Aminooxy Pluronics: Synthesis and Preparation of Glycosaminoglycan Adducts

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Novel biomaterials have been prepared in which glycosaminoglycans (GAGs) are chemically modified to create amphiphilic multiblock copolymers that are able to adhere to hydrophobic surfaces and can self-assemble into cross-linker-free hydrogels. First, the triblock poly(ethylene oxide)−polypropylene oxide copolymers (Pluronics) were converted into the previously unknown aminooxy (AO) derivatives. Both mono-AO and bis-AO Pluronics (AOPs) were synthesized and fully characterized in order to prepare tetrablock and pentablock copolymers, respectively. Second, the AOPs were coupled to the uronic acid carboxylates of heparin (HP) and hyaluronic acid (HA) using carbodiimide chemistry in order to give the previously undescribed amidooxy GAG derivatives. The coupling chemistry was confirmed using a newly prepared fluorescent AO reagent. Third, AOP-heparin and AOP-fluorescently labeled heparin were shown to adsorb efficiently to polystyrene surfaces, as determined by IL-8 based ELISA and fluorescence measurements, respectively. Fourth, AOP-linked fluorescently labeled HA was shown to adsorb efficiently to plastic surfaces. Finally, three different AOPs were evaluated for self-assembling hydrogel formation by AOP−HA pentablock polymers. In short, AOP−GAG adducts are semisynthetic amphiphilic biomacromolecules that offer a range of valuable practical opportunities for surface modification, preparation of cross-linker-free hydrogels, and formation of self-assembling mimics of the extracellular matrix.

Introduction

The poly(ethylene oxide)−poly(propylene oxide)−poly(ethylene oxide), or PEO−PPO−PEO, triblock copolymers are members of the Pluronic family of surfactants and have unique properties which have led to numerous commercial applications (Figure 1).[1] The hydrophobic PPO block adsorbs to hydrophobic (e.g., polystyrene) surfaces, whereas the PEO arms provide a hydrophilic coating that is protein-repellent.[2−4] The majority of Pluronics form micelles in water solution with an inner core consisting mainly of the hydrophobic PPO and hydrate PEO forming the corona.[5] Pluronics have low toxicity and are approved by the FDA for direct use in medical applications and as food additives.[1] Surface treatments with Pluronics reduce platelet adhesion, protein adsorption, and bacterial adhesion, as summarized recently by Caldwell.[6] The coating layer, which is rich in dynamic PEO chains, shows low nonspecific adsorption of biomolecules from biological fluids.

Chemically modified Pluronics have been developed to broaden the applications of this class of surface-active polymers.[6−8] For example, chemical modification of the hydroxyl ends of the PEO blocks with 4-nitrophenyl chloroformate[6] permitted coupling to thiol groups of fibronectin and other proteins, with subsequent surface immobilization of these proteins.[9,10] Pluronic F108 [$(PEO)_{129}(PPO)_{56}(PEO)_{129}$], a water- and alcohol-soluble solid, has been used for surface modifications,[11−13] to generate macroporous beads,[14] and for steric stabilization of liposomes.[15,16] Pluronic F127 has been employed to make chondrocyte-seeded hydrogels in vivo, resulting in neocartilage growth of autologous cells in a pig model,[17,18] to promote attachment of human gingival fibroblasts[19] and to deliver indomethacin[20] and insulin[21] in rats. Both F108 and F127 spontaneously form biocompatible hydrogels.

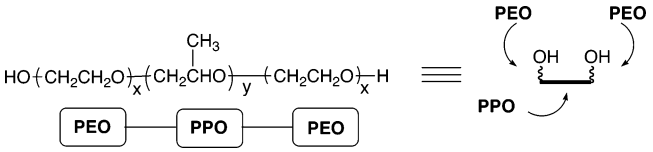

Figure 1. Structures of the Pluronic triblock copolymers

Pluronics show temperature-sensitive self-assembly in aqueous solution in a fashion dependent on molecular composition of the PEO and PPO segments.[22−24] Besides temperature-dependent micellization, the most characteristic property of aqueous Pluronic solutions is gel formation at high concentration.[23] As a result, Pluronics are an important class of surfactants and have widespread industrial applications,[25,26] as well as applications in pharmaceutics (drug solubilization and controlled release),[27−30] bioprocessing,[31] and separations.[32]

Hyaluronan (HA) is a nonsulfated glycosaminoglycan (GAG) consisting of repeating disaccharide units (β-1,4-D-glucuronic acid-β-1,3-*N*-acetyl-D-glycosamine), with molecular weights (MW) up to 5000 kDa. A component of all connective tissues[33] and extracellular matrix (ECM), HA promotes cell mobility and proliferation, morphogenesis,[34] and wound repair.[35] In cartilage, HA forms a scaffold for binding large chondroitin sulfate (CS) proteoglycans (PGs) which are present at 25−50 mg/g.[33] HA retains CSPGs molecules in the ECM through specific protein−HA interactions.

To exploit the potential of Pluronic-based hydrogels in the synthetic ECM technology, we prepared and characterized surface-active adducts of Pluronics with two GAGs, heparin (HP) and HA. First, we present a new chemical modification of Pluronics, based on the introduction of reactive aminooxy groups at the PEO terminus, a method that had been previously

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applied to star-type PEO polymers.[36] The new aminooxy (AO) derivatives of Pluronics, or AOPs, were prepared in two types. First, the mono-methoxy mono-AOPs were prepared as precursors for surface-active tetrablock Pluronic−GAG copolymers. Second, the bis-AOPs were prepared, affording self-assembling pentablock GAG−Pluronic−GAG copolymer units. Since aminooxy groups have a pK_a value of approximately 5, they are nucleophilic at the low pH conditions required for reaction of a carbodiimide with a protonated carboxylic acid. This pK_a value is considerably lower than that of a primary amine (pK_a ∼ 12),[37,38] and much closer to the pK_a values of hydrazides (pK_a 2−4). Thus, as with hydrazides, AO compounds are nucleophiles at the acidic conditions required for reaction with carbodiimide-activated uronic acids of GAGs.[37]

Heparin (HP) is commonly used as an anticoagulant.[39] Heparization has been recognized as the most potent and reliable tromboresistant surface. Several technologies to prepare copolimer-grafted heparin and their coating onto biomedical surfaces have been invented. [40−44] Since the Pluronics are well-recognized for adsorption to plastic surfaces[11−13,45−47] we anticipated that GAG-conjugated AOPs would provide a novel method for coating hydrophobic surfaces with these hydrophilic biopolymers. Thus, herein we describe the synthesis of six AOPs with different molecular compositions and physical properties. We further describe the carbodiimide-mediated conjugation of these AOPs with HA and with HP to produce surface-active multiblock copolymers. We then demonstrate specific applications in surface adsorption and cross-linker-free gelation of particular AOP-GAG adducts.

Materials and Methods

General. Chemicals were obtained from Aldrich, Acros, and BASF and were used without further purification unless otherwise specified. Solvents were reagent-grade and distilled before use: THF was distilled from sodium wire and CH_2Cl_2 was distilled from CaH_2. Reactions requiring anhydrous conditions were carried out in oven-dried glassware (2 h, 120 °C) under inert atmosphere (Ar) unless otherwise indicated. Concentration in vacuo refers to the use of rotary evaporator for solvent removal. Sodium hydride was rinsed free of mineral oil with hexanes three times before use. DEAD refers to diethyl azodicarboxylate, Ph_3P to triphenylphosphine, and PhtOH to *N*-hydroxyphthalimide. NMR spectra were recorded at 400 MHz (^{1}H) and 101 MHz (^{13}C) at ambient temperature. Chemical shifts are reported relative to those of internal chloroform (δ_H 7.24), acetone (δ_H 2.04), for ^{1}H; chloroform (δ_C 77.0), acetone (δ_C 206.0) for ^{13}C. Symbols: s, singlet; d, doublet; t, triplet; bs, broad signal. Coupling constants (*J*) are all reported in Hz.

Fermentation-derived HA (sodium salt, M_w 1.5 MDa) was purchased from Clear Solutions Biotechnology, Inc. (Stony Brook, NY), and was used after degradation in acid and determined by gel permeation chromatography (M_n = 90 kDa, M_w = 190 kDa).[48] 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Unfractionated heparin (UFH, M_n = 10651; M_w = 12 935 kDa; polydispersity 1.214) was purchased from Acros (Houston, TX). Based on the UV data provided by the manufacturer, the protein content was estimated to be less than 5%. BODIPY (BODIPY−FL hydrazide) was purchased from Molecular Probes (Eugene, OR). Human interleukin-8 (IL-8) and biotinylated anti-human IL-8 for enzyme-linked immunosorbent assay (ELISA) as previously described[48] were purchased from Peprotech (Rock Hill, NJ). Dulbecco's phosphate-buffered saline (DPBS), streptavidin horseradish peroxidase conjugate (SA-HRP), 3,3′,5,5′-tetramethyl benzidine (TMB) and 3-[*N*-morpholino]propanesulfonic acid (MOPS) were purchased from Sigma (St. Louis, MO). Stabilguard blocking solution was from Surmodics (Eden Prairie, MN).

Gel permeation chromatography (GPC) analysis was performed using the following system: Waters 515 HPLC pump, Waters 410 differential refractometer, Waters 486 tunable absorbance detector, Ultrahydrogel 1000 columns (7.8 mm i.d. × 300 mm) (Milford, MA). Eluent was 200 mM phosphate buffer (pH 6.5): MeOH = 80:20 (v/v) and the flow rate was 0.3 or 0.5 mL/min. The system was calibrated with standard HA samples from Hyalose (Oklahoma City, OK).

***N*-(5-Dimethylamino-1-naphthalenesulfonamido)-2-methylamino Ethanol (3).** To a solution of (2-methylamino)ethanol (**2**) (33 μL, 0.4 mmol) and triethylamine (129 μL, 0.93 mmol) in anhydrous CH_2Cl_2 was added a solution of Dansyl chloride (**1**) (100 mg, 0.37 mmol) in CH_2Cl_2 at 0 °C. The mixture was stirred for 1 h at room temperature (rt). The solvent was then evaporated, and the residual oil obtained was purified using silica gel flash chromatography (CH_2Cl_2:CH_3OH, 95:5). Compound **3** was obtained as a green oil (108 mg, 0.35 mmol, 95% yield). R_f = 0.38 (hexanes:acetone 6:4). ^{1}H NMR ($CDCl_3$): δ 8.52 (d, 1H, J = 8.4), 8.31 (d, 1H, J = 8.4), 8.16−8.14 (m, 1H), 7.55−7.47 (m, 2H), 7.15 (d, 1H, J = 7.2), 3.72 (t, 2H, J = 5.2), 3.31 (t, 2H, J = 5.2), 2.92 (s, 3H), 2.85 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 151.8, 133.6, 130.5, 130.1, 130.0, 129.9, 128.2, 123.1, 119.2, 115.2, 59.9, 51.8, 45.3, 35.2. LRMS (CI) *m*/*z*: (M^+, 100.00). HRMS for $C_{15}H_{21}N_2O_3S$ (M+1): found: 309.1268, calcd: 309.1273.

***N*-(5-Dimethylamino-1-naphthalenesulfonamido)-2-methylamino ethyloxy-1-phthalimide (4).** Compound **3** (67 mg, 0.22 mmol), Ph_3P (74 mg, 0.28 mmol), and *N*-hydroxyphthalimide (46 mg, 0.28 mmol) were dissolved in anhydrous THF (5 mL), and then DEAD (54 μL, 0.28 mmol) was added at 10 °C. The reaction was stirred overnight at room temperature, concentrated in vacuo, and purified by flash chromatography (CH_2Cl_2:CH_3OH, 200:3) to give **4** (60 mg, 0.19 mmol) in 88% yield. R_f = 0.48 (hexanes: acetone 6:4). ^{1}H NMR ($CDCl_3$): δ 8.59 (d, 1H, J = 8.8), 8.41 (d, 1H, J = 8.8), 8.26−8.24 (m, 1H), 7.89−7.79 (m, 4H), 7.63−7.57 (m, 2H), 7.23 (d, 1H, J = 7.6), 4.44 (t, 2H, J = 5.6), 3.75 (t, 2H, J = 5.6), 3.16 (s, 3H), 2.93 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 163.2, 151.6, 134.6, 133.9, 130.4, 130.0, 129.6, 128.7, 128.1, 123.6, 123.1, 119.5, 115.2, 77.3, 47.8, 45.3, 35.9. LRMS (CI) *m*/*z*: 454.4 (M^+, 100.00). HRMS for $C_{23}H_{24}N_3O_5S$ (M^+ + 1): found: 454.1431, calcd: 454.1436.

***N*-(5-Dimethylamino-1-naphthalenesulfonamido)-2-methyl ethyloxyamine (5).** To a solution of **4** (46.7 mg, 0.1 mmol) in CH_2Cl_2 (5 mL) was added hydrazine monohydrate (9.98 μL, 0.21 mmol) at 0 °C. After 1 h stirring at room temperature, the reaction mixture was filtered through a pad of Celite, concentrated, and purified using flash chromatography (CH_2Cl_2:CH_3OH, 200:3). The desired compound **5** was isolated as a greenish oil (31 mg, 0.095 mmol, 95% yield). R_f = 0.43 (hexanes:acetone 6:4). ^{1}H NMR ($CDCl_3$): δ 8.51 (d, 1H, J = 8.8), 8.32 (d, 1H, J = 8.8), 8.16−8.14 (m, 1H), 7.54−7.47 (m, 2H), 7.15 (d, 1H, J = 8.0), 5.44 (bs, 2H), 3.76 (t, 2H, J = 5.2), 3.45 (t, 2H, J = 5.2), 2.86 (s, 3H), 2.85 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 151.6, 134.3, 130.2, 130.1, 130.0, 129.6, 127.9, 123.0, 119.5, 115.1, 72.4, 47.8, 45.4, 34.7. LRMS (CI) *m*/*z*: 324 (M^+, 100.00). HRMS for $C_{15}H_{22}N_3O_3S$ (M^+, 100.00); found: 324.1377, calcd: 324.1382.

***N*-5-Dimethylaminonaphthalene-1-sulfonic Acid (2-Isopropylideneaminooxyethyl)methyl Amide (6).** Treatment of AO-dye **5** with acetone followed by silica purification afforded compound **6**, which was isolated quantitatively as a greenish oil. R_f = 0.53 (hexanes:acetone, 6:4); ^{1}H NMR ($CDCl_3$): δ 8.51 (d, 1H, J = 8.8), 8.32 (d, 1H, J = 8.8), 8.16−8.14 (m, 1H), 7.54−7.47 (m, 2H), 7.15 (d, 1H, J = 7.2), 4.14 (t, 2H, J = 5.6), 3.49 (t, 2H, J = 5.6), 2.92 (s, 3H), 2.85 (s, 6H), 1.78 (s, 2H), 1.72 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 155.3, 151.6, 134.3, 130.2, 130.1, 130.0, 129.6, 127.9, 123.0, 119.5, 115.1, 71.2, 48.6, 45.3, 35.3, 21.7, 15.5. LRMS (CI) *m*/*z*: 364 (M^+, 100.00). HRMS for $C_{18}H_{26}N_3O_3S$ (M^+ + 1): found: 364.1690, calcd: 364.1695.

Pluronic F88 Bis-*O*-phthalimide Derivative (8a). To a stirred solution of Pluronic F88 (13.98 g, 1.16 mmol), Ph_3P (6.1 g, 23.3 mmol), and *N*-hydroxyphthalimide (3.78 g, 23.2 mmol) in dry THF (75 mL) was added DEAD (3.5 mL, 23.2 mmol) at 10 °C. The reaction was stirred overnight at room temperature and then quenched by precipitation of the product with petroleum ether. The crude **8a** was recrystallized four times using two solvent systems: THF/petroleum ether

and THF/diethyl ether (twice for each solvent system). The pure product **8a** was obtained as a loose white solid (12.28 g, 86%). ^{1}H NMR ($CDCl_3$): δ 7.80−7.78 (4H, m), 7.72−7.69 (4H, m), 4.32 (t, 4H, J = 4.4 Hz), 3.83−3.68 (m, 8H), 3.60−3.27 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)−O)$_n$), 2.21 (s, 9H), 1.10−1.08 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$).^{13}C NMR ($CDCl_3$): δ 163.9, 134.2, 128.8, 123.2, 75.2, 75.0, 74.8, 73.1, 72.6, 70.2 (bs), 69.0, 17.2, 17.1.

Pluronic F108 Bis-*O*-phthalimide Derivative (8b). Following the procedure described for the synthesis of compound **8a**, the pure product **8b** was obtained as a flocculent white solid (11.4 g, 74%). ^{1}H NMR ($CDCl_3$): δ 7.79−7.77 (4H, m), 7.71−7.69 (4H, m), 4.32 (t, 4H, J = 4.4 Hz), 3.82−3.75 (m, 8H), 3.70−3.26 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)−O)$_n$), 2.32 (s, 6H), 1.10−1.08 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$)).^{13}C NMR ($CDCl_3$): δ 163.9, 135.0, 128.8, 124.3, 75.5, 73.6, 73.1, 70.8, 17.6.

Pluronic F127 Bis-*O*-phthalimide Derivative (8c). Following the procedure described for the synthesis of compound **8a**, the pure product **8c** was obtained as a flocculent white solid (12.8 g, 83%). ^{1}H NMR ($CDCl_3$): δ 7.80−7.78 (4H, m), 7.71−7.69 (4H, m), 4.32 (m, 4H), 3.82−3.75 (m, 8H), 3.74−3.28 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)−O)$_n$), 1.10−0.96 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$)).^{13}C NMR ($CDCl_3$): δ 163.9, 134.3, 128.8, 123.3, 75.3, 75.2, 75.1, 74.9, 73.2, 72.7, 70.4 (bs), 69.1, 17.3, 17.2.

Pluronic F88 Bis-aminooxy Derivative (9a). Hydrazine monohydrate (0.71 mL) was added to a stirred solution of **8a** (12.38 g) in CH_2Cl_2 (50 mL) at 0 °C. The mixture was stirred for 1.5 h at room temperature, the precipitate was removed by filtration, and the solvent was removed in vacuo. The residue was dissolved in dry THF and reprecipitated using diethyl ether. Re-crystallization using THF/petroleum ether yielded the pure compound **9a** as a white solid (11 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.81−3.78 (m, 8H), 3.71−3.30 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)−O)$_n$), 2.02 (s, 10H), 1.24−0.98 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$); ^{13}C NMR (101 MHz, $CDCl_3$): δ 75.5, 75.3, 75.2, 73.3, 72.9, 70.5 (bs), 17.4, 17.3;^{1}H NMR (acetone-d_6): δ 4.06 (t, 4H, J = 4.8), 3.78−3.26 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**-(CH$_3$)-O)$_n$), 2.07 (12H), 1.05−1.09 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$).

Pluronic F108 Bis-aminooxy Derivative (9b). Following the procedure described for the synthesis of compound **9a**, the pure compound **9b** was obtained as a white solid (10 g, 89%). ^{1}H NMR ($CDCl_3$): δ 3.75−3.72 (m, 8H), 3.60−3.40 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)−O)$_n$), 1.22−0.89 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$); ^{13}C NMR ($CDCl_3$): δ 75.3, 75.1, 75.1, 74.9, 74.5, 73.2, 72.7, 70.3 (bs), 69.4, 17.3, 17.1;^{1}H NMR (acetone-d_6): δ 4.06 (t, 4H, J = 4.8), 3.78−3.28 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)−O)$_n$), 1.05−1.09 (m, (−O−CH$_2$−CH−(C**H**$_3$)-O)$_n$).

Pluronic F127 Bis-aminooxy Derivative (9c). Following the procedure described for the synthesis of compound **9a**, the pure compound **9c** was obtained as a white solid (11 g, 90%). ^{1}H NMR ($CDCl_3$): δ 3.80−3.76 (m, 8H), 3.74−3.24 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)O)$_n$), 1.24−0.92 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$); ^{13}C NMR ($CDCl_3$): δ 75.2, 75.0, 75.0, 74.8, 74.4, 73.0, 72.6, 70.2 (bs), 69.2, 17.2, 17.0; ^{1}H NMR (acetone-d_6): δ 4.06 (t, 4H, J = 4.8), 3.78−3.28 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)−O)$_n$), 1.10−1.09 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$).

Pluronic F88 Mono-methoxy Derivative (10a). To a solution of F88 (0.25 mmol, 3 g) in anhydrous THF was added a suspension of NaH (1.49 mmol, 35.7 mg) in THF at room temperature. The reaction mixture was stirred for 30 min, cooled to 0 °C, and then CH_3I (0.015 mmol, 9.3 μL) was added. The reaction was stirred overnight at 6 °C and then 1 h at room temperature. Methanol was added, and the solvent was removed in vacuo. The residue was redissolved in THF, and pure product was then precipitated with diethyl ether. Compound **10a** was obtained as a white solid (2.8 g, 93%). ^{1}H NMR (acetone-d_6): δ 3.75−3.73 (m, 3H), 3.73−3.36 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)O)$_n$), 3.27 (s, 3H), 1.25−0.92 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$);^{13}C NMR (acetone-d_6): δ 75.5, 75.4, 75.2, 75.1, 73.4, 73.1, 73.1, 70.9, 70.5 (bs), 68.6, 28.4, 17.4, 17.3.

Pluronic F108 Mono-methoxy Derivative (10b). To a solution of F108 (10 g) in anhydrous THF was added a suspension of NaH in dry THF (4.17 mmol, 100 mg) at room temperature. The reaction mixture was stirred for 30 min, cooled to 0 °C, and treated with CH_3I (0.40 mmol, 25 μL). The reaction was stirred overnight at 6 °C and then 1 h at room temperature. Methanol was added, and the solvent was removed in vacuo. The crude product was redissolved in THF, and pure product was precipitated with diethyl ether. Compound **10b** was obtained as a white solid (9.5 g, 95%). ^{1}H NMR (acetone-d_6): δ 3.75−3.74 (m, 7H), 3.73−3.37 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)O)$_n$), 3.28 (s, 3H), 1.26−0.92 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$);^{13}C NMR (acetone-d_6): δ 76.0, 75.9, 75.8, 73.9, 73.7, 73.6, 71.1 (bs), 28.9, 17.9, 17.7.

Pluronic F127 Mono-methoxy Derivative (10c). To a solution of F127 (10 g) in anhydrous THF was added a suspension of NaH in THF (4.38 mmol, 105 mg) at room temperature The reaction mixture was stirred for 30 min, cooled to 0 °C, and treated CH_3I (0.45 mmol, 28 μL). The reaction was stirred overnight at 6 °C and then 1 h at room temperature. Methanol was added, and the solvent was removed in vacuo. The crude product was redissolved in THF, and pure product was precipitated with diethyl ether. Compound **10c** was obtained as a white solid (9.5 g, 95%). ^{1}H NMR (acetone-d_6): δ 3.76−3.74 (m, 4H), 3.73−3.33 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)O)$_n$), 3.28 (s, 3H), 1.26−0.92 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$); ^{13}C NMR (acetone-d_6): δ 75.8, 75.7, 75.5, 73.7, 73.4, 73.4, 70.8 (bs), 28.7, 17.7, 17.6.

Pluronic F88 Mono-methoxy Mono-*O*-phthalimide Derivative (11a). To a stirred solution of **10a** (2.8 g, 0.23 mmol), Ph_3P (608 mg, 2.32 mmol), and *N*-hydroxyphthalimide (378 mg, 2.32 mmol) in THF (50 mL) was added DEAD (365 μL, 23.2 mmol) at 10 °C. The reaction was stirred overnight at room temperature, filtered through a pad of Celite and activated charcoal, concentrated, and precipitated from THF by addition of petroleum ether. The crude product **11a** was re-crystallized four times using two solvent systems: THF/petroleum ether and THF/diethyl ether (twice for each system). The 99% pure product **11a** was obtained as a loose light yellow solid (2.1 g, 75%). ^{1}H NMR (acetone-d_6): δ 7.89−7.87 (m, 4H), 4.34 (t, 2H, J = 4.8), 3.84−3.81 (m, 2H), 3.76−3.71 (m, 5H), 3.70−3.29 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)O)$_n$), 3.28 (s, 3.12H), 1.26−0.92 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$); ^{13}C NMR (acetone-d_6): δ 163.9, 135.3, 130.0, 123.9, 77.6, 76.1, 75.9, 75.8, 75.7, 73.9, 73.8, 73.7, 71.5, 71.2 (bs), 70.2, 69.3, 17.9, 17.8.

Pluronic F108 Mono-methoxy Mono-*O*-phthalimide Derivative (11b). Following the procedure described for the synthesis of compound **11a**, the 91% pure product **11b** was obtained in the form of a loose yellow solid (8.3 g, 83%). ^{1}H NMR (acetone-d_6): δ 7.90−7.86 (m, 4H), 4.35−4.33 (m, 2H), 3.84−3.82 (m, 2H), 3.76−3.71 (m, 6H), 3.72−3.29 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)O)$_n$), 3.28 (s, 2.44H), 1.26−0.92 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$); ^{13}C NMR (acetone-d_6): δ 163.9, 135.3, 130.0, 123.8, 76.0, 75.9, 75.7, 73.9, 73.7, 73.6, 71.1, 70.1, 28.9, 17.8, 17.7.

Pluronic F127 Mono-methoxy Mono-*O*-phthalimide Derivative (11c). Following the procedure described for the synthesis of compound **11a**, the 90% pure product **11c** was obtained in the form of a loose yellow solid (6.8 g, 67%). ^{1}H NMR (acetone-d_6): δ 7.90−7.85 (m, 4H), 4.35−4.33 (m, 2H), 3.84−3.81 (m, 2H), 3.75−3.73 (m, 6H), 3.71−3.43 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)O)$_n$), 3.28 (s, 3.63H), 1.26−0.93 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$); ^{13}C NMR (acetone-d_6): δ 163.9, 135.6, 130.0, 123.8, 77.5, 76.0, 75.9, 75.8, 73.9, 73.7, 73.6, 71.4, 71.1, 70.15, 69.2, 17.9, 17.8.

Pluronic F88 Mono-methoxy Mono-aminooxy Derivative (12a). Hydrazine monohydrate (34 μl, 0.69 mmol) was added to a stirred solution of **11a** (2.1 g, 0.17 mmol) in CH_2Cl_2 (30 mL) at 0 °C. It was stirred for 1.5 h at room temperature, and the precipitate was removed by filtration. After removal of solvents, the residue was dissolved in THF and the product was precipitated using diethyl ether. Re-

crystallization using THF/petroleum ether led to the 97% pure compound **12a** as a flocculent white solid (1.87 g, 90%). ^{1}H NMR (acetone-d_6): δ 4.06 (t, 2H, J = 4.8), 3.76−3.74 (m, 7H), 3.65−3.28 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)O)$_n$), 3.28 (s, 3.13H), 1.26−0.92 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$); ^{13}C NMR (acetone-d_6): δ 75.9, 75.8, 75.7, 73.8, 73.6, 73.6, 71.4, 71.1 (bs), 70.0, 28.9, 17.8, 17.7.

Pluronic F108 Mono-methoxy Mono-aminooxy Derivative (12b). Following the procedure described for the synthesis of compound **12a**, the pure product **12b** was obtained as a light yellow solid (8.3 g, 93%). ^{1}H NMR (acetone-d_6): δ 4.06 (t, 2H, J = 4.8), 3.75−3.71 (m, 7H), 3.71−3.28 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)O)$_n$), 3.28 (s, 2.83H), 1.25−0.93 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$); ^{13}C NMR (acetone-d_6): δ 76.0, 75.7, 75.7, 73.9, 73.6, 73.6, 71.4, 71.1, 70.0, 28.9, 17.8, 17.7.

Pluronic F127 Mono-methoxy Mono-aminooxy Derivative (12c). Following the procedure described for the synthesis of compound **8a**, the 92% pure product **12c** was obtained as a light yellow solid (6.3 g, 92%). ^{1}H NMR (acetone-d_6): δ 4.06 (t, 2H, J = 4.8), 3.76−3.73 (m, ~6H), 3.71−3.28 (bs, (C**H**$_2$−C**H**$_2$−O)$_n$, (−O−C**H**$_2$−C**H**−(CH$_3$)O)$_n$), 3.28 (s, 3.48H), 2.79−2.77 (bs, 3H), 1.26−0.93 (m, (−O−CH$_2$−CH−(C**H**$_3$)−O)$_n$); ^{13}C NMR (acetone-d_6): δ 76.0, 75.9, 75.8, 73.9, 73.7, 73.6, 71.1, 28.9, 17.8, 17.7.

Fluorescamine-Labeled 12b AOP (13). A solution of **12b** (88 mg) and fluorescamine (2 mg, 0.0072 mmol) in anhydrous CH_3CN (2 mL) was stirred for 1 h, and the solvent was evaporated. The residue was dissolved in THF and precipitated with petroleum ether to yield 75 mg of **13** as a yellow powder. TLC of the resulting crystals showed the absence of any starting fluorescamine reagent. A solution of labeled AOP **13** in CH_2Cl_2 was characterized by green fluorescence when activated by long wavelength UV. The absorption spectrum showed: λ_{max} = 350 nm (CH_3OH) and the fluorescence excited at 350 nm was λ_{em} = 460 nm (CH_3CN).

NDA-Labeled 12b AOP (14). To a stirred suspension of 2,3-naphthalene dialdehyde (5 mg, 0.027 mmol) in CH_3OH (1 mL) at room temperature was added NaCN (1.3 mg, 0.027 mmol). The reaction mixture became homogeneous and turned pale yellow. Within 1 min, a solution of 394 mg of **12b** in CH_3OH (2 mL) was introduced by syringe. The fluorescent reaction mixture was stirred for an additional 1 h and then evaporated to dryness under reduced pressure. The residue was dissolved in THF and precipitated with petroleum ether. The resulting crystals were washed with diethyl ether to yield 350 mg of a lightly yellow powdery product **14**. TLC of the crystalline product showed the absence of any starting NDA reagent. A solution of **14** in CH_2Cl_2 was characterized by blue fluorescence when excited with long wavelength UV. The absorption spectrum showed: λ_{max} = 354 nm (CH_3OH) and the fluorescence excited at 354 nm was λ_{em} = 400 nm (CH_3CN).

Fluor-Labeled HA using EDC Chemistry. HA (50 mg) was dissolved in 20 mL of water to give 0.25% (w/v) solution. Then, a solution of 5 mg of dye **5** in 20 mL of ethanol was added. Next, 100 mg of NHS was added, and the solution pH was adjusted to 4.75 by adding 0.01 M HCl. Finally, 50 mg of EDC was added, and the solution pH was maintained at 4.75 by adding 0.01 M HCl. After 12 h, the solution was transferred to dialysis tubing (3500 Da cutoff) and dialyzed exhaustively against 100 mM NaCl, followed by dialysis against distilled water. The solution was then centrifuged, and the supernatant was lyophilized to give the fluorescent HA-dye **7**. GPC analysis showed only a high-mass fluorescent product (retention time: t_R = 14 min), which coeluted with the peak for HA itself as monitored at 210 nm. The UV spectrum for **7** did not show a discrete absorbance maximum. The excitation and emission spectra for an aqueous solution of the HA-dye conjugate **5** were determined using a fluorescence plate reader to as λ_{ex} = 320 nm and λ_{em} = 640 nm.

Aminooxy Pluronic (AOP)−heparin (HP). Bis-aminooxy−Pluronic **9b** (120 mg, 14,600 Da, bis-AO−F108) and 80 mg of UFH were dissolved in 8 mL of water. The pH was adjusted to 4.75 followed by addition of 3.1 mg of EDC. The pH was maintained at 4.75 by addition of HCl for 2 h, and then the reaction was stirred overnight at room temperature. Next, the reaction was loaded onto a diethylaminoethyl (DEAE, DE23 fibrous cellulose, Whatman, Florham Park, NJ) ion-exchange column containing a 15 mL bed volume (1 dry gram of DE23 fibrous cellulose) that had been preequilibrated with 0.1 M, pH 6.0 sodium acetate buffer. Unreacted EDC and **9b** were washed out using the same acetate buffer and AOP−HP conjugate was eluted by 1.0 M NaCl in 0.1 M sodium acetate buffer pH 6.0. The eluate was dialyzed against water, and the purity of AOP−HP was monitored by gel permeation chromatography (GPC). The purified AOP−HP was lyophilized, and the structure was verified by ^{1}H NMR. ^{1}H NMR (D_2O): δ 5.30−4.85 (bm, HP+**9b**), 4.30−3.80 (HP+**9b**), 3.80−3.00 (bm, HP+**9b**), 1.89 (s, HP), 1.00 (s, **9b**).

EDC-Mediated Synthesis of AOP−HA Hydrogel. HA (100 mg) was dissolved in 50 mL of distilled water to give 0.2% (w/v) solution, and then mono- (or bis-) aminooxy−Pluronic (**9a−c** or **12a−c**) (1.0 g) was added and dissolved under magnetic stirring. Next, the solution pH was adjusted to 4.75, and EDC (100 mg) was added. The solution pH was maintained at 4.75 by adding 0.01 M HCl until a gel formed.

EDC-Mediated Immobilization of HP on Surface-Adsorbed AOP. HP was immobilized on a polystyrene surface by coupling UFH to a preadsorbed AOP. Thus, 200 μL/well of a 5 mg/mL aqueous solution of bis AO−F108 (**9b**) was loaded into 96-well polystyrene plate (E&K Scientific, Campbell, CA) and incubated at room temperature overnight; excess AOP was removed using five rinses with water. Then, 10 mg/mL of EDC in 0.1 N MOPS buffer (pH 4.7) was added to each well (100 μL/well), together with 100 μL/well of UFH or BODIPY−FL−HP in 1:2 serial dilutions in the same MOPS buffer. Each reaction was allowed to proceed for 3 days at room temperature, and then each well of the plate was washed five times with MOPS buffer. For BODIPY−FL−HP, the immobilization was measured directly with fluorescence spectrometry (λ_{ex} = 503 nm; λ_{em} = 512 nm).

For nonfluorescently labeled HP, immobilized HP was quantified using a human interleukin-8 (IL-8)-based ELISA. In brief, after incubation with 200 μL/well Stabilguard at room temperature for 2 h, 200 μL/well human IL-8 (0.5 μg/mL) was added to each well and incubated 1 h, and then rinsed three times with DPBS. Next, biotinylated anti-human IL-8 (1 μg/mL) was added (200 μL/well), incubated 1 h, and rinsed three times with DPBS. Then, 200 μL/well SA-HRP (1:1000 dilution with DPBS) was added, incubated 30 min, rinsed three times with DPBS, and 200 μL/well of theTMB substrate was added. When the desired color intensity was achieved, the reaction was quenched with 200 μL/well of 1 M H_2SO_4 and the plate was read at 450 nm to measure the quantity of HP immobilized.

EDC-Mediated Immobilization of Fluorescent HA on Surface-Adsorbed AOP. Bis-aminooxy−Pluronic F108 (**9b**) (1.0 g) was dissolved in distilled water to give a 0.5% (w/v) solution. Then, 0.1 mL of solution was added into each well of a 96-well plate. After 12 h at room temperature, each well was washed five times with distilled water. Then, 0.1 mL of a solution of EDC (10 mg/mL) in 0.1 N MOPS, pH 4.7, was added into each well. Finally, 0.1 mL of 2 mg/mL fluorescein−HA (previously synthesized using EDC and 5-(((2-(carbohydrazino)methyl)thio)acetyl)amino fluorescein (Molecular Probes) in 0.1 N MOPS, pH 4.7), was added. The surface reaction was allowed to proceed for 3 days in the dark. Next, the plate was washed five times with DPBS, and the fluorescence was measured in a plate reader using λ_{ex} = 496 nm and λ_{em} = 520 nm.

Results and Discussion

Carbodiimide-Mediated Condensation of GAGs with an Aminooxy Fluor. Prior to the preparation of the aminooxy Pluronics (AOPs) and the carbodiimide-mediated condensation of AOPs with GAGs, we required a proof of concept experiment. As with hydrazides, the aminooxy NH_2 group retains its nucleophilicity in acidic aqueous media, thereby permitting

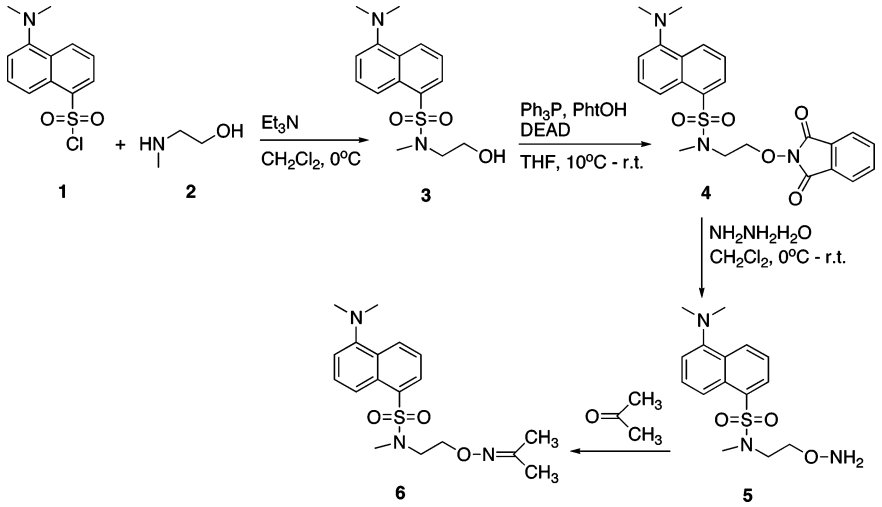

Figure 2. Synthesis of AO-terminated dye **5.** Experimental details are found in the Materials and Methods.

efficient condensation with carboxylic acids that have been activated as *O*-acyl urea adducts by reaction with a water-soluble carbodiimide. Thus, to test the possible formation of an amidooxy bond (−ONH−CO−) to a GAG, we synthesized a fluorescent aminooxy-terminated molecule that could be reacted with the carboxylic group of HA (Figure 2). We postulated that chemical shifts of a fluor-labeled AO derivative would be similar to the desired AO Pluronic and would facilitate identification using NMR. Dansyl chloride (**1**) is a nonfluorescent dye until it undergoes reaction with a primary amino group. Reaction of **1** with (2-methylamino)ethanol (**2**) afforded compound **3** in 95% yield. Condensation of alcohol **3** with *N*-hydroxyphthalimide (PhtOH) was performed under Mitsunobu reaction conditions,[49,50] which gave (after chromatography on silica gel) pure **4** in 88% yield. Deprotection with hydrazine monohydrate gave *N*-alkoxyamine **5** in 95% yield.

Compound **5** exhibited a greenish fluorescence when irradiated with long wavelength UV. Dansyl−AO (**5**) reacted rapidly with acetone to form compound **6**, caused shifting of the $-C\mathbf{H}_2O-$ signal observed in the ^{1}H NMR spectrum from 3.76 ppm for compound **5** (t, 2H, $J = 5.2$) to 4.14 ppm for compound **6**. (The complete NMR spectra for key new compounds are provided as Supporting Information). Since the aminooxy ONH_2 proton resonance in water can be broad and obscured by solvent, this simple expedient allowed rapid and sensitive detection of free aminooxy groups. We anticipated that this behavior would be important in characterizing both the free AOPs and the GAG−AOP adducts.

AO-dye **5** was condensed with HA in aq. ethanol using EDC at pH 4.75. The HA-dye conjugate **6** was purified by exhaustive dialysis, and then lyophilized to give a greenish solid. Only a high-mass fluorescent product peak was observed in the GPC spectrum, indicating all small molecule impurities had been efficiently removed during dialysis. Excitation and emission spectra for an aqueous solution of the HA-dye conjugate **6** were determined using a fluorescence plate reader: $\lambda_{ex} = 320$ and $\lambda_{em} = 640$ nm (Figure 3). This experiment confirmed the notion that aminooxy-containing compound could be readily condensed with carboxylic acids of GAGs to generate amidooxy linked adducts.

Synthesis and Characterization of Bis- and Mono-AO Pluronics. Three different Pluronics were selected for the preparation of aminooxy derivative for our study: F88 ($PEO_{103}-PPO_{39}-PEO_{103}$) (**7a**), F108 ($PEO_{132}-PO_{50}-PEO_{132}$) (**7b**), and F127 ($PEO_{100}-PPO_{65}-PEO_{100}$) (**7c**). This selection included

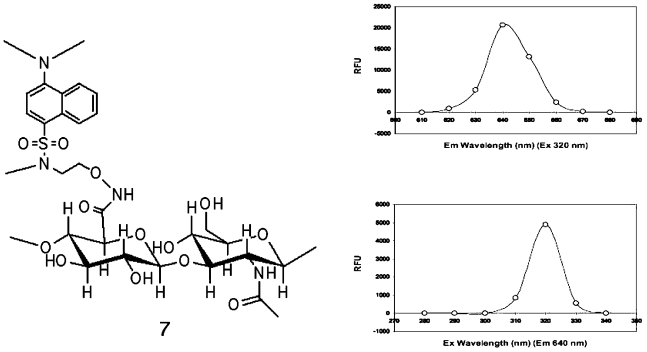

Figure 3. Right, chemical structure of HA-dye conjugate **7**; left, absorption and emission spectra of **7**.

different PPO/PEO ratios (from 0.19 to 0.3) and different molecular weights: 11,400, 14,600 and 12,600 Da. In addition, both F108 and F127 are known to spontaneously form biocompatible hydrogels, a feature compatible with our goal of generated self-assembling GAG−AOP hydrogels.

The bis-aminooxy derivatives of each of the three Pluronics (**9a**−**9c**) were synthesized using a Mitsunobu reaction in the presence of excess (20 equiv) of phthalimide, triphenyl phosphine, and diethyl azodicarboxylate (Figure 4A). The crude products **8a**−**8c** were precipitated from the reaction mixtures with petroleum ether and re-crystallized four times using two solvents systems: THF/diethyl ether followed by THF/petroleum ether. The pure Pluronic derivatives **8** were analyzed by ^{1}H NMR at 400 MHz using $CDCl_3$ as the lock solvent. This choice of NMR solvent gave well-resolved terminal methylene peaks for the bis-phthalimide derivative **8a**−**8c**. As expected, the chemical shifts of the terminal methylenes were similar for both the fluorescent compound **4** (4.44 ppm) and Pluronic derivative **8**. This resonance (4H, 4.33 ppm, triplet, $J = 4.4$) was integrated and compared to the multiple signal of phthalimide aromatic rings (8H, 7.81−7.73 ppm). Next, hydrazinolysis of the phthalimide afforded the bis-aminooxy Pluronics **9a**−**9c**. This method was applied to each of the three Pluronics, leading to the three bis-AOP derivatives in high yields (over 80%).

To obtain monovalent AOPs that could be employed to immobilize a GAG chain on a plastic surface, we also prepared monomethoxy mono-AO Pluronic derivatives (Figure 4B). Careful calculation of the number of equivalents required for a given molecular weight allowed monomethylation to be achieved in a two-step protocol. The amounts of CH_3I required for the

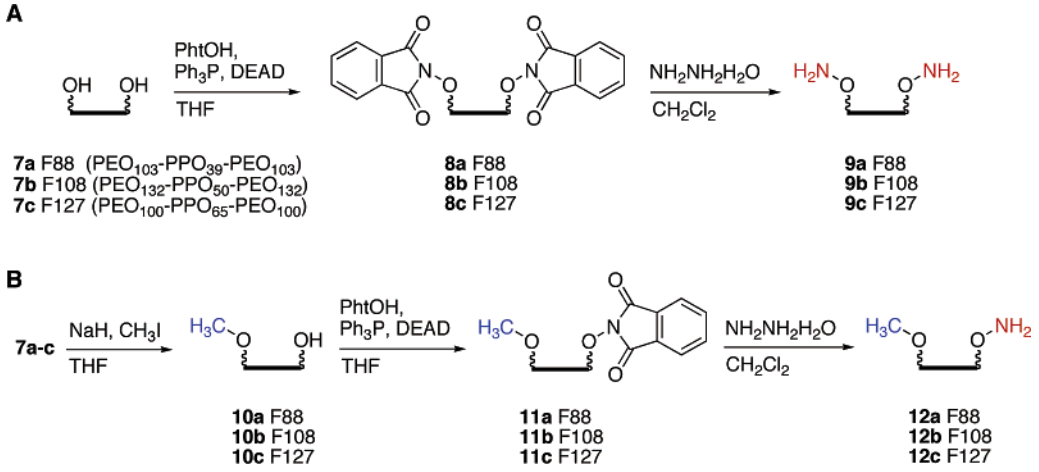

Figure 4. (**A**) Synthesis of bis-AO Pluronics; (**B**) synthesis of mono-MeO mono-AO Pluronics.

Table 1. Synthesis of Mono(aminooxy) Derivatives of Pluronics

mono-AO Pluronic	Pluronic	proportion of Pluronic/ CH_3I[a]	purity based on ^{1}H NMR[b]	yield after 3 steps
12a	F88	2 g/6.25 μL	97−99%	60%
12b	F108	2 g/5 μL	91−97%	71%
12c	F127	2 g/5.6μL	90−92%	54%

[a] For all Pluronics, 6 equiv of NaH was used. [b] ^{1}H NMR was performed in acetone-d_6 for compounds **11** and **12**.

crucial methylation step are shown in Table 1. First, each Pluronic **7** was methylated with approximately 1 equiv. of iodomethane in the presence of 6 equiv of sodium hydride to give the monomethyoxy Pluronics **10**. Second, a Mitsunobu reaction on the **10a**−**10c** using a 10-fold excess of each reagent gave the phthalimidoxy adducts **11a**−**11c**. In analogy to the characterization of the bis-AO derivatives, the pure monomethoxy AOPs were analyzed by ^{1}H NMR. The most reliable results were obtained for monomethoxy mono phthalimide compounds **11** when the ^{1}H NMR analyses were performed in acetone-d_6. The C**H**$_3$O− protons (singlet, 3.28 ppm) were integrated and compared to the multiplet signal of methylene −C**H**$_2$O−Pht group (m, 4.35−4.33 ppm).

Third, the phthalimides **11a**−**11c** were hydrazinolyzed to afford the final compounds **12a**−**12c**. The homogeneity was also verified by ^{1}H NMR in acetone-d_6 by comparative integration of methylene −C**H**$_2$−O−NH=C(CD$_3$)$_2$ and methyl −O−**CH**$_3$ signals. Note that the acetone-d_6 acted as a derivatizing agent as well as the solvent, producing the corresponding oximes. The methylene protons adjacent to the oxime were thus shifted downfield from the broad −OCH$_2$CH$_2$− signal; the shift also confirmed the presence of the aminooxy function in **12a**−**12c**. Finally, the purity of compounds **12a**−**12c** was calculated to be 90−99% (Table 1); the primary contaminants that could not be readily removed were the expected bis-methoxy derivative (for compound **12a**) and the expected bis aminooxy derivatives (for compounds **12b** and **12c**).

Another way to confirm existence of −ONH$_2$ groups of the AOPs was simply converting the mono-AOPs to their fluorescent versions. Thus, AOP **12b** was coupled with two nonfluorescent reagents, fluorescamine and NDA,[51,52] which are commonly used to obtain fluorescent products by reaction with primary amine-containing compounds and amino acids. The resulting adducts of **12b** with these reagents were fluorescent biopolymers, characterized by green fluorescence. Fluorescamine derivative **13** showed an absorbance maximum for excitation of λ_{max} = 350 nm in CH$_3$OH, with green fluorescence at λ_{em} = 460 in CH$_3$CN. The NDA derivative **14** showed an absorbance maximum for excitation of λ_{max} = 354 nm in CH$_3$OH, with blue fluorescence at λ_{em} = 400 in CH$_3$CN.

Preparation of HP−AOP and HA−AOP. EDC chemistry is widely used with carboxyl-containing biomacromolecules, in particular carboxyl-containing glycosaminoglycans (GAGs) and peptides.[53−57] Even the carboxylic acids of HP, which contains a high density of strongly basic O and N sulfates, is readily converted to hydrazide derivatives using EDC.[58] At pH 4.75, the carboxylic acid is protonated, leading to formation of an *O*-acyl isourea as the reactive intermediate. In the absence of a suitable nucleophile, HA−*O*-acyl isourea adducts can rearrange to the stable *N*-acyl ureas.[59,60] When nucleophiles are present in sufficiently high concentration, addition to the activated *O*-acyl isourea occurs, giving the urea byproduct and the corresponding carboxylate derivative. With hydrazides, the corresponding GAG−hydrazides are readily prepared under mild conditions.[37,61] As with hydrazides, aminooxy groups remain nucleophilic at pH 4.75, and the intermediate GAG−*O*-acyl isourea can react efficiently to afford the GAG−amidooxy adducts.

Since the molecular sizes of the HP−AOP conjugate and bis-AO Pluronic were similar, we took advantage of the highly anionic nature of the adduct and used DE23 anion exchange chromatography to first remove the unreacted AOP, followed by dialysis to remove charged small molecule contaminants. The ^{1}H NMR spectrum of the final product shows numerous resonances between δ 1.0 and δ 3.0−4.0 that are due to the overlap of the bis-AOP reagent with heparin adduct at those chemical shifts. Two additional resonances appeared at δ 2.77 (**I**) and at δ 1.20 (**II**). (For NMR spectra, see the Supporting Information.) These signals correspond to a side-product arising from the rearrangement of the initially formed *O*-acylisourea from the reaction of heparin with EDC to an *N*-acyl urea.[60] This peak was greatly increased in the ^{1}H NMR spectrum of the product obtained from the reaction of heparin and EDC when no additional nucleophiles were added (data not shown). The large contribution of this side reaction is consistent the slow diffusion of the 15-kDa AOP, thus decreasing the rate of reaction with the intermediate *O*-acyl isolurea relative to the 210-Da nucleophiles such as DTP.[53]

The EDC-mediated reaction of AOPs with HA was used to prepare hydrogels. We anticipated that both chemical and physical cross-linking could occur in these hydrogels, and that the hydrophobic association of PPO blocks could create hydrogels with unique biological and physical properties. In

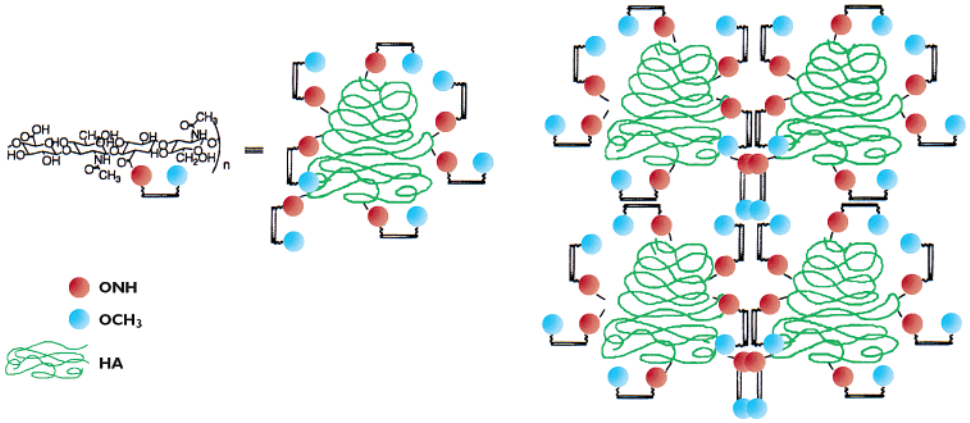

Figure 5. Depiction of the proposed self-assembly of bis-AO−F108 Pluronic-HA into a hydrogel.

Table 2. Water Loss of HA-AOP Hydrogel after 24 h Incubation at 37 °C

	bis-AO−F127	bis-AO−F108	bis-AO−F88
weight loss (%)[a]	59.3	18.4	0

[a] The gels were synthesized at room temperature, and then incubated at 37 °C 150 rpm for 24 h.

particular, we wished to investigate the effects of temperature and Pluronic type on gelation. Therefore, the mono-AOPs (**12a**−**12c**) were added to a 0.2% (w/v) solution of 190 kDa HA, and the solution pH was adjusted to 4.75. After ca. 5 min stirring at room temperature, a gel formed for all three Pluronics. No gel formed under the same conditions without the mono-AO derivatives of Pluronics, and no reaction occurred in the absence of EDC. This result suggests that the mono-functionalized AOP formed a covalent amidooxy linkage to HA and that the physical association of PPO sequences contributed to the gel formation.

In the absence of the covalently attached GAGs, the temperature-sensitive gelation of a Pluronic solution is attributed to the hydrophobic association of PPO blocks and occurs within a defined range of Pluronics.[1] Under our experimental conditions, the mono-AO derivatives of F88, F108, and F127 were all readily dissolved in distilled water at room temperature to give 2% (w/v) solution. Under these conditions of temperature and concentration, the intermolecular PPO−PPO interactions are insufficient to form a physically gel. However, following covalent attachment of AOPs **12** to HA, a single HA molecular chain would possess numerous AOPs bound as amidooxy derivatives of the glucuronic acid moieties. As a result, both entanglement of the GAG chains and inter- and intramolecular PPO−PPO interactions increased leading to the spontaneous formation of a physical gel.

In unmodified Pluronics, the increase in hydrophobic interaction with increasing temperature results in gelation at sufficiently high concentrations. Normally, F127 solutions form physical gels at 37 °C if the concentration exceeds 10% w/v; at 4−24 °C, a solution would persist. For the Pluronics with short PPO sequence (e.g., F88 and F108), hydrophobic associations are inadequate to produce a physical gel in this temperature range. Thus, the formation of gels for all three adducts of F88, and F108 and F127 AOPs with HA even at low concentrations and at room temperature was noteworthy. The three gels formed at room temperature from bis-AO−F88−HA, bis-AO−F108−HA, and bis-AO−F127−HA were incubated at 4 and 37 °C, and gel swelling was monitored. Surprising, no gel dissociation occurred at 4 °C. However, the PPO sequence length did influence the gel swelling at 37 °C, and the water was expelled from the gel as the additional hydrophobic interactions resulting from increased temperature from room temperature to 37 °C. This effect was most prominent for bis-AO−F127−HA gel, in which the Pluronic with the longest PPO block was employed (Table 2).

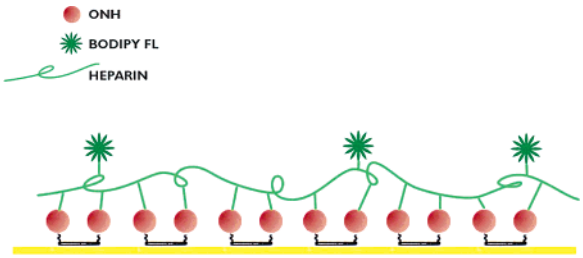

Figure 6. Representation of the surface immobilization of BODIPY−FL labeled heparin as the bis-AO−F108 Pluronic conjugate.

Surface Immobilization of AOP Adducts of HP and HA on Plastic. The hydrophobic PPO patches of Pluronics promote adsorption to plastic surfaces.[11−13,45−47] We tested the hypothesis that EDC-mediated conjugation to an immobilized AOP could be utilized to adsorb HP to the wells of an ELISA plate (Figure 6). For this purpose, a 96-well polystyrene plate was first coated with bis-AOP F108 (**9b**) followed by reaction with either UFH or BODIPY−FL-HP using EDC in MOPS buffer at pH 4.7 to form the amidooxy linkage. The MOPS buffer was selected because its pI value of 4.7 allows the correct pH for the EDC chemistry to be maintained without addition of acid as the reaction progressed. This approach has significant potential to improve the efficiency of surface immobilization of hydrophilic GAGs to plastic surfaces. The loading of Pluronic to the polystyrene surface was rapid and the adsorption was robust.[62] The immobilized HP was quantified by ELISA (using binding to human IL-8), and BODIPY−FL-HP was quantified using fluorescence spectrometry. The background of IL-8 in these studies varied depending on the incubation time but was generally under $A \sim 0.6$. All readings were corrected for the nonspecific binding measured as background.

Figure 7 shows immobilization of HP using the human IL-8 based ELISA as described.[48] The quantity of immobilized HP was concentration-dependent, with minimal binding observed for controls without F108, EDC, or heparin. The additional increase in immobilized HP plateaued with increasing heparin concentration, because the binding capacity of human IL-8 to heparin had been reached.[48] Therefore, to further quantify the immobilization of heparin, we used BODIPY−FL-HP to directly quantify immobilized HP.

Figure 8 shows a concentration-dependent increase in BODIPY−FL−HP bound to the surface to which bis-AO F108 had been preadsorbed. As a standard, 1 mg/mL BODIPY−FL−

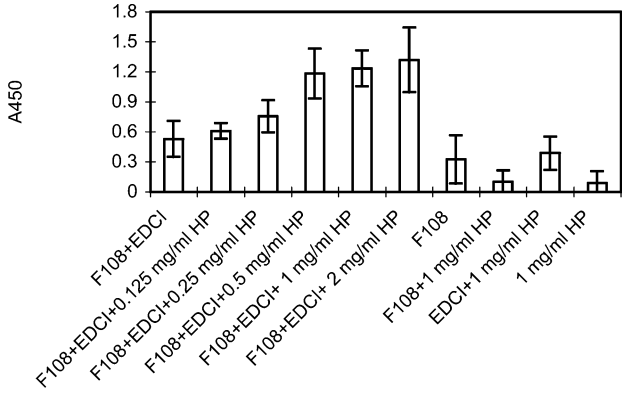

Figure 7. ELISA-based measurement of the immobilization of UFH (HP) using EDC-mediated coupling with bis-AOP **9b**.The quantity of HP immobilized was measured using a human IL-8-based ELISA. Heparin immobilization showed a strong dose-dependence on heparin concentration, with significant increase compared to other controls. Controls included bis-AOP **9b** ("F108") plus EDC without HP (far left), bis-AOP **9b** alone, bis-AOP **9b** plus HP without EDC, EDC plus HP with no bis-AOP **9b**, and HP alone.

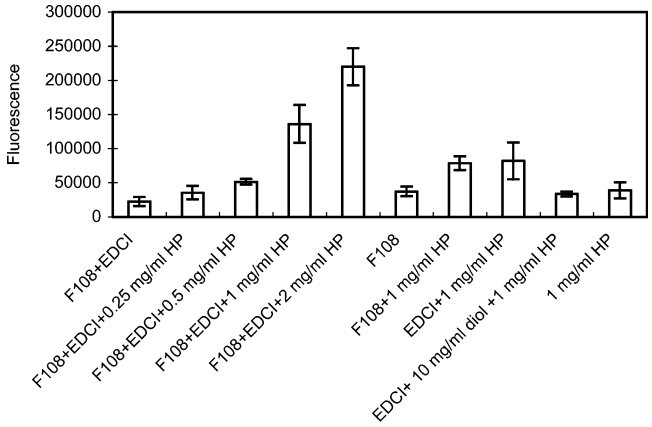

Figure 8. BODIPY−FL−HP immobilization through bis-AOP **9b** with EDC catalysis. Immobilized BODIPY−FL−HP was measured using fluorescent spectrometry, and showed dose-dependence on BODIPY−FL−HP concentration. Similar controls were performed as in Figure 7. Without bis-AOP **9b**, we noted that EDC also bound to surface and adsorbed BODIPY−FL−HP; this effect could be minimized by adding 3-aminooxy-butane-1,2-diol to consume the reactive EDC and make a water-soluble adduct that failed to promote BODIPY−FL−HP adsorption.

HP in MOPS was also measured by fluorescence spectrometry. Based on the reading of that as a benchmark, we calculated that the immobilization efficiency of BODIPY−FL−HP by bis-AO F108 (**9b**) throughout the concentrations tested was in the range of 40−50%. The efficiency is thus the ratio of fully immobilized heparin (calibrated from the 1 mg/mL BODIPY−heparin reading) to total heparin added.

The controls, F108-AOP (**9b**) + heparin (or BODIPY−FL-HP) without EDC, as well as EDC + heparin (or BODIPY−FL-HP) without F108-AOP (**9b**), gave modest background attachment. We formulated several potential explanations for the control background results. First, we ruled out a slow reaction or association between **9b** and HP in the absence of EDC. However, it was possible that hydrophobic adsorption of EDC onto the surface, followed by the reaction with HP (the "side reaction" in AOP−HP bioconjugation), could cause attachment. We tested this hypothesis by reducing the background of HP immobilization in the presence of a 10 mg/mL solution of 3-aminooxy-butane-1,2-diol. Reaction of EDC with an excess of a readily soluble aminooxy compound dramatically reduced HP reaction with EDC (Figure 8, column 9).

Following the same EDC coupling protocols, BODIPY−FL−HA was condensed with surface-adsorbed bis-AO−F108 (**9b**). The fluorescence was measured using $\lambda_{ex} = 496$ nm and $\lambda_{em} = 520$ nm. The results were consistent with those observed for heparin/IL-8 experiments and confirmed that BODIPY−FL−HA was immobilized on the plastic surface as a result of the formation of covalent amidooxy bonds to the adsorbed AOP (data not shown).

Conclusions

Herein we introduced a facile and efficient method of preparing bis-AO and mono-AO derivatives of the Pluronics F88, F108, and F127. Based upon simple chemical transformations and purifications, we obtained novel reactive derivatives of these triblock polymers in good overall yields. The purity and the degree of derivatization were calculated using ^{1}H NMR performed in $CDCl_3$ and acetone-d_6, with oxime formation providing chemical shift changes that permitted quantification of the modified end groups. In addition, the synthesis of mono-MeO mono-AO derivatives of Pluronics (**12**) has been, to the best of our knowledge, the first attempt to perform mono-derivatization of this group of triblock copolymers. We also confirmed the chemical structures of the newly synthesized AOPs by further derivatization fluorescamine and NDA in order to produce fluorescent biopolymers with characteristic blue and green fluorescence.

The new AOPs were successfully coupled with HP and HA to produce new biomaterials with unusual physical properties. With mono-AOPs, even low concentrations of AOP−GAG adducts formed physical gels after 5 min in the presence of HA and EDC at pH 4.75. No gels formed under the same conditions in the absence of the AOPs. The conjugation of the AO-modified Pluronics to HA adds hydrophobic patches that are capable of inter- and intramolecular self-association, thereby producing large aggregates and physically crosslinked gels. The hydrophobic patches may also contribute to the hydrophobic interactions with plastic surfaces, since the AOP−GAGs were effectively adsorbed to the plastic. Thus, the gelation of AOP−GAGs will compete with the potential for surface adsorption. The AOP−HA gels exhibit temperature-sensitive swelling, and thus have potential utility in temperature-responsive drug delivery systems. Taken together, these results demonstrate the utility of AOP-precoated plates for immobilization of GAGs and other hydrophilic polymers that have carboxylic acid groups.

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Supporting Information Available. Complete NMR spectra for key new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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