Synthesis and Characterization of DOPA-PEG Conjugates

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Introduction

Mussel adhesive proteins (MAPs) are remarkable underwater adhesive materials which form tenacious bonds between certain marine organisms and the substrates upon which they reside.1 These proteins are secreted by the organisms as fluids which undergo a crosslinking or hardening reaction which leads to the formation of a solid adhesive plaque. One of the unique features of MAPs is the presence of L-3,4-dihydroxyphenylalanine (DOPA), an amino acid which is believed to be responsible for both adhesion and crosslinking characteristics of MAPs.2,3 Oxidation of DOPA to the o-quinone can lead to crosslinking (e.g. by Michael addition with a primary amine), whereas the catechol form of DOPA is believed responsible for adhesion to substrates.4,5 However, complexation between DOPA and metal ions could also contribute to bulk crosslinking as well as adhesion. For example, Fe3+ forms extremely stable coordination complexes with a variety of catechols, and bis- and tris-catecholate complexes have been observed to form between Fe3+ and a MAP from Mytilus edulis.6,7

Recently, DOPA-containing synthetic polyphenides have been synthesized by copolymerization of N-carboxyanhydride monomers of lysine and DOPA.8,9 The water soluble copolymers were found to form gels in the presence of oxidizing agents, and adhesion to metal substrates was observed. Our interest is in developing DOPA-containing synthetic polymeric systems for use as biomaterials. We hypothesize that the adhesive, metal ion binding, and crosslinking characteristics of DOPA can be exploited for development of new hydrogels as tissue adhesives and drug delivery matrices. In this paper we describe our preliminary efforts toward the synthesis and characterization of linear and branched DOPA-poly(ethylene glycol) conjugates (DOPA-PEGs).

Experimental

Materials. N-Hydroxysuccinimide ester of PEG (PEG-NHS, 3.4K MW, 1.018 Mw/Mn), PEG-bis-amine (PEG-NH2, 3.4K MW) as well as a branched PEG-amine (4-arm, 10 K MW) were purchased from Avanti Polar Lipids (Birmingham, AL). Dicyclohexylammonium salt of Boc-L-Dopa was purchased from Shearwater Polymers (Birmingham, AL). HBTU and HOBT were purchased from Sigma (St. Louis, MO). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). HBTU and HOBT were purchased from Advanced ChemTech (Louisville, KY).

Synthesis of Linear CO2H-DOPA-PEG (1). Acid terminated DOPA-PEG was obtained by reacting PEG-NHS (0.294 mmole) and L-DOPA (2.94 mmole) in 15 mL of 5% NaHCO3 under N2 bubbling for 45 minutes. The organic layer was washed with 1N HCl and the solution was treated with TFA (5 mL) for 30 min. The product was purified by dissolution in benzene and precipitation with ether. 1H NMR analysis revealed that 80% of the PEG endgroups contained DOPA residues.

Synthesis of Linear Boc-DOPA-PEG (2). Boc-protected linear DOPA-PEG was synthesized by reacting PEG-NH2 (0.294 mmole), Boc-L Dopa dicyclohexylammonium salt (1.18 mmoles), HOBTr(2.35mmole) and triethylamine (2.35 mmoles) in 10 ml of a 50:50 mixture of dimethylformamide (DMF) and DCM. HBTU(1.18 moles) and DCM(5 mL) were then added. After 30 minutes at room temperature the coupling reaction was complete as determined by a negative Kaiser test, and DCM(60 mL) was added. The solution was washed with saturated sodium chloride solution, 5% NaHCO3, 1N HCl and water and then dried with rotary evaporator. The crude product was purified by dissolution in DCM or benzene and precipitation with ether. A Kaiser test on the final product also was negative.

Synthesis of Branched Boc-DOPA-PEG (4). Boc-protected branched DOPA-PEG was synthesized by reacting four arm PEG-NH2 (0.2 mmole), Boc-L-Dopa dicyclohexylammonium salt (1.0 mmoles), HOBTr(3.2 mmoles) and triethylamine (3.2 mmoles) in DMF(5ml) and DCM(5 mL). HBTU(1.6 mmoles) and DCM(5 mL) were added. After 30 minutes at room temperature the coupling reaction was complete as determined by a negative Kaiser test.

Extraction and purification of the product was as described above for DOPA-PEG. 1H NMR analysis revealed that 80% of the PEG endgroups contained DOPA residues.

Synthesis of Linear and Branched NH2-DOPA-PEG (3 and 5). Amin terminated DOPA-PEG 3 and 5 were obtained from the treatment of 2 and 4 with TFA. In a typical reaction, Boc-DOPA-PEG (1.0 g) was dissolved in DCM (5 mL) and treated with TFA (5 mL) for 30 min. The product was isolated by the precipitation with ether. Kaiser test on the final product was positive.

Complexation of DOPA-PEG with Fe3+. Separately, a 25 mM FeCl3 solution in pH 7.0 buffer (50mM Bis-Tris), and a 15 mg/ml solution of I in pH 7.0 buffered NaCl (.15M NaCl, 50mM Bis-Tris), were bubbled with argon for 20 minutes. 1ml of the DOPA-PEG solution was transferred into a quartz cuvette, sealed, and purged briefly again with Ar. Aliquots of the Fe3+ solution were then added to the cuvette with stirring, and the visible light absorbance spectrum (U-1010, Hitachi Instruments) was obtained to detect complexation between DOPA and Fe3+.

Preparation of Liposomes Containing Fe3+ (Fe-liposomes). Fe-liposomes were prepared by modification of a previously described protocol for preparation of CaCl2 liposomes.10 A dry film of dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) (9:1 DPPC:DMPC) was hydrated with 25 mM FeCl3 in pH 7.0 buffered NaCl (.1M NaCl, 50mM Bis-Tris, 451 mosm), and processed as described previously to yield Fe-liposomes suspended in Fe3+-free NaCl (0.245 M, 449 mosm). The Fe-liposomes were suspended to a final lipid concentration of 20 mg/ml in 0.245 M NaCl, and stored at 20ºC until use. Dynamic light scattering analysis (N4Plus, Beckman-Coulter Corp.) of the Fe-liposomes yielded an average particle size of 1.55 µm. The [Fe3+] in the final liposome suspension was determined by ICP to be 16mM.

Thermally Triggered Complexation of PEG-DOPA with Fe3+. 136µl of Fe-liposome suspension was introduced into a Ar-purged water jacketed quartz cuvette, after which 1ml of I (15mg/ml) in buffered NaCl (.15M NaCl, 50mM Bis-Tris, pH 7.0) was added. The spectrophotometer baseline was recorded with the suspension in both reference and sample beams, after which the absorption spectrum was recorded after the sample was incubated for 30 minutes at 20ºC and 30ºC. Following this, the temperature of the sample cuvette was changed to 37ºC and the absorbance spectrum recorded after 5, 10, 15, 20, 25 and 30 minutes of incubation.
Results and Discussion

Mefp1, a MAP from the mussel Mytilus edulis, was previously found to form highly stable complexes with Fe\(^{3+}\). The Mefp1 complexes range in color from pink to purple, corresponding to tris- and bis-catecholate complexes between DOPA and Fe\(^{3+}\), respectively. Although the physiologic significance of Fe-DOPA complexes is not known, it has been speculated that Fe\(^{3+}\) binding could lead to crosslinking or curing of a Mefp1-containing lacquer in mussel byssus. By synthesizing DOPA-PEG conjugates, we seek to accomplish two goals. The first is to exploit the formation of intermolecular Fe\(^{3+}\)-DOPA complexes as a route to crosslinking of PEG-based hydrogels. The second is to introduce bioadhesive moieties into PEG-based hydrogels for the purposes of tissue repair as well as adhesion to and drug delivery across tissue interfaces.

Several linear and branched DOPA-PEG conjugates with acid (1), amine (3 and 5), and Boc-protected (2 and 4) endgroups were synthesized. The Fe\(^{3+}\) complexation behavior of these molecules was studied using the spectrophotometric approach of Taylor et al. Briefly, this method consisted of recording the visible light absorbance of the DOPA-PEG polymers as Fe\(^{3+}\) is introduced into the system. Addition of Fe\(^{3+}\) to DOPA-PEG solution at 20°C results in the formation of purple complexes during the first hour (data not shown). As can be seen in Figure 1, peak maxima range from 580nm at low [Fe\(^{3+}\)] to 589nm at higher [Fe\(^{3+}\)]. At this point it is not yet known whether the observed complexes represent the bis (DOPA:Fe\(^{3+}\)=2), tris (DOPA:Fe\(^{3+}\)=3), or some other form of the complex. Although qualitatively similar to the spectra reported by Taylor et al. for the bis-complexes with Mefp1, the observed peak maxima of the complexes with DOPA-PEG are at higher wavelengths. Thus, the exact nature of the complexes is not known at this point. In the case of DOPA-PEG 1, the presence of the acidic endgroups complicates this analysis since they could participate in complex formation along with the DOPA residues. It is also not known whether the complexes of DOPA-PEG with Fe\(^{3+}\) are intra- or intermolecular. Experiments are in progress to investigate these effects and to determine the impact of DOPA-PEG endgroups in general on Fe\(^{3+}\)-DOPA complexation.

Figure 1. Complexation of Fe\(^{3+}\) with DOPA-PEG 1. Aliquots of 25mM FeCl\(_3\) solution were sequentially added to a cuvette containing 1ml of DOPA-PEG 1 (15mg/ml) in BIS-TRIS buffer: A) 0, B) 10, C) 20, D) 30, E) 40, F) 50, G) 60, H) 70, I) 80 µl FeCl\(_3\). Each solution was equilibrated for 30 minutes prior to measurement.

We also investigated the possibility of thermally triggering complexation by stimuli-induced release of Fe\(^{3+}\) from lipid vesicles. Our approach to accomplish this mirrors our earlier work in which calcium-loaded lipid vesicles were used to form calcium phosphate mineral and polymer hydrogels. In our previous systems, liposomes were designed to release entrapped ions when heated from ambient to body temperature, a result of an increased lipid bilayer permeability at the lipid chain melting temperature. In this work, we have entrapped a 25mM FeCl\(_3\) solution within liposomes, and suspended these liposomes in Fe\(^{3+}\)-free buffer. The resulting liposome suspension had a light orange color which reflects the color of the entrapped FeCl\(_3\) solution.

When Fe-liposomes were combined with a clear solution of 1 at 20°C, the suspension maintained a faint orange color for at least several hours when maintained at 20°C. As shown in Figure 2, little or no color change occurred when the suspension was heated to 30°C, however when heated to 37°C a purple complex with a peak maximum at 578nm rapidly formed during the first few minutes. This behavior can be explained by the thermally triggered release of Fe\(^{3+}\) from liposomes, resulting in the complexation of Fe\(^{3+}\) with DOPA-PEG. The peak maximum shifted to 594nm and increased significantly in intensity during the first 30 minutes at 37°C.

Figure 2. Thermally triggered complexation of Fe\(^{3+}\) with DOPA-PEG 1. 136µl of Fe-liposomes were combined with 1ml DOPA-PEG 1 in BIS-TRIS buffer. From bottom to top: 30 minutes at 20°C, 30 minutes at 30°C, and 0, 5, 10, 15, 20, 25, and 30 minutes at 37°C.

Conclusions

Linear and branched DOPA-PEG conjugates with acid, amine, and Boc-protected endgroups were synthesized. The linear DOPA-PEG with acidic endgroups was found to form complexes with free Fe\(^{3+}\) as determined by spectrophotometric analysis. Similar complexes between Fe\(^{3+}\) and DOPA-PEG were rapidly formed when a DOPA-PEG solution containing Fe-liposomes was heated to 37°C. Ongoing experiments are aimed at elaborating the nature of the complexes formed (i.e. bis versus tris) and whether the formation of intermolecular complexes between PEG-bound DOPA endgroups can result in physical gelation.

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References

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