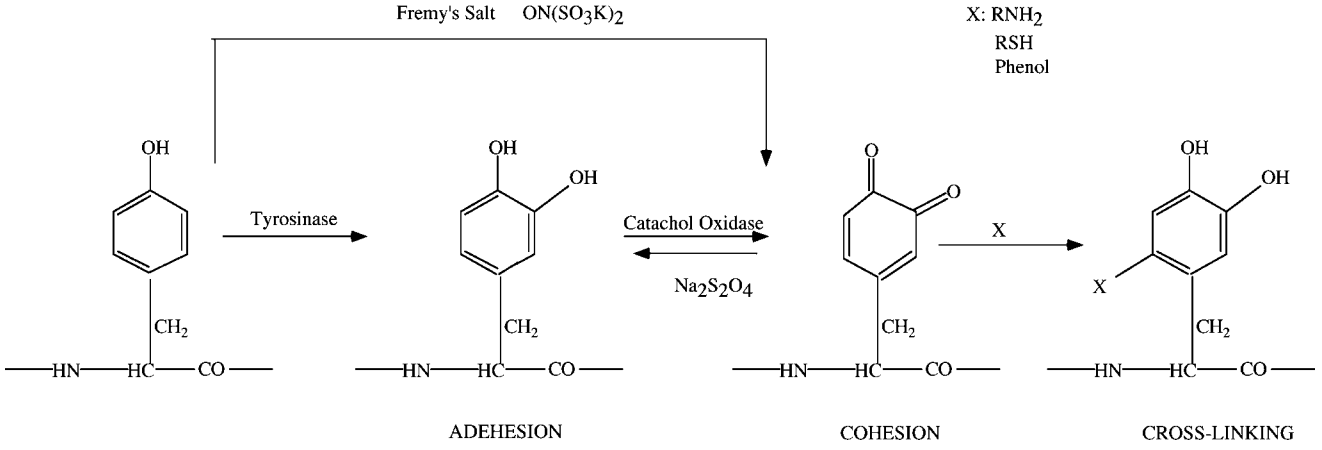
Scheme 1. Conversion of Tyrosine to DOPA-o-quinone and DOPA



In this study we show that Fremy's salt oxidation induces polymerization of peptides and proteins containing tyrosines. Polymerization and cross-linking are enhanced by the presence of lysines, cysteines, and tyrosines. Cross-linking and polymerization also occur between peptides and proteins that interact in biological systems.

RESULTS

Based on our previous results in which tyrosine derivatives are oxidized with Fremy's salt to DOPA-o-quinones and can then be reduced to DOPA with sodium dithionite, we treated acetyl-tyrosine in phosphate buffer (PB) with a 5-fold molar excess of Fremy's salt and recorded the optical spectra before and after reduction with dithionite. A shift from 275 nm (Tyr) to 280 nm (DOPA) was observed (Figure 1). Mass spectrometry confirmed the quantitative conversion of the Acetyl-Tyr (Da 223) to Acetyl-DOPA (Da 239).

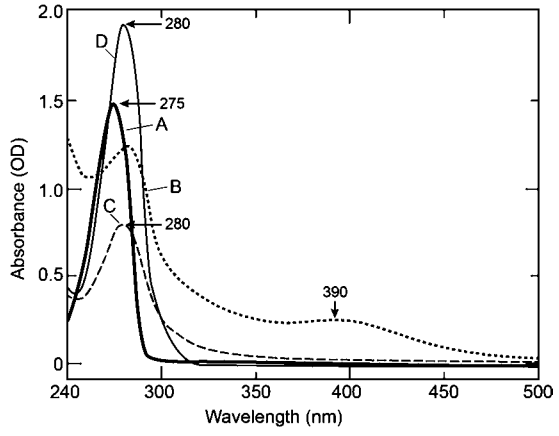


Figure 1. Spectroscopy of *N*-acetyl tyrosine nonoxidized (A), oxidized with Fremy's salt (B), and reduced with dithionite (C); DOPA control (D).

The ability of Fremy's salt oxidized tyrosine derivatives to convert to DOPA-o-quinone and to polymerize and cross-link was tested, starting with low-molecular-weight peptides, through oligo-peptides to large proteins consisting of several subunits.

Reaction of Fremy's Salt with Small Tyrosine Derivatives and Peptides. Polydopamine (PD) was introduced by Messersmith et al.[9] and has since been extensively studied. PD was prepared by oxidative self-polymerization of dopamine using either sodium periodate or oxidizing enzymes such as horseradish peroxidase or mushroom tyrosinase in Tris buffer, which contain primary amino groups. We explored the possibility to produce PD by Fremy's salt oxidation of tyramine. A range of PD polymers of molecular weights as high as 120 kDa was observed on SDS-PAGE following tyramine oxidation. There were also some larger molecules, which did not penetrate the gel (Figure 2).

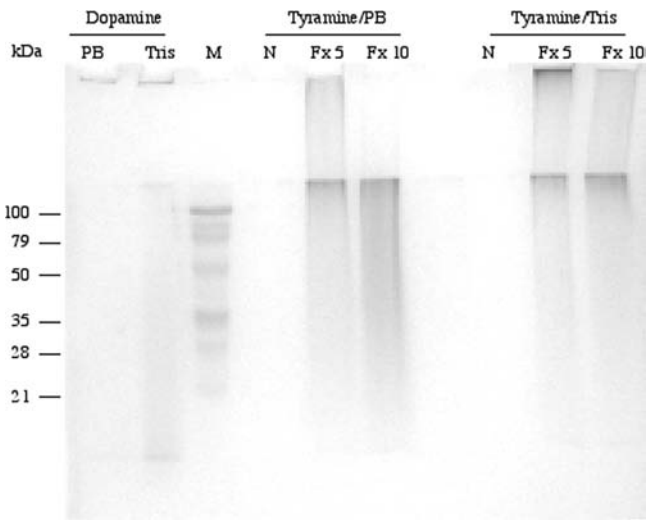


Figure 2. SDS-PAGE (15%) pattern of tyramine after oxidation with Fremy's salt in phosphate buffer and in Tris buffer. Nontreated (N), Fremy's salt-treated (F). Fremy's salt was used at a 5- or 10-fold molar ratio over tyramine. Molecular weight markers (M). Staining was performed with Coomassie blue.

Tris buffer, which contains primary amines, did not interfere with polymer formation and in some cases even enhanced the polymerization. We cannot exclude the possibility of its incorporation into the polymers.

p-Amino-D-phenylalanine oxidation with Fremy's salt also led to polymer formation (Figure 3). The polymer is presumably poly PD, since it had previously been shown that Fremy's salt converts aniline to *para*- or *ortho*-quinones.[10]

The involvement of amino groups in the polymerization process was examined by preparing PEG-BT, which does not contain amino groups and is similar in structure to biotin-

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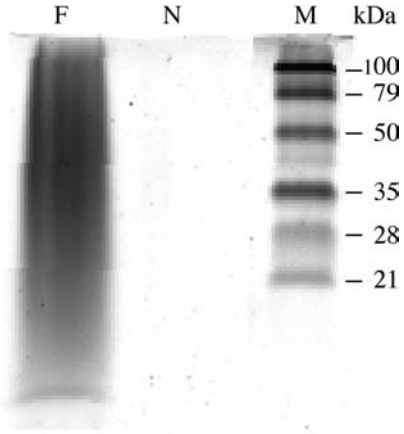

Figure 3. SDS-PAGE (15%) pattern of *p*-amino D-phenylalanine (N), Fremy'salt oxidized *p*-amino D-phenylalanine (F), molecular weight markers (M). Staining was performed with Coomassie blue.

PEG_4-DOPA (PEG-BD) previously studied.[11] PEG-BT in PB was treated with Fremy's salt for time periods from 15 min to 16 h. The reaction was followed using spectroscopy. The solution became brownish, confirming the presence of DOPA-o-quinone. After dialysis against water it was analyzed by MS. PEG-BT yielded homopolymers of different molecular weights as high as 20 kDa, all equivalent to the sum of repeating subunits of either catechol- or *o*-quinone-containing starting material (620−630 Da) (Figure 4).

Dithionite reduction, following Fremy's salt oxidation, of PEG-BT (610 Da) resulted in the formation of only low amounts of PEG-BD (626 Da), due to the high polymerization rate of PEG-BD-o-quinone formed.

Fremy's Salt Oxidation of Oligopeptides. Based on MAP repeats, we purchased two peptides: Ala-Lys-Pro-Ser-Tyr-4Hyp-4Hyp-Thr-Tyr-Lys (Glue-2) and the same peptide in which Lys 2 was blocked with a trifluoroacetyl group (Glue-3). The peptides were treated with a 5-fold molar excess of Fremy's salt in PB. After 15 min the optical spectra indicated the formation of a DOPA-o-quinone in both peptides, with a peak at about 450 nm. The peak gradually decreased and changed to a shoulder at around 350 nm (Figure 5).

Since the spectroscopy absorption peaks of acetyl-DOPA and acetyl-DOPA-o-quinone are known to be quite stable,[8] the change in spectrum of the DOPA-o-quinone peptides may indicate peptide polymerization. To verify polymerization, SDS-PAGE of the oxidized peptides was run after no further change in spectrum was observed (Figure 5 inset a). Molecular weights as high as 120 kDa and 80 kDa were observed for the decapeptide and its TFA-blocked derivative, respectively. These results were further established by MS (data not shown). The difference in molecular weight between the polymerized decapeptide and its acetylated analogue may suggest the involvement of lysine in the polymerization.

It was claimed that lysine is not involved in cross-linking and multimer formation of DOPA containing peptides.[12] However, most of the above reactions were performed in Tris buffer which may have interfered with lysine interactions and consequently disrupted polymerization.[13] Upon testing oxidation of the decapeptides with Fremy's salt in Tris buffer, a significant drop in the degree of polymerization was observed, as compared to that in PB (Figure 5 inset b). These results clearly indicate the involvement of amino groups and particularly lysines in the polymerization of the peptides.

To further clarify the role of lysine in the polymerization, following oxidation, we examined the behavior of the B-chain of porcine insulin, which contains two tyrosines and one lysine, as well as that of the acetylated B-chain. This peptide bears primary structural similarity to MAP but in a reverse order (Tyr-Thr-Pro-Lys-Ala). Upon reaction with Fremy's salt, the B-chain of insulin was polymerized while the acetylated chain was not, which clearly indicates lysine participation in polymerization and cross-linking (Figure 6).

Reaction of Fremy's Salt with Proteins. To investigate polymerization of proteins, lysozyme, RNase, rabbit IgG, avidin, and streptavidin were incubated with a 5−20-fold molar excess of Fremy's salt/tyrosine residues, for 16 h.

Lysozyme oxidation proceeded through oligomer formation, followed by high-molecular-weight polymers, aggregates, and finally precipitation, depending on the concentration of Fremy's salt and time of incubation. SDS-PAGE analysis showed molecular weights ranging from oligomers through polymers,

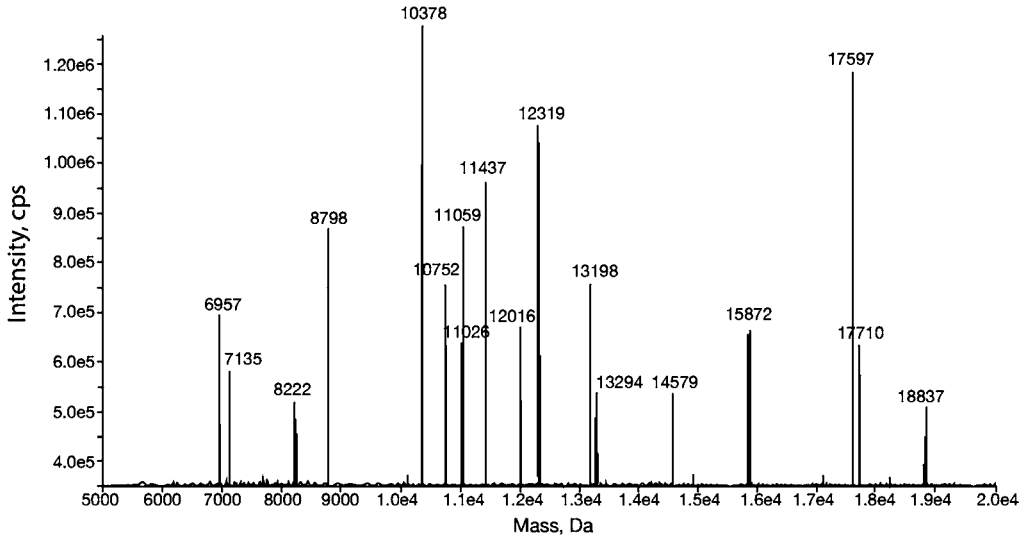

Figure 4. MS of biotin-PEG4-tyramine after Fremy's salt oxidation. Analysis was done using Bruker reflex III by MALDI-TOF MS.

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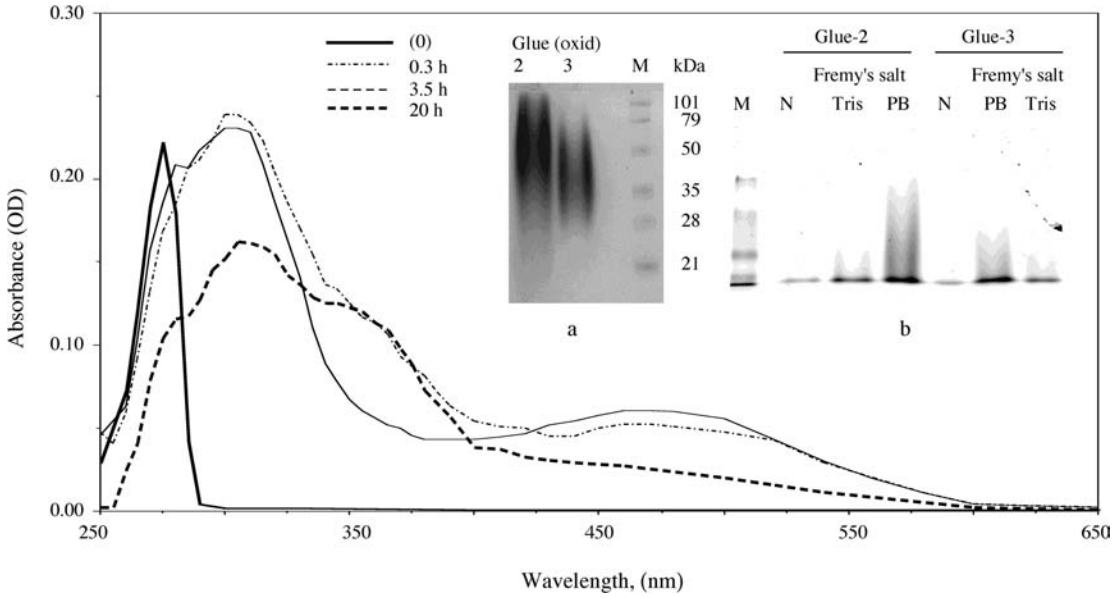

Figure 5. Change in optical spectrum of Glue peptides upon Fremy's salt oxidation. SDS-PAGE (15%) pattern after Fremy's salt oxidation (inset a). SDS-PAGE (12%) pattern of oxidation carried out in either PB or tris buffer (inset b). Molecular weight markers (M), nonoxidized peptide (N).

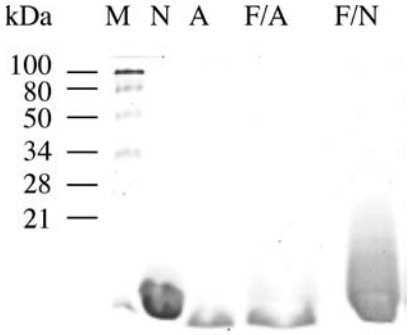

Figure 6. SDS-PAGE (15%) pattern of B-chain of insulin: Fremy's salt oxidized (F), Native, non-acetylated (N), acetylated (A).

exceeding 100 kDa, the latter stuck on top of the gel. MS analysis showed peaks as high as 130 kDa (Figure 7).

In order to check the involvement of the tyrosines and lysines in this polymerization, we performed amino acid analysis of the soluble fraction and the pellet, as well as of nontreated lysozyme. Of the original 3 tyrosine and 6 lysine residues, there was a drop in residue numbers to 1.4 and 4, respectively, in the soluble material. Only traces of tyrosine were detected in the precipitate after oxidation, and lysine number was the same as in the soluble fraction (Table 1). No change was observed in any other amino acids.

Since the N-terminal part of lysozyme contains 2 lysine and 2 tyrosine residues, we also performed amino acid sequence analysis of the polymerized protein. We found that the N-terminal Lys remained intact. Further sequencing showed that only traces of Tyr 20 were left and Lys13 and Tyr 23 completely disappeared. A minor loss of His 15 was also observed (Table 2).

The oxidized lysozyme had lost its activity after a very short time of oxidation (5−10% residual activity was observed 1 or 10 min after oxidation). RNase contains 6 tyrosine and 10 lysine residues. Its oxidation under the same conditions as lysozyme resulted in a similar polymerization behavior, yielding a dark brown solution followed by precipitation (Figure 8).

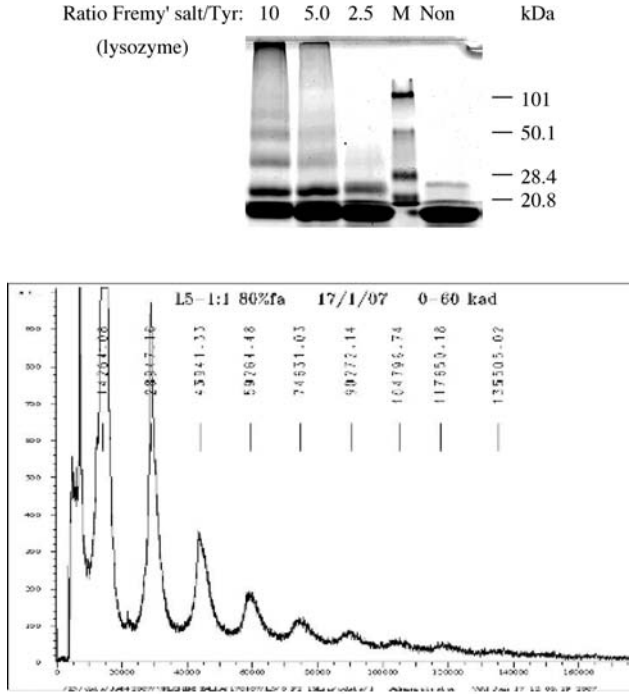

Figure 7. SDS-PAGE (15%) pattern of lysozyme oxidized with Fremy's salt (above), MS (below).

Table 1. Amino Acids Analysis of Fremy's Salt Oxidized Lysozyme[a]

amino acid	no of residues nonoxidized	soluble fraction	insoluble fraction
Lys	6	3.9 (4)	3.8 (4)
Tyr	3	1.4	0.34 (1)
His	1	1	0.94 (1)

[a]Results are represented by number of residues/proteins.

RNase homopolymers retained up to 70% enzymatic activity in the soluble fraction (under a 5 M excess of Fremy's salt for 16 h) as was determined with RNA as substrate. Amino acid

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Bioconjugate Chem. 2015, 26, 502−510

Table 2

(a) Amino Acid Sequence of Hen Egg White Lysozyme		
K-V-F-G-R-C-E-L-A-A-A-M-K-R-H-G-L-D-N-Y-R-G-Y-S-L-G-N-W-V-C-A-A-K-F-E-S-N-F-N-T-E-A-T-N-R-N-T-D-G-S-T-D-Y-G-I-L-E-I-N-S-R-W-W-C-N-E-G-R-T-P-G-S-R-N-L-C-D-I-P-C-S-A-L-L-S-D-I-T-A-V-N-C-A-K-K-I-V-D-G-D-E-M-N-A-W-V-A-W-R-N-C-K-G-T-D-V-Q-A-W-I-R-G-C-R-L		
(b) Amino Acid Sequence of Fremy's Salt Oxidized Lysozyme		
cycle no.	amino acid pmol/cycle	AA
1	188.8	K
2	185.2	V
3	161.2	F
4	68.3	G
5	41.5	R
6	-	C
7	40.7	E
8	46.4	L
9	42.1	A
10	55.9	A
11	58.3	A
12	30.9	M
13	10.3	K
14	28.3	R
15	7.2	H
16	28.6	G
17	34.3	L
18	26.8	D
19	30.6	N
20	8.7	Y
21	12.4	R
22	15.8	G
23	0	Y

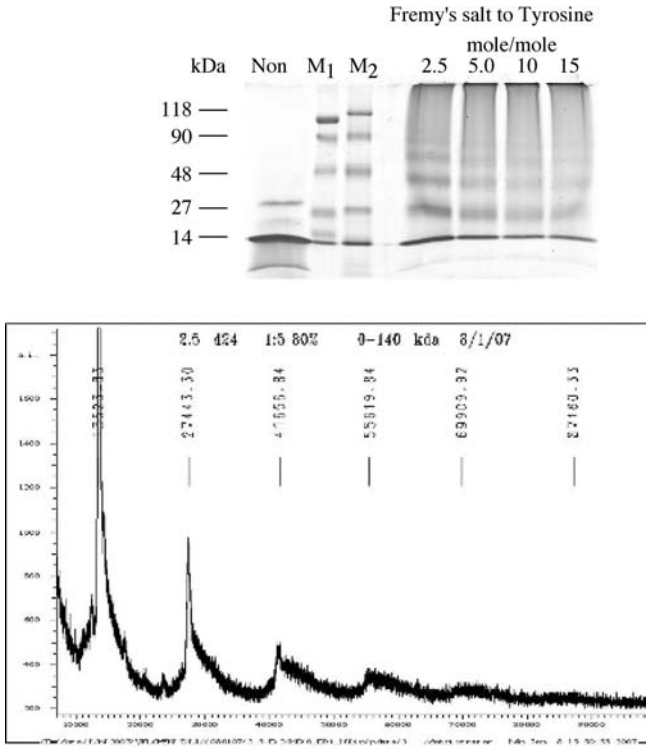

Figure 8. SDS-PAGE (15%) pattern of Fremy's salt oxidized RNase (above) and MS (below). RNase nonoxidized (Non), molecular weight markers (M1, M2).

analysis showed that only Tyr and Lys residues were affected, Tyr numbers dropped from 6 to 3 and Lys from 10 to 8 (Table 3). No change in any other amino acid was observed.

Table 3. Amino Acid Analysis of Native and Fremy's Salt Oxidized Ribonuclease A[a]

amino acid	nonoxidized	Fremy's salt (5-fold molar excess)	Fremy's salt (10-fold molar excess)
Lys	9.8 (10)	8.3 (8)	7.6 (8)
Tyr	5.5 (6)	3.1 (3)	2.4 (3)
His	3.7 (4)	3.7 (4)	3.6 (4)

[a]Results represent number of residues/protein.

In order to check the involvement of Lys residues in the polymerization of RNase, we acetylated the RNase with a 20-fold molar excess of acetic anhydride per Lys. Treatment of the acetylated RNase with Fremy's salt resulted in a lower degree of polymerization, than its nonacetylated counterpart (Figure 9), again corroborating the involvement of lysines in the polymerization of proteins.

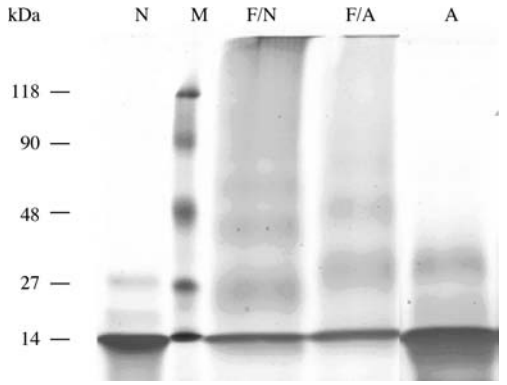

Figure 9. SDS-PAGE (12%) pattern of Native RNase (N) and acetylated RNase (A) oxidized with Fremy's salt (F).

Rabbit IgG was treated with Fremy's salt, applied to SDS-PAGE, and stained with goat anti-rabbit IgG-alkaline phosphatase (GaR-AP). No precipitate was observed. A single band of about 150 kDa was obtained (calculation was extrapolated from marker molecular weight, whereas non-treated IgG showed heavy and light chains, which indicates that chemical inter cross-linking occurred and probably no polymerization (Figure 10).

Avidin and streptavidin are tetramers of identical subunits. Avidin contains one tyrosine residue per subunit, and streptavidin, six. Avidin was treated with Fremy's salt at various molar excess ratios over tyrosine residues and analyzed on SDS-PAGE. The electrophoretic pattern of the oxidized avidin was similar to that of the nontreated protein. Intra cross-linking in the subunit cannot be excluded since MS results show a difference in molecular weight of 16 Da, 14 291 Da (non-treated), and 14 307 Da (oxidized). This means that one oxygen atom/avidin was incorporated and an *o*-quinone formed.

This was not the case with streptavidin. Quantitative SDS-PAGE analysis of streptavidin showed that the nontreated protein contained 91% monomers, and about 8% dimers, whereas Fremy's salt-treated streptavidin contained 36% monomers and 31% dimers. Additionally, we observed trimers,

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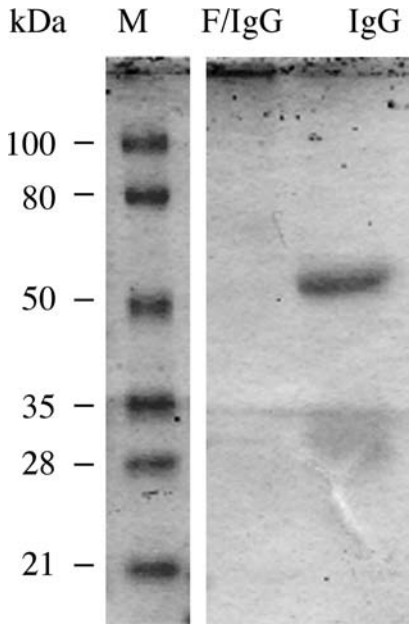

Figure 10. SDS-PAGE (12%) pattern of rabbit immunoglobulin (IgG) oxidized with Fremy's salt (F/IgG). Staining was done with Goat anti-rabbit-alkaline phosphatase.

tetramers, and some larger polymers at different quantities (Table 4). Biotin decreased polymerization to a certain extent (Figure 11).

Table 4. Distribution (%) of Streptavidin (STA) Oligomers before and after Fremy's Salt Oxidation (± Biotin), Obtained from Quantitative Scanning of SDS-PAGE Bands

(STA) n	monomers	dimers	trimers	tetramers	pentamers
STA (Native)	91.1	8.9			
STA/Fremy's salt	35.6	30.8	13.9	4.4	15.3
STA+ Biotin/ Fremy's salt	51.0	25.8	8.1	5.3	9.8

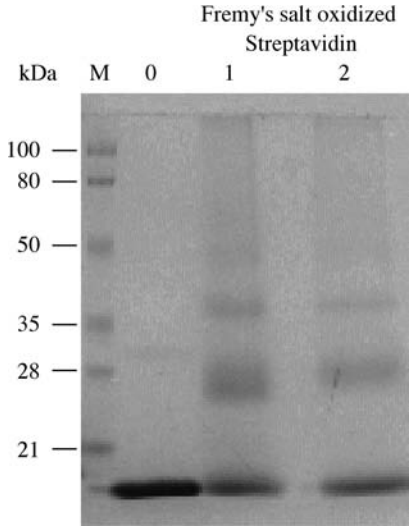

Figure 11. SDS-PAGE (12%) pattern of streptavidin and biotinyl-streptavidin upon Fremy's salt oxidation. Nonoxidized streptavidin (0), Fremy's salt oxidized streptavidin (1), oxidized streptavidin in the presence of biotin (2).

Recently it has been shown that peptides and proteins containing DOPA, oxidized with periodate, and converted to DOPA-o-quinones, cross-link if they interact in biological systems. No such cross-linking occurred when noninteracting peptides/proteins were used.[11,14]

We prepared PEG-BT, a reagent similar to PEG-BD used above, employing Fremy's salt as an oxidant in order to confirm the above observation. In our case, PEG-BT was oxidized to biotinyl-DOPA-o-quinone and was added to any of the following: lysozyme, RNase, avidin, and streptavidin. The oxidized PEG-BT polymerized before having a chance to interact with the proteins. When avidin was incubated with PEG-BT, followed by oxidation with Fremy's salt, a covalent coupling of the PEG-BT with avidin was obtained. A change of +630 Da was observed between the nonoxidized protein, 14 293 Da, and the oxidized protein, 14 923 Da. No such change occurred without the addition of Fremy's salt, thus confirming the finding that only interacting molecules cross-link upon *o*-quinone formation. To further substantiate this, we biotinylated lysozyme and RNase and incubated each of these proteins with avidin or streptavidin, followed by Fremy's salt oxidation. A shift to high-molecular-weight polymers of both proteins was observed in contrast to their nonbiotinylated counterparts (Figure 12). The gels were blotted and stained

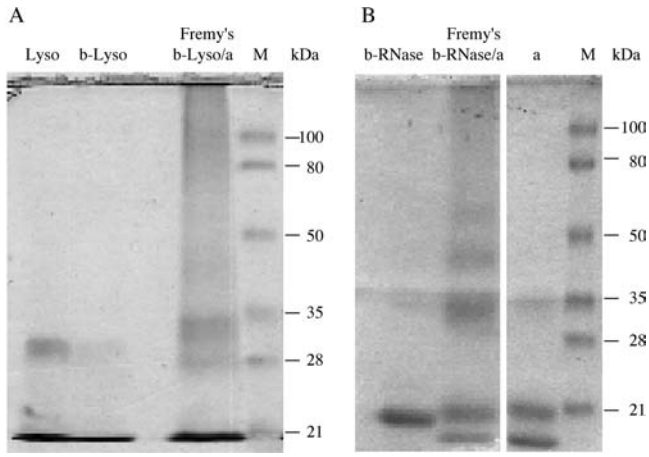

Figure 12. SDS-PAGE (12%) pattern of Fremy's salt oxidized avidin in the presence of biotinyl lysozyme (A) and biotinyl RNase (B). Lysozyme nonoxidized (Lyso), biotinyl lysozyme (b-Lyso), biotinyl RNase (b-RNase), avidin (a), molecular weight markers (M).

with biotinyl-alkaline phosphatase to identified avidin, confirmed the presence of avidin in the higher-molecular-weight bands (data is not shown).

DISCUSSION

The aim of this study was to contribute toward the development of protein- or polypeptide-based biological adhesives, by studying the behavior of interacting proteins following tyrosine oxidation with Fremy's salt. The approach we chose was to mimic the enzymatic mechanism by which marine organisms produce moisture-resistant adhesives, by employing chemical means.[1] The principle by which these adhesives are formed is a two-step enzymatic conversion of tyrosine-rich proteins to DOPA-proteins first, followed by catechol oxidation to DOPA-o-quinones, which tend to polymerize and cross-link.[2]

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It has been shown that not all tyrosines in proteins can be enzymatically converted to DOPA.[15] Tyrosinase is probably able to modify only highly specific tyrosines, as it is known that in enzymes[15,16] and other biorecognition systems the binding or active site is small.[17,18] It was therefore suggested that MAP, consisting of 80 repeating units of tanden linear penta- through decapeptides, may have evolved multispecific enzyme recognition sequences.[13]

Considering the fact that it is very easy to incorporate tyrosine residues to any protein by genetic engineering,[19] but not DOPA,[7] we have attempted in this study to incorporate DOPA or DOPA quinone, which are accountable for the adhesive properties, by oxidation of tyrosine residues of various proteins. At present, there is no chemical method by which a tyrosine can be converted directly to DOPA. We showed, several decades ago, that peptide tyrosines can be converted chemically by Fremy's salt oxidation to DOPA-o-quinones.[8] Almost all the proteins that we examined after Fremy's oxidation resulted in either polymerization or cross-linking, followed, in some cases, by precipitation upon longer incubation. Amino acid analysis of lysozyme and RNase revealed that only tyrosine and lysine residue numbers decreased. Only avidin did not polymerize, but since oxygen was incorporated after oxidation, it is likely that intra-cross-linking occurred. We found that Tris buffer interferes with the polymerization of polypeptides, which also indicates the involvement of amino groups in polymerization. However, it does not prevent Fremy's salt induced polymerization of tyramine, probably due to the fast cyclization of *o*-quinones to hydroxyl-containing indoles. The structure of PD is also assumed to be of polyhydroxy indoles.[20] The fact that PEG-BT by itself undergoes polymerization after Fremy's salt treatment indicates that Lys assists in the polymerization process but is not essential. However, in view of results obtained for larger peptides and proteins manifesting the contrary, lysine residues are definitely involved in cross-linking and may prove to be essential at a later step leading to cohesion/adhesion in nature. Thus, the difference in molecular weight between the decapeptide and the acetylated decapeptide polymers may suggest the involvement of lysine in the polymerization. More evidence is provided from the B-chain of insulin homopolymerization, contrary to the acetylated chain which did not polymerize at all. The same results were obtained for RNase as opposed to acetylated RNase. This indicates that lysine amino groups are important for polymerization and cross-linking of DOPA-o-quinones in proteins.

We determined which tyrosine and lysine residues were involved in the polymerization of lysozyme. Interestingly, the N-terminal lysine was not involved. It is somehow shielded from reaction or is far removed from the tyrosines.

We have established that oxygen was incorporated into the proteins following Fremy's salt treatment, thus proving the formation of *o*-quinones. We have observed incorporation of one oxygen atom per one avidin subunit, one Tyr/subunit, and several oxygens in RNase.

Attempts to reduce the DOPA-o-quinone-proteins to DOPA-protein with dithionite produced inconclusive results due the tendency of the *o*-quinones to polymerize or cross-link instantaneously, moreso in the proteins than the peptides tested. Future approaches to enable chemical introduction of DOPA into proteins will necessitate stabilization of *o*-quinones or devising in situ reducing agents. However, when a need for protein polymerization arises, tyrosine, which is easy to incorporate into a peptide chain, can be used and oxidized as described.

Recently, it has been shown that peptides containing DOPA can cross-link with interacting protein partners, upon periodate oxidation, which forms DOPA-o-quinones. These peptides do not interact with irrelevant proteins, but precipitate as a result of forming large homopolymers.[11,21] While the above studies were done with PEG-BD, we used a similar molecule PEG-BT in order to investigate coupling to proteins.

Upon oxidation with Fremy's salt, we could not detect any coupling between irrelevant protein, but found a very fast polymerization of PEG-BT, which was converted to PEG-BD-quinone, yielding molecular weights as high as 20 kDa (Figure 4). It is likely that the same reaction occurred with PEG-BD.[11,21] When PEG-BT was first incubated with avidin, and then reacted with Fremy's salt, one PEG-BT molecule was covalently bound per one subunit of avidin. We thus confirmed that DOPA peptides can cross-link with interacting proteins.[14]

Interestingly, a surprising observation was claimed in the same study: that 1,2-diols, such as sugars[14] on proteins, do not interfere with the periodate-mediated cross-linking of BD. This is not surprising since the product of periodate oxidation of 1,2-diols is iodate, which is also a potent oxidant of catecols to *o*-quinones.[22]

In summary, we have shown that peptides and proteins containing tyrosines can be directly converted to DOPA-o-quinones and polymerized or cross-linked when there are nucleophiles such as amino group of lysine, thiol group of cysteine, and phenol group of tyrosine in close proximity. We have also shown covalent cross-linking between IgG subunits, streptavidin subunits, and cross-linking between avidin and biotinylated lysozyme and biotinylated RNase, thus indicating that interacting proteins and subunits readily cross-link after oxidation with Fremy's salt.

It was impossible at this stage of the study to control the equilibrium of the oxidation reduction toward the stabilization of DOPA intermediates and convert the proteins to a biological glue. However, it might be possible to obtain glues by converting tyrosine-rich proteins to DOPA, using tyrosinase, adsorbing them to solid surfaces, and then using Fremy's salt to oxidize tyrosines that have not been enzymatically converted (see Scheme 1).

Since many of the tyrosine-containing proteins in cells are involved in protein−protein interactions, and due to the fact that Fremy's salt is a small molecule, which may penetrate cell membranes and cross-link interacting protein tyrosines, in situ cross-linking within the cell or on the cell surface can be studied, using Fremy's salt oxidation.

EXPERIMENTAL PROCEDURES

Materials and Methods. The peptide AKPSYP*P*TYK (P* = hydroxyl proline), and its trifluoro acetylated derivative (Lys'2 modified), Fremy's salt, bovine pancreatic ribonuclease A (RNase), Hen egg-white lysozyme, IgG, avidin, and streptavidin were obtained from Sigma Israel. Biotinyl-dPEG4-*N*-hydroxysuccinimid ester (NHS-dPEG4-biotin) was purchased from Quanta Biodesign, Powell, Ohio 43065. Biotinylation of the proteins was performed as previously described.[23]

Oxidation of Proteins with Fremy's Salt. Fremy's salt (freshly prepared, 50−100 mM) was added at increasing molar ratios (2.5−10-fold molar excess/tyrosine) to protein solutions (1−10 mg/mL) in 0.05 M phosphate buffer, pH 8.0 (PB), and

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Bioconjugate Chem. 2015, 26, 502−510

left for 16−20 h at room temperature. The reaction mixtures were subjected to UV−vis spectroscopy, MS analysis, and SDS-PAGE (12% or 15%). Gels were either stained with Coomassie Brilliant Blue R250 Stain or transblotted and stained with alkaline phosphatase conjugated Goat anti-rabbit IgG or biotinylated alkaline phosphatase. Reduction of the *o*-quinone to DOPA-derivatives was done by addition of solid sodium dithionite in molar excess over Fremy's salt.

Proteins Acetylation. Acetic anhydride (10−20-fold molar excess/lysine) was added to the protein in the presence of sodium carbonate (0.12%) and left for 30 min at room temperature. The acetylated protein was diluted with PB.

Biotin-dPEG$_4$-Tyramine (PEG-BT). Biotin-dPEG4-NHS (50 mg) was dissolved in 1 mL dimethylformamide and tyramine (13 mg) was added. The reaction was left for 5 h and precipitated with ether, washed with ether, and filtered. The product was TLC pure. Rf 0.3 using methanol:chloroform (2:8). HRMS-EI ($C_{29}H_{46}N_4O_8S$) calculated: 610, observed: 610.21.

Enzymatic Activity of RNase. RNase activity was measured by using the substrate, yeast RNA (1% RNA in Na acetate buffer pH 5.0) at 37 °C. The reaction was stopped with 25% perchloric acid in uranyl acetate and cooled in an ice bath for 5 min. The supernatant was collected after centrifugation (4 °C, 14 000 rpm, 15 min), diluted with water (1:10), and measured at 260 nm.[24]

Enzymatic Activity of Lysozyme. Lysozyme activity was measured by using *Micrococcus luteus* cell walls as substrate (0.03% in Na acetate buffer pH 5.0) at 25°. The kinetics of turbidity loss at 450 nm was followed and compared to the activity of nonmodified lysozyme. Monitoring was done by using Varian-Cary 219 UV−vis spectrophotometer (Varian, Palo Alto, CA, USA).

Sequencing. N-terminal sequence analysis was performed using Procise 492 Protein Sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Mass Spectrometry. Mass spectra of polymers were determined using mass spectrometer API-3000 (Applied Biosystems AB Sciex, Foster City, CA, USA) or by using Bruker reflex III by MALDI-TOF MS (Bruker Daltonik, Bremen, Germany). Smaller peptide mass spectra were determined using Micromass Platform LCZ 4000 ionization mode: ESI-Electrospray ionization (Micromass, Manchester, UK).

Quantitative SDS-PAGE. Analysis was performed by Imagescanner (Amersham Bioscience) using *LabScan 5.0* software application (GE Healthcare) according to LabScan user manual.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

GaR-AP, goat anti-rabbit IgG- alkaline phosphatase; NHS-dPEG$_4$-biotin, biotinyl-dPEG$_4$-*N*-hydroxysuccinimide ester; MAP, mussel's adhesive protein; PB, phosphate buffer; PD, polydopamine; PEG-BD, biotin-PEG$_4$-DOPA; PEG-BT, biotin-PEG$_4$-tyramine; RNase, bovine pancreatic ribonuclease A

REFERENCES

(1) Lee, B. P., Messersmith, P. B., Israelachvili, J. N., and Waite, J. H. (2011) Mussel-inspired adhesives and coatings. *Annu. Rev. Mater. Res. 41*, 99−132.

(2) Fullenkamp, D., Barrett, D. G., Miller, D. R., Kurutz, J. W., and Messersmith, P. B. (2014) pH-dependent cross-linking ocatechols through oxidation via Fe3+ and potential implications for mussel adhesion. *RSC Adv. 4*, 25127−25134.

(3) Nicklisch, S. C., and Waite, J. H. (2012) Mini-review: the role of redox in Dopa-mediated marine adhesion. *Biofouling 28*, 865−877.

(4) Yu, J., Hwang, M., and Deming, T. J. (1999) Role of L-3,4-dihydroxyphenylalanine in mussel adhesive proteins. *J. Am. Chem. Soc. 121*, 5825−5826.

(5) Waite, J. H., and Tanzer, M. L. (1980) The bioadhesive of *Mytilus byssus*: a protein containing L-dopa. *Biochem. Biophys. Res. Commun. 96*, 1554−1561.

(6) Brubaker, C. E., Kissler, H., Wang, L. J., Kaufman, D. B., and Messersmith, P. B. (2010) Biological performance of mussel-inspired adhesive in extrahepatic islet transplantation. *Biomaterials 31*, 420−427.

(7) Alfonta, L., Zhang, Z., Uryu, S., Loo, J. A., and Schultz, P. G. (2003) Site-specific incorporation of a redox-active amino acid into proteins. *J. Am. Chem. Soc. 125*, 14662−14663.

(8) Dukler, S., Wilchek, M., and Lavie, D. (1971) Oxidation of tyrosine and its peptides with potassium nitrosodisulphonate. *Tetrahedron 27*, 607−614.

(9) Lee, H., Dellatore, S. M., Miller, W. M., and Messersmith, P. B. (2007) Mussel-inspired surface chemistry for multifunctional coatings. *Science 318*, 426−430.

(10) Zimmer, H., Lankin, D. C., and Horgan, S. W. (1971) Oxidation with potassium nitrosodisulfonate (Fremy's radical). The Teuber reaction. *Chem. Rev. 71*, 229−246.

(11) Lim, H. S., Cai, D., Archer, C. T., and Kodadek, T. (2007) Periodate-triggered cross-linking reveals Sug2/Rpt4 as the molecular target of a peptoid inhibitor of the 19S proteasome regulatory particle. *J. Am. Chem. Soc. 129*, 12936−12937.

(12) Burzio, L. A., and Waite, J. H. (2001) Reactivity of peptidyl-tyrosine to hydroxylation and cross-linking. *Protein Sci. 10*, 735−740.

(13) Burzio, L. A., and Waite, J. H. (2000) Cross-linking in adhesive quinoproteins: studies with model decapeptides. *Biochemistry 39*, 11147−11153.

(14) Liu, B., Burdine, L., and Kodadek, T. (2006) Chemistry of periodate-mediated cross-linking of 3,4-dihydroxylphenylalanine-containing molecules to proteins. *J. Am. Chem. Soc. 128*, 15228−15235.

(15) Cory, J. G., and Frieden, E. (1967) Differential reactivities of tyrosine residues of proteins to tyrosinase. *Biochemistry 6*, 121−126.

(16) Schechter, I., and Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun. 27*, 157−162.

(17) Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968) Staphylococcal nuclease: size and specificity of the active site. *Science 162*, 1491−1493.

(18) Alon, R., Bayer, E. A., and Wilchek, M. (1990) Streptavidin contains an RYD sequence which mimics the RGD receptor domain of fibronectin. *Biochem. Biophys. Res. Commun. 170*, 1236−1241.

(19) Jennissen, H. P., and Laub, M. (2007) Development of an universal affinity fusion tag (Poly-Dopa) for immobilizing recombinant proteins on biomaterials. *Materalwiss. Werkstofftech. 38*, 1035−1039.

DOI: 10.1021/bc5006152
Bioconjugate Chem. 2015, 26, 502−510

(20) Liebscher, J., Mrówczyński, R., Scheidt, H. A., Filip, C., Hădade, N. D., Turcu, R., Bende, A., and Beck, S. (2013) Structure of polydopamine: a never-ending story? *Langmuir 29*, 10539−10548.
(21) Burdine, L., Gillette, T. G., Lin, H. J., and Kodadek, T. (2004) Periodate-triggered cross-linking of DOPA-containing peptide-protein complexes. *J. Am. Chem. Soc. 126*, 11442−11443.
(22) Itoh, Y., Kakuta, T., Hirano, M., and Morimoto, T. (1979) A facile synthesis of 4,5-dialkoxy-o-benzoquinones by the oxidation of catechol with iodates in alcohol. *Bull. Chem. Soc. Jpn. 52*, 2169−2170.
(23) Bayer, E. A., and Wilchek, M. (1990) Protein biotinylation. *Methods Enzymol. 184*, 138−160.
(24) Crook, E. M., Mathias, A. P., and Rabin, B. R. (1960) Spectrophotometric assay of bovine pancreatic ribonuclease by the use of cytidine 2′:3′-phosphate. *Biochem. J. 74*, 234−238.

DOI: 10.1021/bc5006152
Bioconjugate Chem. 2015, 26, 502−510