ENZYMATIC AND NON-ENZYMATIC PATHWAYS TO FORMATION OF DOPA-MODIFIED PEG HYDROGELS

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Introduction

Marine mussels are known to secrete unique adhesive proteins that form strong bonds with various substrates in the presence of water.1 These mussel adhesive proteins (MAPs) are secreted as fluids and can undergo rapid curing to form adhesive plaques in a wet environment. The presence of the unusual amino acid 3,4-dihydroxyphenylalanine (DOPA) in MAPs is believed to impart strong adhesive characteristics and cross-link formation.2 Although the mechanisms of these processes are not fully understood, it is believed that the oxidized o-DOPA quinone is responsible for the cross-linking of the adhesive proteins.2

We are investigating the preparation of conjugates between poly(ethylene glycol) (PEG) and DOPA for potential use as bioadhesives. PEG was chosen because of its nontoxic, non-immunogenic, and non-antigenic properties.3 When immobilized chemically or physically on polymer surfaces, it renders the surfaces highly resistant to biological fouling due to protein and cell adhesion. Conjugation of PEG to biomolecules is known to increase the solubility and molecular weight while having little effect on the chemical and biological properties of the conjugated biomolecules.3

In a previous report,4 we investigated the synthesis and metal ion complexation properties of simple PEG-DOPA constructs. In this study, PEG-DOPA molecules of varying molecular weight and architecture were synthesized using standard peptide coupling chemistry and investigated for their ability to form cross-linked hydrogels under oxidative conditions. Chemical and enzymatic oxidizing reagents such as sodium periodate (NaIO4), horseradish peroxidase/hydrogen peroxide (HRP/H2O2), and mushroom tyrosinase/oxygen (MT/O2) were added to aqueous solutions of PEG-DOPA to induce gelation. Reaction conditions were studied in an effort to understand the cross-linking mechanisms and to develop rapidly hardening adhesive hydrogels. The process of cross-linking was monitored using UV/vis spectroscopy and gel permeation chromatography in concert with multiplex laser light scattering (GPC-MALLS).

Experimental

Materials. Bi-functional bis-PEG-amine (MW 3400) and monofunctional methoxy-PEG-amine (MW 5000) were purchased from Shearwater Polymers and the branched 4-armed-PEG-amine (MW 10k) was obtained from SunBio PEG-Shop.

Synthesis. Two four-armed PEG’s (I, II), two linear bis-PEG’s (III, IV), and one linear methoxy-PEG (V) were synthesized using standard carbodiimide coupling chemistry as described previously.5 Amine-terminated PEG’s were reacted with tert-butylxycarbonyl- (Boc) protected DOPA in 50% DCM/DMF using HOBt, HBTU, and triethylamine as catalysts. The reaction solution was extracted with saturated NaCl solution, 5% NaHCO3, 1 N HCl, and water and then dried with a rotary evaporator. The crude products were further purified by eluting with methanol through a Sephadex® LH-20 column followed by precipitation in cold methanol. Boc was removed by reacting in 50% TFA/DCM for 30 minutes. The DOPA coupling efficiency was determined to be greater than 85% by both 1H NMR spectra and the DOPA assay of Waite and Benedict.5

Determination of Gelation Time. To an aqueous solution of DOPA-modified PEG, oxidizing reagents (horseradish peroxidase (HRP) and hydrogen peroxide (H2O2), mushroom tyrosinase (MT) and oxygen, or sodium periodate (NaIO4)) were added to induce gelation. Gelation time was qualitatively determined to be when the mixture ceased flowing, as measured by inversion of a vial containing the fluid.

Results and Discussion

Gelation Time. As shown in Figure 1, periodate-induced gelation of N-Boc-protected polymers exhibited a strong dependence on the ratio of NaIO4 to DOPA concentration. Although the minimum gelation time occurred at NaIO4/DOPA < 1 (one minute at NaIO4/DOPA = 0.6 for I and seven minutes at NaIO4/DOPA = 0.75 for III), rapid gelation was observed at around equimolar NaIO4/DOPA (under 6 minutes at 0.52 < NaIO4/DOPA < 1.4 for I and under 13 minutes at 0.75 < NaIO4/DOPA < 1.2 for III). Methoxy-PEG-(N-Boc-DOPA), V, did not gel over the extent of periodate concentration tested but an increase in viscosity after addition of the oxidizing agent was apparent. The deprotected PEG-(DOPA), II, exhibited an exponential decrease in gelation time with increasing periodate concentration, suggesting a different cross-linking mechanism from the N-Boc-protected species.

Figure 1. Qualitative gelation time of NaIO4-induced cross-linking of 130 mg/ml of I, II, and III in 10 mM PBS, pH 7.4.

Enzymatic oxidation of PEG-DOPA polymers demonstrated exponentially decreasing gelation time with increasing enzyme concentration as seen in Figure 2. I was demonstrated to gel much faster than II and III. I gelled within 2 minutes with a HRP concentration of 7,000 units/ml. In contrast, III and II required more than 20 and 90 minutes, respectively, to gel at the same polymer and peroxidase concentration. Although the oxidation of

UV/vis. DOPA-modified PEG was dissolved in 10 mM PBS solution with pH = 7.4. After adding the oxidizing reagents, the absorbance spectra of the solutions were measured at wavelengths from 200 to 700 nm over time at a scanning rate of 800 nm/min. All samples were blanked against PBS buffer and recorded at room temperature using a Hitachi U-2010 UV/vis spectrophotometer.

Gel Permeation Chromatography. The extent of polymerization was examined by GPC-MALLS on a DAWN EOS (Wyatt Technology) using Shodex-OH Pak columns in an aqueous mobile phase (50mM PBS, 0.1M NaCl, pH=6.0). The experimentally determined dn/dc value of V (0.136) was used.
V did not result in gel formation, the polymer mixtures demonstrated a dramatic increase in viscosity. When H2O2 of concentrations between 0.24 and 3.6 M was added to I in the absence of HRP, no gelation occurred. MT-induced gelation exhibited a similar exponential dependence on the enzyme concentration.

UV/vis. To understand the mechanisms behind the cross-linking of N-Boc-protected PEG-DOPA, the oxidation process was monitored over time using UV/vis. In periodate-mediated oxidation, DOPA quinone (λmax = 392 nm) formed initially and transformed further into α,β-dehydroDOPA (λmax = 326 nm). α,β-dehydroDOPA was suggested to be a precursor to insect cuticle sclerotization9 and our DOPA-modified PEG could conceivably cross-link through a similar mechanism. In peroxidase-mediated oxidation, emergence of peaks with λmax of 274 and 480 nm suggested the formation of 5,5'-di(3,4-dihydroxyphenylalanine) (diDOPA) as the possible coupling mechanism through aryloxy radical formation on the DOPA phenyl ring. The model catechol of diDOPA has absorbance peaks with λmax of 268 and 420 nm at pH 3 and 274 and 510 nm at pH 9.8 MT-induced oxidation revealed intermediates that were present in both the periodate- and peroxidase-mediated experiments. This finding suggested that the MT-based functionality in the intramolecular reaction was prevented by the presence of the Boc group. Intramolecular cyclization of the amine terminus in the oxidized DOPA. This mechanism through aryloxy radical formation on the DOPA phenyl ring.7

Figure 2. Qualitative gelation time of HRP-mediated cross-linking of 130 mg/ml of I, II, and III in 0.24 M H2O2, 10 mM PBS, pH 7.4

To understand the mechanisms behind the cross-linking of N-Boc-protected PEG-DOPA, the oxidation process was monitored over time using UV/vis. In periodate-mediated oxidation, DOPA quinone (λmax = 392 nm) formed initially and transformed further into α,β-dehydroDOPA (λmax = 326 nm). α,β-dehydroDOPA was suggested to be a precursor to insect cuticle sclerotization9 and our DOPA-modified PEG could conceivably cross-link through a similar mechanism. In peroxidase-mediated oxidation, emergence of peaks with λmax of 274 and 480 nm suggested the formation of 5,5'-di(3,4-dihydroxyphenylalanine) (diDOPA) as the possible coupling mechanism through aryloxy radical formation on the DOPA phenyl ring. The model catechol of diDOPA has absorbance peaks with λmax of 268 and 420 nm at pH 3 and 274 and 510 nm at pH 9.8 MT-induced oxidation revealed intermediates that were present in both the periodate- and peroxidase-mediated experiments. This finding suggested that the MT-based functionality in the intramolecular reaction was prevented by the presence of the Boc group. Intramolecular cyclization of the amine terminus in the oxidized DOPA. This mechanism through aryloxy radical formation on the DOPA phenyl ring.7

Figure 3. UV/vis spectroscopy of 0.4 mg/ml of II (0.13 mM DOPA) oxidized with 0.13 mM NaIO4 in 10 mM PBS, pH 7.4. The arrows indicate the progression of the spectra with time. A indicates the unoxidized II.

Oxidation of deprotected PEG-DOPA polymers did not result in the same spectra as their N-Boc-protected counterparts. As shown in Figure 3, periodate-induced oxidation led to the initial formation and decay of two characteristic peaks with λmax of 299 and 463 nm, which was followed by the emergence of a peak at λmax = 324 nm. The initial two peaks compared favorably with DOPAchrome (λmax = 302, 473 nm), resulting from intramolecular cyclization of the amine terminus in the oxidized DOPA. This intramolecular reaction was prevented by the presence of the Boc group in the N-Boc-protected species as evident by the absence of DOPAchrome characteristic peaks in their UV/vis spectra (not shown). DOPAchrome further transformed into 2-carboxylated 5,6-dihydroxyindole (λmax = 320 nm), which is believed to be a precursor to the formation of melanin, high molecular weight polymers cross-linked from catechol amines.9 Based on the observed intermediates, melanogenesis appeared to be the cross-linking mechanism for Boc-deprotected species. Similar UV/vis spectra were observed for both the HRP/H2O2 and MT/O2-mediated oxidation, which suggest a similar coupling mechanism.

Gel Permeation Chromatography. Periodate-mediated polymerization of mono-substituted mPEG-(N-Boc-DOPA), V, was examined by GPC-MALLS. The effect of varying NaIO4:DOPA ratio on the degree of polymerization after 24 hours of incubation is shown in Figure 4. The greatest extent of polymerization occurred at equimolar of NaIO4 and DOPA while significant fractions of multimers were detected at NaIO4:DOPA between 0.5 and 2. Comparing the results from GPC with gelation time experiment (Figure 1), rapid gelation of I and III was observed at NaIO4:DOPA around unity, suggesting that extensive cross-linking of DOPA end groups at this composition contributed to rapid gel formation. Covalently coupling of multiple DOPA residues creates a branching point for network formation of linear bifunctional III. However, III requires a higher degree of polymerization than tetrafuctional I, which is evident by the narrower range of periodate concentrations in which rapid gelation was observed.

Figure 4. GPC traces of V 24 hours after the addition of NaIO4 in 50 mM PBS, 0.1 M NaCl, pH 6.0.

Conclusions

PEG-DOPA polymers with high coupling efficiencies were synthesized using standard peptide chemistry. Addition of different oxidizing reagents such as NaIO4, HRP/H2O2, and MT/O2 resulted in the formation of DOPA-modified PEG hydrogels. The gelation time of the DOPA-modified polymers could be controlled by the architecture of PEG, oxidation chemistry of DOPA functionalities, and the type and concentrations of the oxidizing reagents utilized. Based on the observed intermediates, possible cross-linking mechanisms were proposed. Oxidation of N-Boc-protected PEG-DOPA led to coupling chemistry similar to that of quinone tanning and insect cuticle sclerotization. PEG modified with deprotected DOPA resulted in cross-linking similar to mammalian melanogenesis which could have accounted for the lengthy gelation time. The results described here indicate that PEG conjugated with DOPA is capable of rapid gelation in situ under mild physical conditions. The adhesive character of these gels is currently being evaluated.

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References
(2) Yu, M., Hwang, J., Deming, T.J. *JACS.* 1999, 875, 301.

Polymer Preprints 2001, 42(2), 152