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Bioconjugate polymer by photo-iniferter approach: Hydrophilic random or block copolymer-coated surface

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ABSTRACT

The fabrication of a high-performance polymer material is important, since it would have the potential of being utilized as a highly effective platform for bioconjugation. In the present study, hydrophilic random or block polymers were designed by a photo-iniferter approach. As typical monomers, *n*-butyl methacrylate (BMA) and acrylic acid (AA) were selected to prepare a surface modifier on a substrate. As surface modifiers, the monomers were copolymerized in block and random sequences, and the resulting polymer-coated surfaces were compared in terms of surface wet ability and the bioconjugation of the fluorescence-labeled protein. The most favorable characteristic of the resulting polymers was that they offered a dual function: the stability of the adsorbed polymer layer and bioconjugation via functional groups. In the present study, we focused on the physical properties of the polymer-coated surface. Bioconjugation and cell viability were also evaluated.

1. Introduction

An approach to create a bioconjugate polymer bearing a cell membrane structure is to use polymer materials comprising a phosphorylcholine polar group with functional groups for bioconjugation [1–4]. In particular, Ishihara et al. have reported the successful synthesis of phospholipid monomers with high yield and excellent purity [5]; the biointerfaces composed of the phospholipid polymer materials facilitated (i) the suppression of non-specific protein adsorption, (ii) the retention of biomolecular activity, and (iii) easy flowing in micro devices [6–10]. By this approach, the biomolecules could be conjugated on the platform of the phosphorylcholine polar group-enriched surface, just as they could be conjugated in the case of a cell membrane surface. In order to precisely mimic the components on the cell membrane, the fabrication of highly versatile and robust biointerfaces is important.

In the present study, we synthesized hydrophilic random or block polymers as surface modifier by using a photo-iniferter approach. The fundamental polymer design in this polymer consists of three strategies: (i) polymer design to cover a versatile substrate, (ii) conjugation of bioactive molecules, and (iii) alteration of surface wet ability by changing pH. The important factor in creating a bioconjugate surface is designing a polymer that is composed of hydrophobic and hydrophilic parts. The hydrophobic part facilitates the stabilization of the polymer coating on versatile substrates, and the hydrophilic part stabilizes the coating at the water–surface interface with the conjugation of bioactive molecules. In the present study, *n*-butyl methacrylate (BMA) and acrylic acid (AA) were selected as typical monomer units. The favorable characteristics of BMA and AA are as follows: (i) stability of polymer-coated layer, (ii) chemical modification for bioconjugation, and (iii) suppression of non-specific protein adsorption. In particular, AA unit is dominant for the conjugation of bioactive molecules. In the present study, bioconjugation is one of the important aspects along with surface wet ability. Further, living radical polymerization was performed using a photo-iniferter. Generally, living radical polymerization by photo-iniferter proceeds via initiation, propagation, primary radical termination, and transfer to the initiator [11]. The photo-iniferter approach enables to polymerize sequentially after coating the resulting polymer on the substrate. A block polymer comprising BMA and AA was synthesized by the living radical polymerization, and a random copolymer was also synthesized by the conventional radical polymerization. The polymer design, block and random sequences, is discussed along with the effect of the surface wet ability by changing pH and the conjugation of fluorescence-labeled protein.

2. Experimental section

2.1. Materials

Sodium *N,N*-diethyldithiocarbamate trihydrate and benzyl chloride were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. BMA, trimethylsilyl acrylate (TMSA), and AA were purchased from Wako

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Pure Chemical Industries, Ltd., Osaka, Japan, and they were distilled at reduced pressure before use. Tetraethylthiuram disulfide (TD) was purchased from Sigma-Aldrich Corp., MO, USA; 2,2′-azobisisobutyronitrile (AIBN) was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. The other organic solvents were purified by the usual method.

2.2. Instruments

The chemical structure of the compounds was confirmed using ^{1}H NMR (JNM-GSX, 400 MHz; JEOL, Tokyo, Japan). Gel permeation chromatography (GPC) measurements were performed on an HLC-8120GPC (Tosoh Corp., Tokyo, Japan) with TSKgel SuperH2000 and TSKgel SuperH4000 columns (Tosoh Corp.) to determine the molecular weights and distributions. Tetrahydrofran (THF) was used as the eluent. The flow rate was 0.6 mL/min and universal calibration with poly (methyl methacrylate) (PMMA) standards (Shodex Standard M-75; Showa Denko K.K., Tokyo, Japan) was performed. Static contact angle measurements were performed using a DropMaster 500 (Kyowa Interface Science Co., Ltd., Saitama, Japan) with HCl (10 mmol/L) and NaOH (10 mmol/L) solutions. To monitor the changes in weight on a substrate, a quartz crystal microbalance (QCM) substrate with a parent frequency of 9 MHz (USI Co., Ltd., Fukuoka, Japan) was used and the change in the frequency was monitored using a frequency counter (53131A; Agilent Technologies, Santa Clara, CA, USA).

2.3. Synthesis of benzyl N,N-diethyldithiocarbamate

Benzyl *N,N*-diethyldithiocarbamate (BDC) was synthesized by a method previously reported by Otsu et al. [11–13]. In brief, benzyl chloride (5.10 g, 40.3 mmol) in ethanol (10 mL) was added to sodium *N,N*-diethyldithiocarbamate trihydrate (8.20 g, 36.4 mmol) in ethanol (40 mL) for 30 min in the dark and stirred at 0 °C for 1 h. The mixture was stirred for another 21 h at room temperature. The crude product was checked by thin-layer chromatography (TLC). Ethyl acetate/hexane (1/3 by volume) was used as the eluent, and an Rf value of 0.32 was obtained. The crude product was recovered and the solvent was evaporated. The resulting solution was distilled under reduced pressure (4 mm Hg, 140 °C). The chemical structure of BDC was confirmed using ^{1}H NMR (400 MHz, DMSO-d_6): (ppm) 1.13–1.25 (m, 6H, –N–CH_2–CH_3), 3.66–4.03 (q, 4H, –N–CH_2–), 4.51 (s, 2H, –CH_2–phenyl), 7.21–7.42 (m, 5H, –phenyl).

2.4. Synthesis of poly(BMA) macroiniferter

BDC (33.8 mg, 0.14 mmol) was dissolved in THF (4.4 mL) with TD (41.8 mg, 0.14 mmol) as the capping reagent. BMA (2.0 g, 14.1 mmol) was added to the solution and mixed well. After purging with nitrogen gas to remove the oxygen in the solution, the flask was shielded with a three-way stop-cock. A high-pressure mercury lamp (UVL-400P; Riko, Chiba, Japan) was used and photo irradiation (distance = 20 cm) was performed for 12 h at room temperature. Wavelength of the irradiation light was over 280 nm. The crude product was purified by reprecipitation using methanol at 0 °C. The precipitate was dried under reduced pressure. The resulting compound was dissolved in benzene and freeze-dried. The composition of the obtained macroiniferter was confirmed using ^{1}H NMR and GPC. ^{1}H NMR (400 MHz, chloroform-*d*): (ppm) 0.74–1.15 (br, 6H, –CH_2–CH_3 and –C–CH_3 in BMA), 1.15–1.33 (br, 6H, –N–CH_2–CH_3 in BDC), 1.33–1.51 (br, 2H, –CH_2–CH_2–CH_3 in BMA), 1.51–1.71 (br, 2H, –CH_2–CH_2–CH_3 in BMA), 1.71–2.26 (br, 4H, –C–CH_2– in BMA, –CH_2–phenyl in BDC), 3.66–4.15 (br, 6H, –O–CH_2– in BMA, –N–CH_2– in BDC), 7.08–7.30 (br, 5H, –*phenyl* in BDC).

2.5. Synthesis of poly(BMA-block-AA) using macroiniferter

The obtained macroiniferter was dissolved in THF (6.6 mL) and the mixture was purged with nitrogen gas. TMSA was added to the solution and nitrogen gas purging was performed. The flask was shielded as mentioned earlier. Photo irradiation (distance = 10 cm) was carried out for 24 h at −30 °C. The crude product was purified by the following protocol. First, the crude product was added to HCl (1 mol/L)/MeOH (1/1 v/v) solution for deprotecting the trimethylsilyl (TMS) groups for 12 h at room temperature. After the concentration of the solvent, the crude product was added to methanol at 0 °C and maintained at 4 °C to remove the unreacted poly(BMA) macroiniferter. The supernatant in methanol was concentrated in an evaporator and the mixture was added to THF at 0 °C and maintained at 4 °C to remove poly(AA) homopolymer through a side reaction. The supernatant in THF was concentrated and added to acetonitrile at 0 °C and maintained at −20 °C to precipitate poly(BMA-*block*-AA). The resulting compound was dissolved in benzene and freeze-dried. The composition of the obtained poly(BMA-*block*-AA) was confirmed using ^{1}H NMR and GPC. ^{1}H NMR (400 MHz, DMSO-d_6): (ppm) 0.66–1.08 (br, 6H, –CH_2–CH_2–CH_3 and –C–CH_3 in BMA), 1.08–2.02 (br, 8H, –CH_2–CH_2–CH_3, –CH_2–CH_2–CH_3, and –C–CH_2– in BMA, –CH–CH_2– in AA), 2.02–2.41 (br, 1H, –CH–CH_2– in AA), 3.81–4.00 (br, 2H, –O–CH_2– in BMA).

2.6 Synthesis of poly(BMA-random-AA)

The desired amounts of BMA and AA were placed in a flask, and AIBN in ethanol (20 mL) was added to the mixture. The final concentrations of the monomers and the initiator were 20 mmol and 0.2 mmol, respectively. After purging with nitrogen gas, the shielded flask was maintained at 60 °C for 24 h. After the polymerization, the product was added to a large amount of hexane to obtain the polymer. The precipitate was filtered and dried under reduced pressure. The composition of the obtained poly(BMA-*random*-AA) was confirmed using ^{1}H NMR and GPC. ^{1}H NMR (400 MHz, DMSO-d_6): (ppm) 0.60–1.15 (br, 6H, –CH_2–CH_2–CH_3 and –C–CH_3 in BMA), 1.15–2.41 (br, 9H, –CH_2–CH_2–CH_3, –CH_2–CH_2–CH_3, and –C–CH_2– in BMA, –CH_2–CH– and –CH_2–CH– in AA), 3.80–4.05 (br, 2H, –O–CH_2– in BMA).

2.7. Preparation of polymer-coated substrates and their physical properties

Poly(BMA-*block*-AA) was dissolved in methanol to a final concentration of 0.2 wt.%. The resulting random polymer was dissolved in ethanol in a similar manner. QCM gold sensor plates and polyethylene terephthalate (PET) discs (Wako Pure Chemical Industries Ltd., diameter: 14 mm) were used as substrates, and they were dip-coated in the abovementioned polymer solution for 3 min. The polymer-coated substrates were then dried with nitrogen gas and maintained in a desiccator before use. They were evaluated in terms of changes in weight, wet ability, conjugation of fluorescence-labeled protein, and cell viability.

The polymer-coated QCM sensor plate was evaluated for its stability in phosphate buffered saline (PBS; pH 7.4, 10 mmol/L) at room temperature. After immersion for a specific time, the plate was rinsed with ultrapure water and dried with nitrogen gas. The change in the frequency of the QCM sensor plate was monitored and plotted as a function of the immersion time.

The wet ability of the surface was characterized by measuring the static contact angle. The polymer-coated substrate was first immersed in PBS for 1 h. After drying in nitrogen gas flow, the static contact angle on the substrate was measured. A droplet (0.5 μL) was added on the polymer-coated substrate using a micro syringe. For this surface characterization process, two types of solution were prepared—HCl (10 mmol/L, pH 2) and NaOH (10 mmol/L, pH 12)—and the change in wet ability in response to ionization–deionization of the carboxyl groups was evaluated.

2.8. Conjugation on polymer-coated substrates

Bioconjugation was performed using fluorescence-labeled bovine serum albumin (FITC-BSA, A-9771; Sigma-Aldrich, Corp.). As condensing