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The effect of molecular mobility of supramolecular polymer surfaces on fibroblast adhesion

Ji-Hun Seo [a,b], Nobuhiko Yui [a,b,*]

[a] Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo 101-0062, Japan
[b] JST-CREST, Tokyo 102-0076, Japan

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ABSTRACT

The effect of hydrated molecular mobility of polymer surfaces on cell adhesion behavior was investigated. ABA-type block copolymers composed of polyrotaxane (PRX) and hydrophobic anchoring terminal segments were synthesized as a platform of molecularly mobile surfaces. The result of QCM-D measurement in water revealed that the molecularly mobile PRX block copolymer surfaces were higher in hydrated molecular mobility than the corresponding random copolymer surfaces with similar content of hydrophobic methoxy groups. The number of adhering fibroblasts depended on the amount of fibronectin adsorbed from serum but was independent of the molecular mobility. However, the morphology of the adhering fibroblasts was strongly dependent on the extent of molecular mobility in water. These results indicate that molecular mobility on polymer surfaces is one of the significant considerations for regulating cellular responses.

1. Introduction

Regulation of cellular responses on artificial materials is a critical issue in the fields of cell biology, biomaterials science, regenerative medicine, etc. Because cells determine their physiological activity by communicating with adhering substrates, a well-defined surface property is essential for regulating cellular responses [1,2]. Various studies have been carried out to find the critical factors that are responsible for cellular responses, such as adhesion morphology, directionality, differentiation rate, and even stem cell differentiation [3–5]. All these studies have clarified that cellular responses on artificial materials are affected by various physicochemical factors such as polarity, roughness, and chemical composition [6]. Recently, it was further clarified that cells can sensitively respond to not only the physicochemical properties but also the mechanical properties of materials surfaces, such as bulk stiffness [7]. Although these studies have been successfully carried out for clarifying the factors that affect cellular responses, one of the important factors, hydrated molecular mobility of the surfaces, still remains an unexplored research area. Living cells are known to communicate with their environments via their surfaces not statically but dynamically, thus inducing hectic and dynamic motions such as pinocytosis [8]. This dynamic nature of the cell surface allows the cell to continuously remodel the extracellular matrix (ECM) and change its functionality [9]. Therefore, a materials property in response to external signals, especially cell-induced dynamic signals, should be considered as a definite factor in designing biomaterials. Recently, surface analysis using quartz crystal microbalance-dissipation (QCM-D) equipment is gaining recognition as a useful method for measuring changes in hydrated molecular mobility in the microenvironment near a materials surface [10]. Highly dynamic molecular segments such as grafted polymer chains or weakly cross-linked hydrogels in water are known to have a high value of energy dissipation in response to microvibration (external signal) of the surfaces [11]. Therefore, it may be hypothesized that the ability of materials surfaces to respond to the dynamic motion of cell surfaces in a physiological environment could be indirectly obtained by measuring the dissipation signals in water in response to microvibration. If a meaningful relationship between the energy dissipation factor and cellular responses is obtained, the paradigm in defining the property of the biomaterials surface could be changed from static and stationary terms to dynamic and time-dependent terms for understanding dynamic communication between cells and materials. In order to realize this hypothesis, a materials lineup possessing a wide range of hydrated molecular mobility is required. From this point of view, polyrotaxane (PRX) block

* Corresponding author. Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo 101-0062, Japan. Tel.: +81 3 5280 8020; fax: +81 3 5280 8027.
E-mail address: yui.org@tmd.ac.jp (N. Yui).

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copolymer is considered a good model materials of extremely dynamic surfaces in water because of the mobile nature of α-cyclodextrin (α-CD) threaded onto a polyethylene glycol (PEG). Hydrated molecular mobility of the PRX block copolymers can be easily modulated by changing the number of α-CDs threaded on the PEG backbone [12]. Previously, we have successfully synthesized PRX block copolymers that can induce non-specific protein adsorption on their surface [13]. However, the relationship between hydrated molecular mobility and cell adhesion behavior remains an unexplored area. In this study, we synthesized a variety of PRX block copolymers with different numbers of CDs and methylation degrees to modulate the hydrated molecular mobility of their surfaces in an aqueous environment. The relationship between adhesion behavior of fibroblasts and molecular mobility in water, estimated by QCM-D measurement, was investigated.

2. Materials and methods

2.1. Materials

2-Methacryloyloxyethyl phosphorylcholine (MPC) was obtained from NOF Co. (Tokyo, Japan), and 4-(benzodithioyl)-4-cyanopentanoic acid (CTA) was synthesized according to a previously reported method [14]. α-CD, *n*-butyl methacrylate (BMA), sodium hydride, iodomethane, α,α′-azobisisobutyronitrile (AIBN), and all the organic solvents were purchased from Tokyo Kasei Co. (Tokyo, Japan) and used as received. PEG (average molecular weight of 20 kDa) (PEG 20k) was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA), and the hydroxyl end group was substituted with amine groups by using a previously reported method [15]. 2-Methoxyethyl methacrylate (MEA) and 2-hydroxyethyl methacrylate (HEMA) were also purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) and were distilled by passing them through a basic alumina column to remove an inhibitor prior to use.

Goat polyclonal antibody to mouse IgG conjugated with horseradish peroxidase (HRP) was purchased from Abcam Inc. (Cambridge, MA, USA), and mouse monoclonal anti-fibronectin antibody (clone FN-15) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Cell Counting Kit #8 and Hoechst 33258 were purchased from Dojindo Lab (Kumamoto, Japan). Alexa Fluor 546 Phalloidin and other biological reagents were purchased from Gibco Invitrogen Corp. (Grand Island, NY, USA).

2.2. Synthetic process of polymer samples

PEG 20k macro CTA was synthesized as follows: 1 g of PEG 20k bis-amine (0.050 mmol) and 0.018 g of dimethylamino pyridine (0.15 mmol) were dissolved in 5 mL of dichloromethane. To this, 0.14 g of CTA (0.50 mmol) and 0.082 g of water soluble carbodiimide (0.50 mmol) were added and stirred for 12 h at room temperature. Next, fresh dichloromethane was added, and the mixture was re-precipitated in cold diethyl ether. The crude product was then dissolved in water, and dialysis was carried out (MWCO 3500) for a day, followed by lyophilization.

The obtained PEG macro CTA (0.35 g) was then mixed with 3.5 g of α-CD in 25 mL of water at room temperature until a light pink and turbid precipitate was formed. The precipitate was then isolated by centrifuging and washed again with 25 mL of water, followed by a repeat of centrifuging. The pink-colored pseudo-PRX macro CTA was finally obtained as an inclusion complex after lyophilization.

Synthesis of PRX block copolymers: The typical synthetic process of PRX block copolymer was as follows: 0.600 g of pseudo-PRX macro CTA was allowed to react with 0.354 g of MPC (1.20 mmol) and 0.633 g of BMA (4.45 mmol) monomer in 7 mL of ethanol/toluene (1/1) mixed solvent by using 0.820 mg of AIBN (5.00 μmol) as an initiator. The heterogeneous solution was bubbled with an argon (Ar) atmosphere for 15 min prior to placement in a 60 °C oil bath. After 24 h, 15 mL of fresh mixed solvent was added to the solution, and the precipitate was obtained by centrifuging. The obtained polymer was sequentially washed with ethanol, acetone, dimethyl sulfoxide (DMSO), and acetone to remove residual monomers and α-CD. The final precipitate was then dried at 40 °C *in vacuo*, and the polymer was obtained as a white powder. Two different PRX block copolymers containing a different number of α-CDs were synthesized by changing the in feed ratio of α-CD.

Methylation of PRX block copolymers: 200 mg of the synthesized PRX block copolymer was heterogeneously dissolved in 7 mL of dehydrated DMSO. To this, 0.155 g of sodium hydride (6.3 mmol) was added under an Ar atmosphere and mixed for 30 min at room temperature. Next, 0.102 g of iodomethane (0.719 mmol) was slowly added to the mixture and stirred for 3 h at room temperature. After the pH was neutralized, the mixture was transferred to a dialysis tube (MWCO 20000), and the dialysis process was carried out for 3 days. The methylated PRX block copolymer was then obtained by lyophilizing. PRX block copolymers having different degrees of methylation were obtained by changing the methylation time from 1 h to 3 h.

Synthesis of random copolymers containing methoxy and hydroxyl groups: Random copolymers containing the same weight composition of methoxy and hydroxyl groups as the PRX block copolymers were synthesized as follows: 0.633 g of BMA (4.45 mmol), 0.354 g of MPC (1.2 mmol), 0.637 g of HEMA (4.9 mmol), and 1.106 g of MEA (8.51 mmol) were dissolved in 8 mL of toluene/ethanol (1/1 vol%) mixed solvent with 0.4 mg of AIBN. After being bubbled with dry Ar, the mixture was sealed and kept in a 60 °C oil bath for 24 h. The reaction mixture was precipitated in cold diethyl ether, and the precipitant was transferred to a dialysis tube (MWCO 10000) with water for 2 days of dialysis followed by lyophilization. Random copolymers with different compositions were synthesized by changing monomer compositions to produce the same weight compositions of methoxy and hydroxyl groups as those of the PRX block copolymers.

2.3. Surface characteristics

The synthesized polymer (5 mg) was initially dispensed in 5 mL ethanol. Next, 5 mL of water was added to prepare 0.05 wt% of clear polymer solution. Each polymer solution (30 μL) was then uniformly cast on a Cell Desk™ (Sumitomo Bakelite Co., Japan) and glass bottom dish for confocal laser microscope observation, and dried in a clean box at room temperature for a day. Each polymer surface was stabilized in water for a day prior to surface characterization and other biological evaluations.

QCM-D monitoring on the polymer surfaces was carried out by using the Q-sense E1-HO device (Q-sense AB, Gothenburg, Sweden). The molecular mobility at the hydrated surfaces was estimated as follows: The Au sensor was cleaned by applying an O_2 plasma treatment for 5 min and a sequential washing with acetone and ethanol, followed by drying with an Ar blowing device. The sensor was placed in an open-type chamber equipped with the QCM-D apparatus at 25 °C. The resonance frequency at 35 MHz ($f_{\text{gold, dry}}$) and the dissipation energy ($D_{\text{gold, dry}}$) were then measured. Subsequently, $f_{\text{gold, wet}}$ and $D_{\text{gold, wet}}$ in a hydrated state were measured with the bare gold in contact with pure water. After the water was removed, 30 μL of each polymer solution was dropped on the surface. After the surface was dried, the resonance frequencies ($f_{\text{sample, dry}}$ and $f_{\text{sample, wet}}$) and dissipation energies ($D_{\text{sample, dry}}$ and $D_{\text{sample, wet}}$) of the coated surface in both dry and hydrated states were measured using the same procedure as above.

2.4. Evaluation of fibronectin density on the block copolymer surface

Enzyme-linked immunosorbent assay (ELISA) was carried out to estimate the surface density of fibronectin, representative cell adhesive protein. Initially, each polymer surface was brought in contact with 10% fetal bovine serum (FBS) for 1 h at 37 °C. After 3 rinses with PBS, each sample was brought in contact with 2 μg/mL of the primary antibody (anti-fibronectin) solution for 1 h at room temperature. After 4 rinses with PBS, the samples were allowed to react with 8 μg/mL of the secondary antibody conjugated with HRP in bovine serum albumin (BSA)-pretreated 24-well plates for 2 h. After 6 rinses with PBS, 0.5 mL of solution (mixture of 10 mL guanylic acid buffer [pH 3.3], 0.125 mL of 3,3′,5,5′-tetramethylbenzidine [44 mM], and 0.018 mL of H_2O_2) was added to each sample surface in the BSA-pretreated well. After the reaction was quenched with 2N sulfuric acid, the absorbance at 450 nm in each resulting solution was measured by a microplate reader (Multiskan FC; Thermo Fisher Scientific, St. Herblain, France).

2.5. Evaluation of adhesion behavior of fibroblast

The cell adhesion test using NIH3T3 mouse fibroblast was performed on each polymer surface. Approximately 1.0×10^5 cells in 1.0 mL of minimum essential medium (Invitrogen Corp. Carlsbad, CA, USA) supplemented by 10% FBS was incubated on the polymer surfaces for 3 h. After a rinse with fresh medium, the surface adhering cells were observed using an optical microscope, and the number of adhering cells was counted by a Cell Counting Kit #8 (Dojindo, Tokyo, Japan).

For fluorescent microscope observation, the cells on the polymer surfaces were stained as follows: Each substrate was carefully washed with fresh PBS and fixed with 4.0% paraformaldehyde for 10 min at room temperature. After being washed with fresh PBS, the cells were permeabilized with 2.5% Triton X-100 for 10 min and rinsed again with PBS. Alexa Fluor 546 Phalloidin (diluted 1:200) and Hoechst 33258 solution were allowed to sequentially react in the dark for 1 h at room temperature after gentle washing with PBS. The samples were then washed with PBS and observed by a confocal laser microscope (FV10i; Olympus, Tokyo, Japan). The projected cell area and best-fit ellipse aspect ratio of stained fibroblasts were calculated using ImageJ software. The aspect ratio was defined as the ratio of short axis divided by long axis of the best-fit ellipse.

3. Results

Two series of PRX block copolymers with different numbers of α-CD molecules (5–8% CDs threading determined by ^{1}H NMR to the maximum stoichiometric value of α-CD/PEG inclusion complex,