Synthesis and Characterization of Thermo- and pH-Sensitive Block Copolymers Bearing a Biotin Group at the Poly(ethylene oxide) Chain End

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ABSTRACT: A poly(ethylene oxide)-*block*-poly(dimethylamino ethyl methacrylate) block copolymer (PEO-*b*-PDMAEMA) bearing an amino moiety at the PEO chain end was synthesized by a one-pot sequential oxyanionic polymerization of ethylene oxide (EO) and dimethylamino ethyl methacrylate (DMAEMA), followed by a coupling reaction between its PEO amino and a biotin derivative. The polymers were characterized with ^{1}H NMR spectroscopy and gel permeation chromatography. Activated biotin, biotin-NHS (*N*-hydroxysuccinimide), was used to synthesize biotin-PEO-PDMAEMA. In aqueous media, the solubility of the copolymer was temperature- and pH-sensitive. The particle size of the micelle formed from functionalized block copolymers was determined by dynamic light scattering. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 3552–3558, 2006

Key words: H_2N-PEO-PDMAEMA; anionic polymerization; block copolymer; biotin; site-specific drug delivery

INTRODUCTION

There is a growing interest in the development of novel drug delivery systems to reach an increased bioavailability of drugs to the targeted cells and to reduce their side effects, especially in the field of cancer chemotherapy and gene therapy.[1–3] Nanospheric particles have been proven as efficient drug delivery systems for intravenous administration because of their comparatively long bloodstream circulation. In recent years, novel approach in the field of polymeric drug delivery systems was introduced by the formation of polymeric micelles and subsequently by functionalized polymeric micelles. A double hydrophilic block copolymer (DHBC) is a diblock copolymer, which consists of two water-soluble blocks of different chemical nature. A temperature or pH change as well as complexation with appropriate molecules can induce micellization. These pH or temperature sensitive and biocompatible block copolymers can be utilized as potential carriers for hydrophobic drugs or DNA.[3–7]

Among the nanospheric particles, functionalized polymeric micelles are expected to find a wide application in the fields of drug delivery and diagnosis, since the possibility of coupling to bioactive substances is provided. A certain number of functional groups on the outer shell of the micelle allow an immobilization of biologically active substances. This is a great advantage for utilizing this particular type of nanosphere in the biomedical field. Therefore, we need to combine DHBCs with biomolecules as they display unique recognition, which is very important for targeted drug delivery systems.[3,8–11] The strong noncovalent bond of biotin–avidin is usually utilized[12–15] by taking advantage of the avidin–biotin interaction, which is the strongest known noncovalent biological interaction (association constant 10^{15} M^{-1}). Bond formation between biotin and avidin (or streptavidin) is very rapid and, once formed, this binding is unaffected by wide extremes of pH, temperature, organic solvents, or other denaturing agents. Moreover, the complex is also resistant to enzymatic proteolysis within the digestive tract.[16] Thus, biotin could be covalently coupled to the PEG terminus, enabling the subsequent construction of molecular superstructures. A variety of biotinylated ligands are available, and this type of nanoparticle would allow simultaneous coupling of several ligands, depending on the target.

Poly(ethylene oxide)-*block*-poly(dimethylamino ethyl methacrylate)s (PEO-*b*-PDMAEMAs) are a class of very important double hydrophilic block polymers to be used as drug and gene delivery systems.[17–21] PEO presents attractive physical, chemical, and biological properties such as very low toxicity and good solubility in water. Therefore PEO is utilized for enhancing water solubility and biocompatibility of drugs. PEO-coated nanoparticles have been developed over the past years, since they showed great potential as

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long circulating systems after intravenous administration[4,11,22–26] together with an ability to bypass natural barriers such as the nasal mucosa.[13,27] PDMAEMA is a class of pH/temperature sensitive and biocompatible cationic polymer, which is especially suitable for gene delivery.[28–31]

Nagasaki et al. reported[32] the synthesis of PDMAEMA using potassium 4-vinylbenzyl alcoholate as a functional initiator, which is named as oxyanionic polymerization. Lascelles et al.[33] used oxyanionic polymerization to synthesize many other poly tertiary amine methacrylates. Most ''oxyanionic polymerization'' involved the use of potassium alcoholates, such as monohydroxy-capped poly(alkylene oxide)s, in the role of macroinitiators for the polymerization of DMAEMA resulting in PEO-PDMAEMA copolymers. During the oxyanionic polymerization, the hydroxyl groups, acting as initiators, were transferred to $^{-}O^{-}K^{+}$ by $DMSO^{-}K^{+}$ (where DMSO is dimethyl sulfoxide), then $^{-}O^{-}K^{+}$ initiated the polymerization of DMAEMA.[6,29,34–36] Such oxyanionic initiators do not normally polymerize methacrylate monomers: the Japanese group (Nagasaki et al.) attributed their unexpected success to complexation of the potassium counterion with the nitrogen heteroatom of the DMAEMA. However, this explanation remains speculative; the precise mechanism for this polymerization has not yet been established. However, to our knowledge, there are not any reports about H_2N-PEO-PDMAEMA. Here, a one-pot synthesis of H_2N-PEO-PDMAEMA via oxyanionic polymerization followed by a coupling reaction between its PEO amino and a biotin derivative was reported. The micellar size of the functionalized block copolymers was determined by dynamic light scattering (DLS).

MATERIALS AND METHODS

Potassium di(trimethylsilicon) amide in toluene (0.5*M*, Aldrich Chemicals), biotin (synthesized by our lab, mp: 231.3–232.1°C; $[\alpha]_D^{20} = 90.9°$), anhydrous tetrahydrofuran (prepared in the presence of Na *in situ*). *N*-Hydroxysuccinimide (NHS) and *N*,*N*-dicyclohexylcarbodiimide (DCC) were purchased from Fluka Chemicals (Buchs, Switzerland). DMAEMA was first treated with activated Al_2O_3 and then vacuum distilled from calcium hydride and then stored under a nitrogen atmosphere at −20°C. Ethylene oxide (EO) was stored in a sealed tube made by ourselves at an ambient temperature. All solvents were of analytical grade and were used as received, unless stated differently.

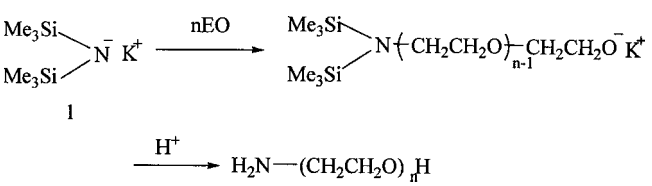
The NMR experiment was done with Bruker DMX500 using *d*-chloroform (> 99.8%, SDS, Peypin, France) as a solvent. The copolymers were analyzed by gel permeation chromatography (GPC) using HPLC-grade THF as mobile phase at a flow rate of 1 mL/min and a GMH-HR M (Viscotek, Houston, TX) column. Polystyrene standards (Polymer Laboratories, Shropshire, UK) were used to determine relative molecular weights. The injected volumes were 0.1 mL and the polymer concentration was 5 mg/mL.

Synthesis of H_2N-PEO

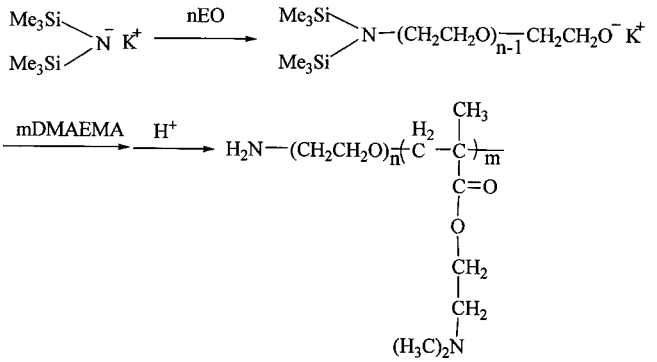
Poly(ethylene oxide)-monoamine (H_2N-PEO-OH) was synthesized as described by Yokoyama et al.,[37] who reported a facile synthetic method of PEO with a primary amino group and a hydroxyl group at each terminal using a commercially available reagent, potassium bis(trimethylsily1)amide ($[(CH_3)_3Si]_2NK$, **1**), as an initiator of EO polymerization. As shown in Scheme 1, EO was polymerized by **1** to obtain PEO with bis(trimethylsily1)amine and potassium oxide terminals (**2**). The terminals were changed into a primary amine and a hydroxyl group, respectively, by subsequent acid treatment. Since the boiling point of EO is 10.7°C, EO cannot be added through a cooled needle. We used a latex pipe to connect the reactor and the store vessel of EO. After the whole systems repeatedly replaced with nitrogen, EO was added directly to the reactor. EO (4.5 mL) was dissolved in tetrahydrofuran (THF, 40 mL) at 0°C. A solution of **1** at a concentration of 0.50*M* in toluene (4 mL, Aldrich Chemicals) was added, and the mixture was stirred in a degassed sealed glass tube at 20°C. After 28 h, the mixture was poured into a four-fold volume of diethyl ether to obtain the precipitated polymer. Polymers obtained were dissolved in 30 mL of THF, followed by an addition of a few drops of 0.1*M* HCl. This solution was stirred for 3 min at room temperature and poured into 300 mL of diethyl ether to obtain the precipitated polymer. The precipitate was washed with diethyl ether and dried in vacuum. The molecule weight was determined by NMR and GPC.

Synthesis of H_2N-PEO-PDMAEMA

Polymerizations were carried out under nitrogen atmosphere in reactors equipped with a magnetic stirring device. A typical procedure for the synthesis of the amino-bearing PEO-*b*-PDMAEMA block copolymers was shown in Scheme 2. H_2N-PEO-PDMAEMA block copolymers were synthesized by a one-pot ani-



Scheme 1 Synthesis of poly(ethylene oxide) with an amino and a hydroxyl group at an each terminal.



Scheme 2 Synthesis of amino functionalized PEO-*b*-PDMAEMA copolymers H_2N-PEO-PDMAEMA.

onic ring opening polymerization of EO followed by adding DMAEMA. EO (11.5 mL, 10 g) was dissolved in tetrahydrofuran (THF, 100 mL) at 0°C. A solution of potassium bis(trimethylsilyl)amide ($[(CH_3)_3Si]NK$, **1**) at a concentration of 0.5*M* in toluene (4 mL) was added, and the mixture was stirred in a degassed sealed glass tube at 20°C for 2 days. The PEO solution was then divided into two equivalent parts. The first one was deactivated by a small amount of methanol and kept for the determination of the molecular weight, the polydispersity, and the end functionality of the PEO, thus prepared. Then the purified DMAEMA (21.5 mL, 20 g) was added into the other part. After 2 h, the reaction was terminated by adding acetic acid to the mixture. The reaction mixture was poured into four-fold of *n*-hexane to precipitate the polymerization product. Heating aqueous alkaline solutions (pH 12) of the crude copolymer mixture to 90°C led to quantitative precipitation of the diblock copolymer, with the PEO homopolymer impurity remaining in solution. The precipitate was dried under vacuum at room temperature. Then the product was treated in the mixed solvent of triethylamine and $CHCl_3$ (1 : 1) to deprotonate at room temperature for 8 h. The reaction mixture was poured into 15-fold of diethyl ether to precipitate the polymerization product, then the precipitate was dried under vacuum at room temperature. This product was analyzed with ^{1}H NMR and GPC.

Synthesis of biotin-*N*-hydroxysuccinimide

Biotin-NHS was prepared from biotin and *N*-hydroxysuccinimide (NHS) by coupling with dicyclohexylcarbodiimide (DCC).[38] Scheme 3 shows the synthesis of Biotin-NHS. DMF was vacuum distilled in the presence of CaH_2. Biotin was dissolved into hot DMF and then NHS and DCC was added. Biotin : NHS : DCC = 1 : 1.2 : 1.2. The mixture was incubated at 50°C for 16 h. The mixture was cooled to room temperature, and the dicyclohexylurea was filtered off. The filtrate was dried on a rotary evaporator. The residue was crystallized from isopropanol. First, the residue was refluxed in isopropanol and then filtered.

Coupling of biotin derivatives to H_2N-PEO-PDMAEMA copolymers

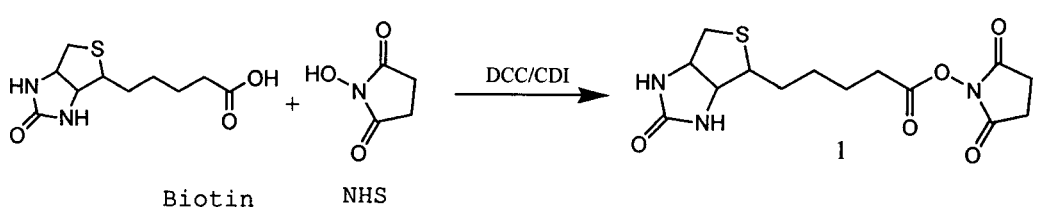
The purified H_2N-PEO-PDMAEMA (1.0 g) was added into CH_2Cl_2 (20 mL). After addition of NHS-biotin (0.25 g, in DMF), the reactants were stirred overnight under nitrogen. The reaction mixture was then extracted with 50 mL 10% $NaHCO_3$ aqueous solution to remove unreacted biotin. The product was isolated from the organic phase and precipitated by the slow addition of diethyl ether (40 mL), which was then filtered on a Buchner funnel and washed with diethyl ether. This product was then analyzed for biotin attachment by ^{1}H NMR spectroscopy. Scheme 4 shows the synthesis of biotin-PEO-PDMAEMA.

The characterization of the particle size of the micelles

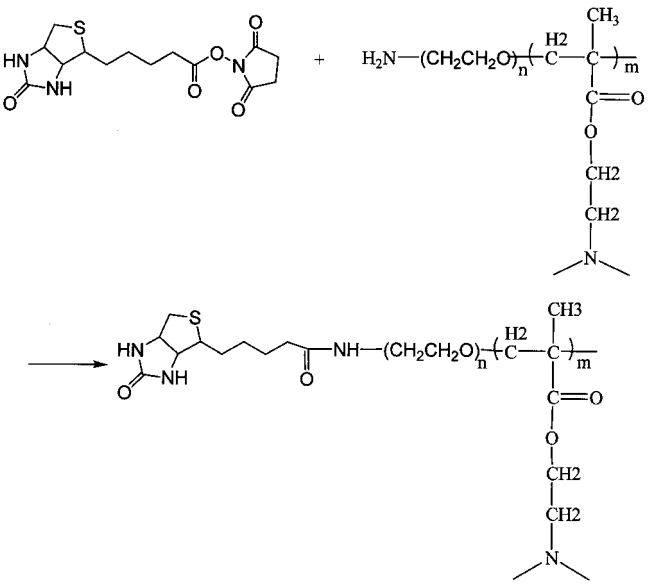
The analysis of the particle size of the micelles was taken on a Brookhaven 90Plus/BI-MAS multi angle particle sizing option (Brookhaven Instruments, USA).

RESULTS AND DISCUSSION

The synthesis of biotin-PEO-*b*-PDMAEMA block copolymers were carried out through the two following steps: (i) synthesis of H_2N-PEO-PDMAEMA; (ii) coupling of the biotin derivatives to these functionalized copolymers. The PEO precursor was analyzed by GPC using PEO standards for the determination of its molecular weight and molecular weight polydispersity. On the basis of the chemical composition of the purified copolymers determined by ^{1}H NMR, molecular weights of copolymers were calculated on the assumption that the block copolymer samples contained neither unreacted PEO nor PDMAEMA homo-



Scheme 3 Synthesis of biotin-*N*-hydroxysuccinimide (NHS).



Scheme 4 Synthesis of biotin-PEO-PDMAEMA.

polymer. The molecular weight of PEO is 5000, and the molecular weight of the copolymer is 15,000, therefore if the samples contain PEO homopolymer, then the GPC results must contain more than one peak. GPC results just contain one peak, hence, it was clearly established from GPC profiles that copolymer samples did not contain a detectable amount of PEO homopolymer. The mean yield of the synthesis of H_2N-PEO-PDMAEMA was above 90%.

Synthesis and characterization of α-amino PEO-*b*-PDMAEMA block copolymers

Most ''oxyanionic polymerization'' involved the use of macroinitiator, such as PEO, and these methods required to remove traces of water of the macromolecule. In our procedure, H_2N-PEO-PDMAEMA was synthesized by a one-pot sequential oxyanionic polymerization of ethylene oxide (EO) and DMAEMA, which does not need such treatments to remove traces of water of the monomer EO. PEO was characterized with ^1H NMR and GPC to determine the molecular weight and molecular weight distribution. Figure 1 shows the NMR spectrum of H_2N-PEO. The peak integral of the signal assigned to the ethylene oxide residues (3.6–3.7 ppm) was compared to those due to the amine in PEO (2.8 ppm), to determine the degree of polymerization of PEO, D. $D = S_1/S_2$. The polymerization degree and molecular weight of PEO were calculated to be 44 and 1950 g/mol, which agreed well with the calculated value (2000) based on the feed molar amount of EO against the initiator. The molecular weight distribution determined by GPC was very narrow (1.17).

DMAEMA was first treated with activated Al_2O_3 and then vacuum distilled from calcium hydride. The antioxidant was removed, thus it was very easy to be self-polymerized without any initiator. Leaving it for 1 day, it will self-polymerize at 25°C. Hence, it needed to be stored under a nitrogen atmosphere at −20°C. When the purified DMAEMA was added, about 3 in 10 times, DMAEMA would be polymerized very quickly in 20 s, and the polydispersity would be very large (> 2.3), which indicated that it was not a living polymerization anymore. We still do not know why, however some procedures will reduce such unexpected results. A magnetic stirring device is needed, and the reaction temperature should be kept below 20°C. DMAEMA should be added at one time.

The synthesis of H_2N-PEO-*b*-PDMAEMA block copolymers were characterized with ^1H NMR and GPC. Here, we have the GPC spectrum of PEO (5000)-PDMAEMA (10,000) in Figure 2. Gel permeation chromatography revealed one peak, indicating of pure material. This polymer is synthesized with low polydispersity (1.24). The results suggest that

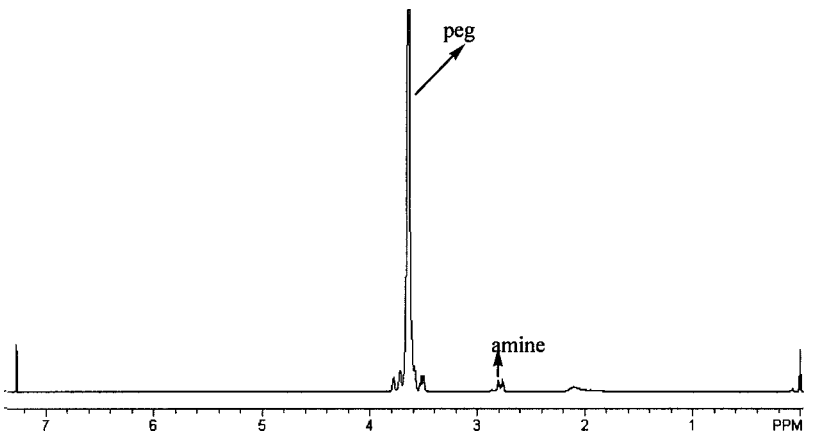


Figure 1 NMR spectrum of H_2N-PEO in $CDCl_3$. The peak at 3.6–3.7 ppm is assigned to the ethylene oxide residues and 2.8 ppm the amine group.

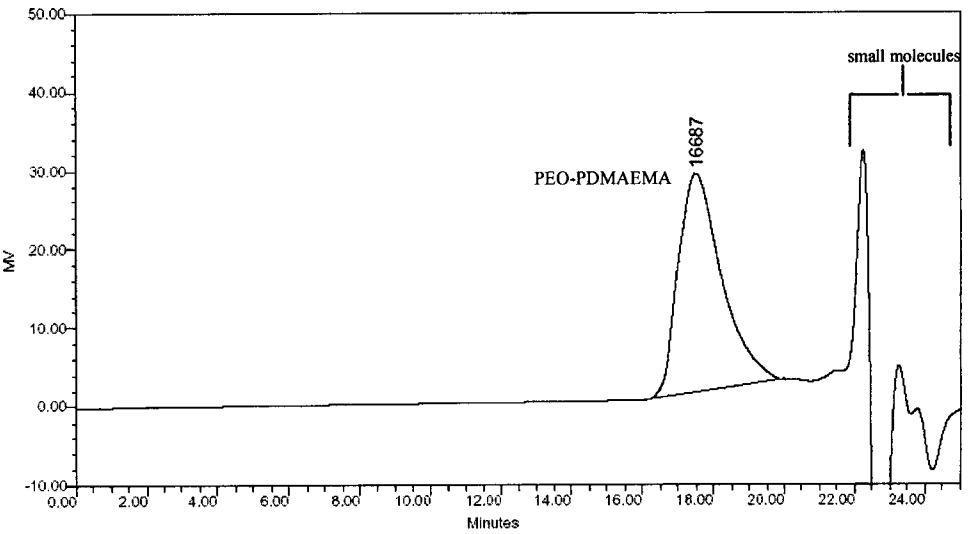


Figure 2 GPC spectrum of PEO (5000)-PDMAEMA (10,000). The results revealed one peak, indicating of pure material.

the oxyanionic polymerization can be used to synthesize H_2N-PEO-PDMAEMA block copolymer.

The molecular weight of the prepolymer (PEO) was determined as described earlier and the results (4890) matched well with the calculated due to the feed amount (5000). Figure 3 shows the NMR spectrum of H_2N-PEO-PDMAEMA. The doublet at 2.8 ppm, which could be assigned to the amine in PEO, could not be seen because the signal in PDAMEMA overwhelmed it. On the basis of the peak intensity ratio of methylene protons (4.1 ppm, $COOCH_2CH_2N(CH_3)_2$) from PDMAEMA to ethylene protons from PEO (3.6 ppm, OCH_2CH_2), the molecular weight of PDMAEMA were calculated to be 9780 g/mol, which agreed well with the calculated value (10,000) based on the feed molar amounts of DMAEMA against PEO prepolymer.

The self-assembly behavior was analyzed with DLS. DLS results showed that at pH = 4, the copolymer did not show any colloid behavior. And at pH = 7, pH = 9, and pH = 12, colloids whose diameters growing up with the temperature were observed. The number mean diameter of H_2N-PEO-PDMAEMA at pH 12 and 25°C is 19.8 nm. DLS confirmed that micellation of PEO-PDMAEMA was pH- and temperature-sensitive.

Coupling of biotin derivatives to α-amino PEO-*b*-PDMAEMA

Biotin binds with high affinity to avidin and streptavidin. The avidin–biotin interaction is the strongest known noncovalent biological recognition ($\sim K_a = 10^{15}\ M^{-1}$).

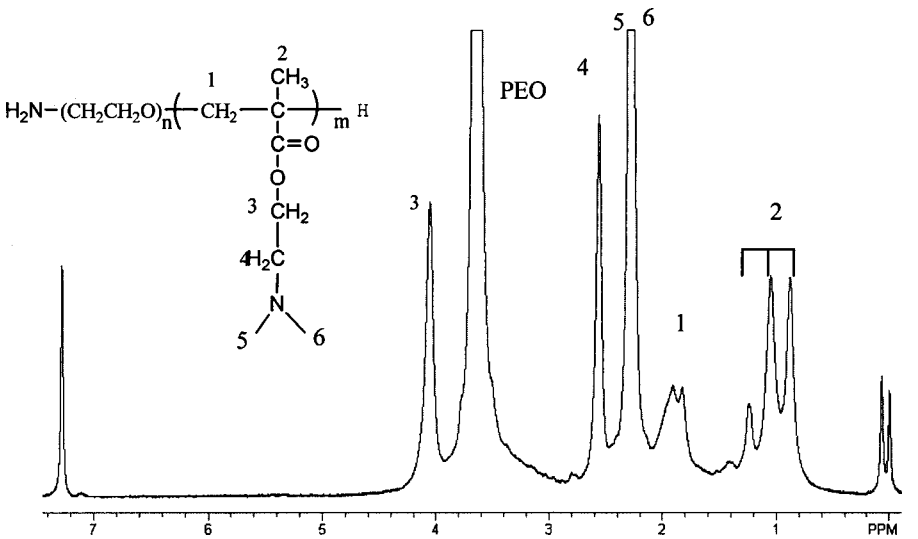


Figure 3 NMR spectrum of H_2N-PEO-PDAMAMA. The molecular weight of PDMAEMA was calculated based on the peak intensity ratio of methylene protons (4.1 ppm, $COOCH_2CH_2N(CH_3)_2$) from PDMAEMA to ethylene protons from PEO (3.6 ppm, OCH_2CH_2).

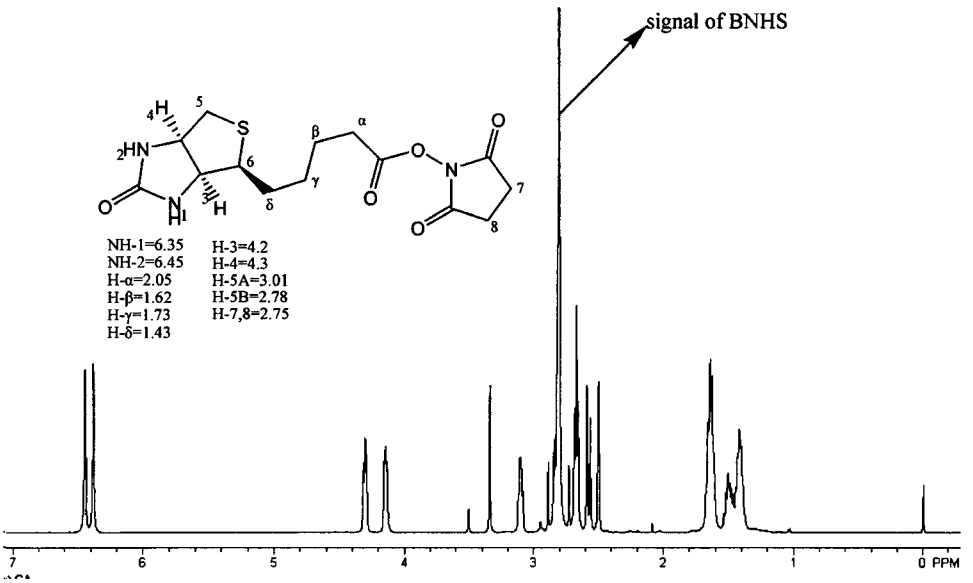


Figure 4 NMR spectrum of BNHS. The peak of 2.8 ppm, which was labeled as the signal of BNHS in Figure 4, composed of two parts, which was H-5B of biotin and H-7, H-8 of NHS. The ratio of H-5B to H-7, H-8 was 1 : 4, which was exactly the same as the ratio of the hydrogen number of the two parts.

Optimal antigen-binding capabilities can be realized using a biotin derivative that has an extended spacer arm reducing steric hindrance. Biotin-NHS is one of activated biotin derivatives. Figure 4 shows the NMR spectrum of BNHS. The peak of 2.8 ppm, which was labeled as the signal of BNHS in Figure 4, composed of two parts, which was H-5B of biotin and H-7, H-8 of NHS. The ratio of H-5B to H-7, H-8 was 1 : 4, which was exactly the same as the ratio of the hydrogen number of the two parts. Therefore this showed the appearance of BNHS.

Biotin-NHS reacts very easily with primary amine. The biotin signal was very weak due to the low amount of this compound in the copolymer. In the NMR spectra of biotin-PEO-PDMAEMA (not shown), the triplet at 2.05 ppm, which can be assigned to the methylene group of chain α in biotin, could not be seen as the signal of PDMAEMA overwhelmed it. The biotin group was identified through the two methine protons (H-3, H-4) from the cyclic biotin structure at 4.3 and 4.2 ppm and two urea protons (NH-1, NH-2) from the cyclic biotin structure at 6.35, 6.45 ppm.

CONCLUSIONS

Well-controlled molecular weight H_2N-PEO-PDMAEMA block copolymers were synthesized with high mean yields via a one-pot sequential anionic polymerization of EO and DMAEMA, followed by a coupling reaction between its poly (ethylene oxide) amino and a biotin derivative. These block copolymers were then successfully conjugated to a biotin derivative (biotin-NHS) for the preparation of bioerodible nanocarriers,[33] with the aim of developing active tissue targeting strategies and drug delivery systems. This route promises good results without the need of complicated purifications. The polymers were characterized by ^{1}H NMR spectroscopy, dynamic light scattering (DLS) and gel permeation chromatography (GPC). Our studies result in a panel of novel macromolecular entities that can be used as starting materials on which to anchor various targeting ligands bearing various chemical functionalities. This way can also be used in the synthesis of other functional DHBCs.

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