Synthesis of Diblock Copolymers Consisting of Hyaluronan and Poly(2-ethyl-2-oxazoline)

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Glycoproteins are ubiquitous constituents of living organisms where they play key functions in mechanisms such as bioadhesion, cell−cell interactions, and recognition phenomena.[1] The chemical structure of most glycoproteins is extremely complex and presents significant interspecies variability. Recognizing the utility of macromolecules that combine in their structure polypeptide and polysaccharide fragments, polymer chemists have prepared synthetic analogues of glycoproteins with controlled composition and architecture. Examples of such biomimetic polymers include polyoxazoline−glycopeptide block copolymers[2,3] as well as comb-type copolymers consisting of a poly(peptide) chain grafted with polysaccharide chains.[4] These polymers have found applications as constituents of targeted drug delivery systems and surface modifiers of various biomaterials.

We introduce here a different class of glycoprotein analogues, where a linear polysaccharide block is linked to a pseudopeptide block. Specifically we describe AB diblock copolymers consisting of a hyaluronan block and a poly(2-ethyl-2-oxazoline) block. The combination of these two polymers offers unique possibilities, in terms of biotechnological applications. Hyaluronan is a natural polymer consisting of alternating *N*-acetyl-*β*-D-glucosamine and *β*-D-glucuronic acid residues linked at the 1−3 and 1−4 positions, respectively.[5] It plays a critical role as signaling molecule in cell motility,[6] wound healing,[7] cancer metastasis,[8] and morphogenesis.[9] Unmodified and modified HA have found various applications in drug delivery systems and surgery.[10−15] Hyaluronan has been found to enhance absorption of drugs and proteins through mucus tissues.[16] It has also been applied, together with the polycation chitosan, in the form of polyelectrolyte multilayer coatings to create biocompatible surfaces and to reduce the thrombogenicity of injured arteries.[17,18]

Poly(2-alkyl-2-oxazolines) are nontoxic neutral hydrophilic macromolecules that show excellent biocompatibility and have found applications as components of drug delivery systems and in antimicrobial formulations.[19] They are synthesized in near-uniform chain length by living cationic polymerization of the corresponding 2-alkyl-2-oxazoline. By selecting appropriate polymerization initiators[20] and quenchers, it is possible to synthesize poly(2-alkyl-2-oxazolines) terminated at one end, or both, with functional groups, such as double bonds,[21] siloxanes,[22] tosylates,[23] hydroxyl groups,[24] amine groups,[21,25] or alkyl chains.[19] Among the various poly(2-alkyl-2-oxazolines), those possessing a methyl or an ethyl side chain have attracted the most attention by far.[26−28] They are water-soluble and can be prepared from commercially available monomers. Aqueous poly(2-ethyl-2-oxazoline) (PEtOz) solutions exhibit a cloud point around 62−65 °C, depending on polymer molecular weight and concentration[26] and on the presence of cosolvents and salts.[27] Recently, several examples of PEtOz-containing diblock copolymers have been described, in which the hydrophilic PEtOz unit was linked to a hydrophobic polymer, such as poly(isobutylvinyl ether),[29] poly(L-lactide), or poly(*ϵ*-caprolactone).[30] Such amphiphilic diblock copolymers form micelles in water and may act as delivery systems for hydrophobic drugs.[31−33]

The methodology adopted in the synthesis of PEtOz-containing diblock copolymers reported to date relies on the sequential living polymerization of two different monomers. This approach, however, is ill-suited to the preparation of HA-*b*-PEtOz since one block, HA, is a natural polymer. A variation of this methodology involving the enzyme-catalyzed synthesis of the polysaccharide block of poly(ethylene oxide)-*block*-amylose was shown to occur in good yields.[34] This method could in principle be applied to the synthesis of HA-*b*-PEtOz, as Kobayashi et al. reported recently the preparation of HA via hyaluronidase-catalyzed ring-opening polymerization of the appropriate disaccharide.[35] We did not pursue it in view of the low overall yield of the elaborate disaccharide synthesis. We opted instead to form HA-*b*-PEtOz via coupling of two preformed polymers: HA and an amine-terminated poly(2-ethyl-2-oxazoline). This approach is viable only if the coupling reaction occurs in high yield and can be carried out in the presence of carboxylic acid and hydroxyl groups, the two types of functional groups present on the HA backbone. We opted for the reductive amination of the HA terminal aldehyde group with a primary amine linked to PEtOz. This reaction was found to be effective in grafting HA to polyamines, such as poly(L-lysine).[4]

The chemistry used to prepare amine-terminated poly(2-ethyl-2-oxazolines) (NH_2-PEtOz) relies on the use of the bifunctional initiator, *N*-[2-(*p*-toluenesulfonyloxy)ethyl]phthalimide, which was obtained in high yield (90%) by reaction of *N*-(2-hydroxyethyl)phthalimide with *p*-toluenesulfonyl chloride and triethylamine in chloroform at room temperature (see Scheme 1, Supporting Information). It was purified by three consecutive recrystallizations from hexane:ethyl acetate = 3:1.[36,37] The polymerization of 2-ethyl-2-oxazoline (99 mmol) was carried out in refluxing acetonitrile using *N*-[2-(*p*-toluenesulfonyloxy)ethyl]phthalimide as initiator (2 mmol) for a period of 24 h. The oxazolinium end groups of the growing polymer chains were quenched by addition of methanolic NaOH to the cooled polymerization mixture. The resulting phthalimide-terminated poly(2-ethyl-2-oxazoline) was isolated by precipitation into anhydrous diethyl ether, followed by 2 days dialysis against distilled water using a Spectra/Por CE membrane (molecular weight cutoff of 5000 g/mol). The experimental number-average molecular weight (M_n = 5500 g/mol) determined by GPC, using DMF as an eluent and a multiangle laser light scattering (MALLS)

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Table 1. Molecular Characteristics of the Polymers Investigated

		GPC		
entry	M_n^{theor}	M_w	M_w/M_n	block ratio[e]
HA		2700[c]	1.26	
PhtN-PEtOz	4900[a]	6800[d]	1.23	
NH_2-PEtOz	4700	6800[d]	1.17	
HA-*b*-PEtOz	9500[b]	10200[c]	1.31	1:9.9

[a] From the ratio of [monomer] to [initiator]. [b] Calculated from GPC results of components. [c] Acetate buffer. [d] DMF. [e] From ^{1}H NMR data.

detector, was close to the value predicted on the basis of the initial monomer/initiator ratio ($M_{n,calc}$ = 4900 g/mol) (Table 1). The ^{1}H NMR spectrum of PhtN-PEtOz in D_2O presents two doublets at 7.58 and 7.26 ppm, attributed to the aromatic protons of the terminal phthalimide group. The degree of polymerization of the polymer was calculated from the integrated areas of these two signals and of the broad singlet at 3.46 ppm, attributed to the methylene protons of the PEtOz backbone. The number-average molecular weight (M_n = 5200 g/mol) derived from integration of the ^{1}H NMR signals is in excellent agreement with the number-average molecular weight determined by GPC.

We set out next to convert the terminal phthalimide group into a primary amine group. This transformation was performed by treatment of PhtN-PEtOz with hydrazine monohydrate in ethanol at room temperature. The success of the reaction was ascertained by GPC/^{1}H NMR analysis to confirm (1) that the molecular weight of the polymer was not affected by the reaction and (2) that the phthalimide group had been removed.

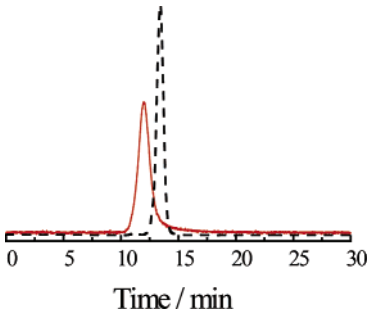
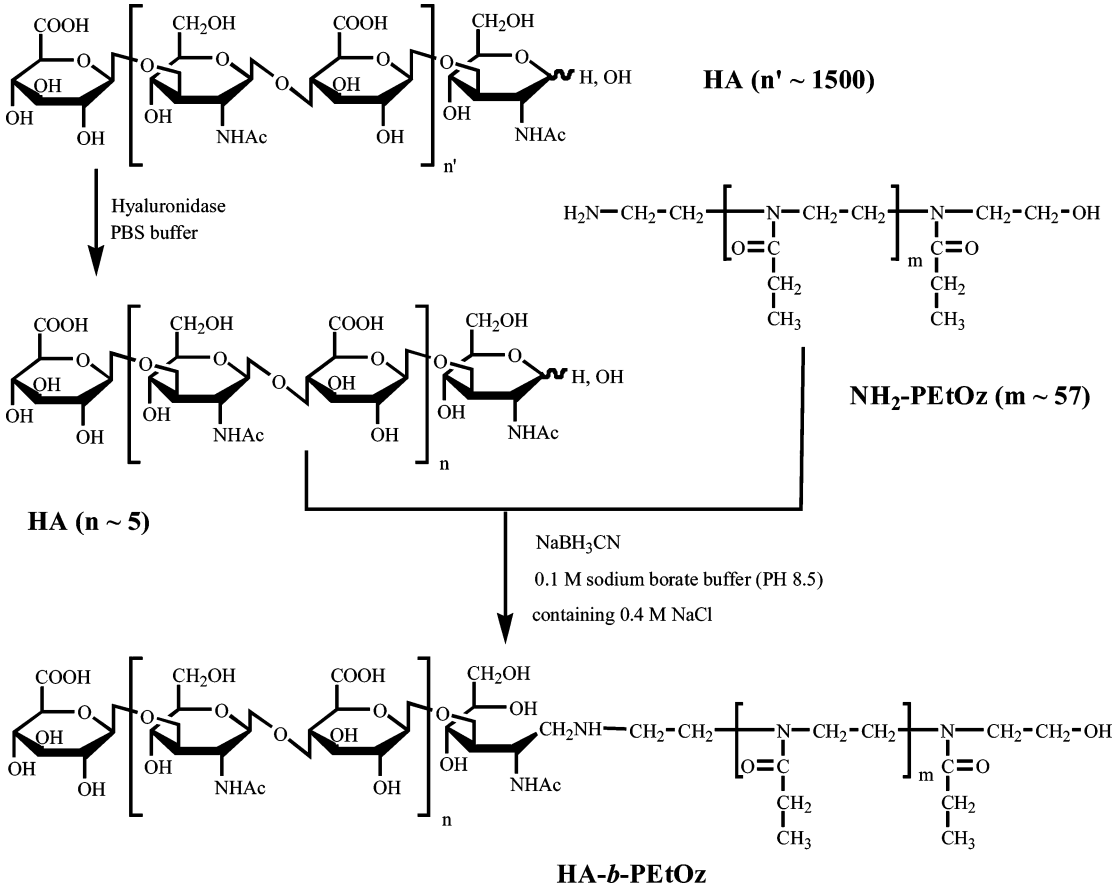


Figure 1. Gel permeation chromatograms of the diblock copolymer HA-*b*-PEtOz (solid line) (M_w = 1.02 × 10^4 g/mol, M_w/M_n = 1.31) and HA (dashed line) (M_w = 2.75 × 10^3 g/mol, M_w/M_n = 1.26): column, Viscotek-8025, Tskgel; eluent, buffer solution, 0.3 M acetic acid, 0.2 M sodium acetate and 0.8 mM sodium azide (pH: 4.5); flow rate, 0.5 mL/min; temperature, 25 °C; and detection, RI and LS.

The GPC-derived number-average molecular weight of the amine-terminated poly(2-ethyl-2-oxazoline) (NH_2-PEtOz) was nearly identical to that of PhtN-PEtOz (Table 1). The polydispersity index (M_w/M_n) remained low as well.[38] No signal was detected in the aromatic region in the ^{1}H NMR spectrum of NH_2-PEtOz (Figure 2b), confirming the successful removal of the phthalimide group.

The hyaluronan sample (Table 1) used for coupling with the NH_2-PEtOz sample was obtained by hyaluronidase degradation (37 °C, 30 h, PBS buffer, pH 7.2) of a commercial HA sample (M_n 590 000 g/mol), followed by purification by size exclusion chromatography over a Sephadex G-75 filter to ensure narrow polydispersity of the sample. Coupling of this HA sample

Scheme 1. Synthesis of the Diblock Copolymer Hyaluronan-*block*-poly(2-ethyl-2-oxazoline) (HA-*b*-PEtOZ) and Structure of the Copolymer



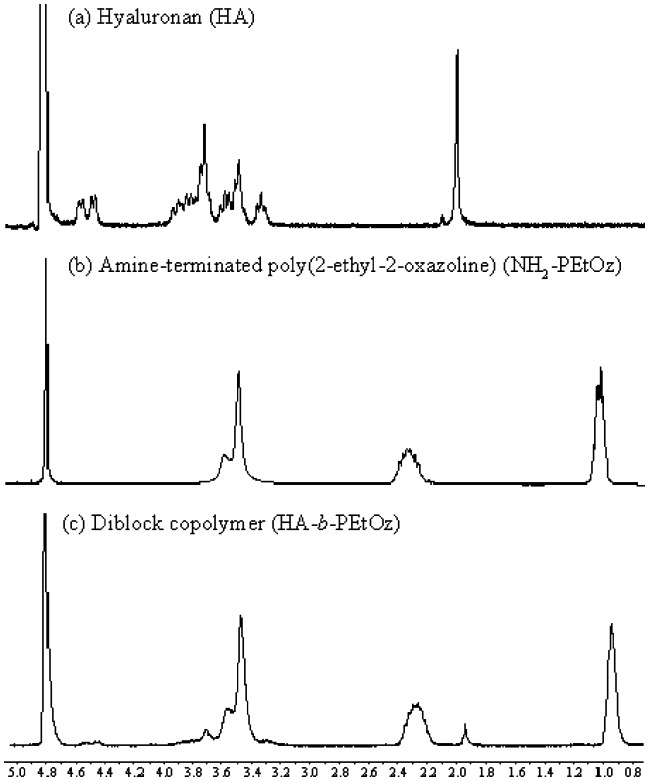


Figure 2. ^{1}H NMR spectra of HA (a), NH_2-PEtOz (b), and HA-*b*-PEtOz (c) in D_2O at room temperature.

with NH_2-PEtOz was performed at 40 °C in an aqueous sodium borate buffer (pH 8.5) for a period of 8 days in the presence of $NaBH_3CN$ (Scheme 1). Purification of the reaction product by extensive dialysis, first against a 0.5 M NaCl aqueous solution and second against deionized water to remove unreacted HA and NH_2-PEtOz, gave, after lyophilization, HA-*b*-PEtOz in 49% yield and 90% purity. The success of the coupling reaction and of the purification procedure was ascertained by GPC analysis. The GPC trace of the coupling product (Figure 1) presents only one peak, of shorter elution time than that of the starting HA sample.[39] The GPC-derived weight-average molecular weight of HA-*b*-PEtOz is 10 200 g/mol ($M_w/M_n = 1.31$), a value slightly higher than the M_w expected on the basis of the M_w of the individual components (HA and NH_2-PEtOz). The purity of the diblock copolymer was determined by analysis of its ^{1}H NMR spectrum (Figure 2c), which presents signals characteristic of HA and of PEtOz (see spectra of HA and PEtOz in Figure 2, a and b, respectively).[40] The molar ratio of HA disaccharide units to PEtOz monomeric units was calculated to be 1:9.9 taking the ratio of the area of the signal at δ 1.99 ppm, attributed to the HA *N*-acetyl methyl protons to that of the signal at δ 1.03 ppm, due to the PEtOz side chain methyl protons. The calculated ratio of the two blocks based on the number-average molecule weight of HA and PEtOz is 1:10.5.

In summary, we report here the preparation of new biomimetic diblock copolymers consisting of two hydrophilic units: a nonionic block (PEtOz) and an anionic block (HA). Preliminary experiments indicate that this anionic copolymer forms colloidally stable particles ($R_h \sim 130$ nm) with the cationic drug diminazene. It is expected to form complexes with other positively charged entities, such as inorganic nanoparticles, enzymes,[41] polycations, and diblock copolymers consisting of a neutral segment and a positively charged segment, such as poly(ethylene glycol)-*block*-poly(L-lysine).[42,43] It may also be used as a temperature-sensitive material, which may undergo heat-induced micellization in water.[44] Both types of micelles will be tools as delivery systems, taking advantage of the biological properties of HA. Conversely, they will also offer new means to explore the biological properties of HA.

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Supporting Information Available: Detailed synthetic procedures and spectral data for all polymers and for the initiator; synthetic scheme for the preparation of NH_2-PEtOz. This material is available free of charge via the Internet at http://pubs.acs.org.

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(37) In a recent publication[19] another bifunctional initiator, 3-[(*tert*-butoxycarbonyl)amino]benzyl-*p*-toluenesulfonate, was used to prepare amine-terminated polyoxazolines.
(38) See Supporting Information for full experiment data.
(39) Even though NH_2-PEtOz is soluble in water and in the acetate buffer used as GPC eluent, it was not possible to analyze it under the same GPC conditions as HA and the diblock copolymer due to irreversible adsorption on the column packing material.
(40) A complete analysis of the ^{1}H NMR spectra of HA, NH_2-PEtOz, and HA-*b*-PEtOz is given in the Supporting Information.
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