Effect of Well-Defined Polymer Brush Surface on Adsorption Force of Bovine Serum Albumin

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When the materials get contact with blood, non-specific protein adsorption occurs on the surface and the protein adsorption layer induces much severe biological responses including cellular reactions. Therefore, evaluating the first protein adsorption is important for preparing the nonbiofouling surfaces. Atomic force microscopy (AFM) makes it possible to directly measure the forces generated from protein-surface interaction in aqueous media down to few piconewtons range. In this study, we evaluated the adsorption force between bovine serum albumin (BSA)-immobilized AFM cantilever and well-defined polymer brush surface with three kinds of hydrophilic monomers, which are 2-methacryloyloxyethyl phosphorylcholine (MPC), poly (ethylene glycol) methacrylate (PEGMA) and 2-hydroxyethyl methacrylate (HEMA) by surface-initiated atom transfer radical polymerization (SI-ATRP) on initiator-immobilized silicon wafer in buffer solution. We found that the protein adsorption forces depended on the both chemical structure and thickness of the polymer brush layer. In particular, the thickness was important to reduce protein adsorption force. From these results, thick polymer brush surface is one of the candidate surfaces with nonbiofouling characteristics.

Key words: Protein adsorption, force curve measurement, surface-initiated atom transfer radical polymerization (SI-ATRP), atomic force microscopy (AFM)

1. INTRODUCTION

Protein adsorption is the first event that occurs on materials surface when foreign materials get contact with blood. Then, platelet adhesion is introduced via adsorbed protein layers, leading to thrombosis formation. Therefore, prevention of protein adsorption on the surface is very important issue on biomaterials development. To obtain the nonbiofouling property, it is necessary to understand the interaction between protein and the surface of materials. Protein adsorption on various surfaces has been investigated [1-3]. However, the relationship between the protein adsorption and surface property has not been clarified systematically. The purpose of this study is to clarify protein adsorption on various hydrophilic polymer brush surface. High density polymer brush surface via surface-initiated atom transfer radical polymerization (SI-ATRP) makes it possible to construct well-defined surface controlling chain length with narrow molecular weight distribution [4]. The atomic force microscopic (AFM) technology is useful for determining the force generated between substrate and cantilever. We evaluated protein-surface interaction in aqueous medium down to the few piconewtons (pN) range [5] using AFM. Effects of the thickness of polymer brush layer, chemical structure and mobility of polymer chains, and the hydrophilicity of the surface on the adsorption force of protein will be discussed.

2. EXPERIMENTS

2.1. Materials

The 2-methacryloyloxyethyl phosphorylcholine (MPC) was synthesized and purified by the previously reported method [6]. Silicon wafers were purchased from Furuuchi Chemical Co., (Tokyo, Japan). The 2-hydroxyethyl methacrylate (HEMA), poly (ethylene glycol) methacrylate (PEGMA), Copper (I) bromide (CuBr), 2,2'-bipyridyl (Bpy), and ethyl-2-bromoisobutyrate (EBIB) were purchased from Sigma-Aldrich Co. (St. Louis, USA) and used as received. All the other reagents and solvents were commercially available in extra-pure grade and were used as purchased.

2.2 Surface preparation

2.2.1 Immobilization of initiator on silicon surface

To prepare the homogeneous monolayer of the initiator on the silicon wafers, monochlorosilane, 3-(2-bromoisobutryl)-decyl dimethylchlorosilane (BrC10DMCS), was synthesized as previously described [7]. Silicon wafers with 10 nm of SiO₂ layer were cut into 1.0 cm x 2.0 cm, rinsed by sonication with hexane, ethanol and acetone, and then etched with oxygen plasma treatment (300 W, 10 min). Reactions of the chlorosilyl groups of the initiator with surface silanol groups of a silicon wafer were performed in a 50 mmol/L toluene solution of BrC10DMCS for 72 h under inert conditions in the presence of triethylamine as a catalyst and acid scavenger. The wafers were removed from the solution, rinsed with toluene and methanol, and dried in vacuo before used for graft polymerization.

2.2.2 Preparation of well-defined polymer brush surfaces

The graft polymerization of monomer from the BrC10DMCS-immobilized surface was performed using an SI-ATRP method under the conditions shown in Table 1. The target degree of polymerization was 10, 20, and 50. After the solution was stirred for 10 min under an argon gas atmosphere, the BrC10DMCS-immobilized silicon wafers were immersed into the solution and at the same time EBIB was added as a free initiator. After polymerization for 24 h, the substrate was rinsed sufficiently with ethanol. The scheme of the reaction is shown in Fig. 1.

Table 1 Polymerization condition.

Monomer	Solvent	[M]	temperature
MPC	MeOH	0.56	R.t
PEGMA	MeOH/THF=1/1	0.50	40 °C
HEMA	MeOH	2.0	R.t

Initiator: Ethyl-2-bromoisobutyrate (EBIB)

[CuBr] / [Bpy] / [Initiator] = 1 / 2 / 1

[M]: Concentration of monomer

[I]: Concentration of EBIB

R.t: Room temperature

[M] / [I] = 10, 20, 50

Fig. 1 Preparation of PMPC, PPEGMA, PHEMA brush surface via SI-ATRP.

2.3 Surface characterization

The static water contact angles (under dry condition) and air contact angles (in aqueous medium) by captive bubble methods were measured using a goniometer (CA-W, Kyowa Interface Science Co., Tokyo, Japan) at room temperature. In the static water contact angles measurement, the samples were completely dried in vacuo before the measurements. Water droplets of 3 μ L were contacted onto the substrates. The data were collected at three positions on each sample. In air contact angles measurement, the samples were immersed in water for 24 h before the measurements. Air bubbles were contacted onto the substrates. The thickness of the PMPC, PPEGMA and PHEMA brush layers under dry conditions was determined by ellipsometry (J. A. Woollam Co., Inc.). The refractive indices (n_r) for SiO₂, PMPC, PPEGMA, and PHEMA were 1.623, 1.488, 1.500, and 1.500, respectively [8].

All the measurements were recorded in air at room temperature. The surface morphologies of the PMPC, PHEMA, and PPEGMA brush layers were observed with a Nanoscope IIIa AFM (Nihon Veeco, Tokyo, Japan) operated in tapping mode. The measurements were performed under ambient conditions using a standard cantilever at a scan rate of 1.0 Hz. Scan size was 500 nm x 500 nm. Immediately prior to the measurements, the samples were rinsed by sonication in ethanol and dried in an air stream. The root-mean square (RMS) surface roughness was calculated from the roughness profiles.

2.4 Protein adsorption measurement

2.4.1 BSA immobilization onto the AFM cantilever

BSA was covalently immobilized on the AFM cantilever through the condensation reaction between the amino groups in the protein and the carboxyl groups on the AFM cantilever [9]. Commercially available 200 μ m-long V-shaped Si₃N₄ cantilevers (OTR8, Veeco NanoProbe Tips) with an announced spring constant of 0.15 N/m were used. Gold film was prepared on the Si₃N₄ cantilever by plasma-sputtering with 3.0 nm adhesion layer of Cr followed by 10 nm of Au. The oxygen-plasma treated Au-coated cantilever (300W, 3min) was reacted with an ethanol solution of 11-mercapto-undecanoic acid (1.0 mmol/L) for 1 h. After rinsing with water, the cantilever was immersed in water solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (WSC) (0.1 mol/L) and N-hydroxysuccinimide (NHS) (0.05 mol/L) for 30 min. The cantilever was washed with water and immersed in the PBS solution of BSA (1 mg/mL) for 1.5 h at 37 °C. After protein immobilization, the cantilever was kept in PBS at 4 °C.

2.4.2 Adsorption force measurement by AFM

A Nanoscope III atomic force microscope (Digital Instruments) was used to measure the adsorption force. Measurement was performed in contact mode in PBS at room temperature, using protein-immobilized cantilever. The scan rate was 1 Hz. In each force measurement, 100 approaching/retracting f-d curves were collected, and the average value was defined as the adsorption force between the protein and the surfaces.

3. RESULTS AND DISCUSSIONS

3.1 Surface characterization

Fig. 2 shows the tendency between α and $180^\circ - \beta$ (the values of α and β are the contact angles under dry and aqueous states, respectively). Both contact angles had the same tendency, indicating that the surface energy of the polymer-grafted surface kept constant in both dry and aqueous states. In addition, PMPC-grafted surface had very high hydrophilicity in thick layer area.

Fig. 3 shows the polymer layer thickness as a function of molecular weight of free polymer determined by ¹H-NMR. A linear increase in the polymer thickness was observed at each polymer, suggesting that the SI-ATRP of each monomer was well controlled. The graft density

Fig. 2 Contact angle as a function of the layer thickness. (Left; under dry condition, right; in aqueous medium)

Fig. 3 Dry thickness as a function of molecular weight determined by ¹H-NMR.

σ of polymer brushes was estimated by following equation,

$$\sigma = \frac{h\rho N_A}{M_n}$$

where, h is the layer thickness determined by ellipsometry, ρ is the density of dry polymer layer (1.30 g/cm³ for PMPC [10], 1.15 g/cm³ for PPEGMA, 1.15 g/cm³ for PHEMA [11], N_A is Avogadro's number, and M_n is the number-average molecular weight of polymer chains on surface, assumed to be the same as that of each polymers in polymerization solution [12]. We calculated M_n from the formula

$$M_n = DP \times M_0 \times C / 100$$

where, DP is the polymerization degree determined by $[Monomer]/[Initiator]$; M_0 , the molecular weight of each monomer; and C , the rate of conversion to the polymer determined by ¹H-NMR measurements. The calculated polymer chain density is; PMPC 0.26 chains/nm², PPEGMA 0.27 chains/nm², PHEMA 0.59 chains/nm². Since a polymer-grafted layer with a graft density of more than 0.10 chains/nm² forms a high-density brush structure [13, 14], this result indicated that well-packed polymer brush surfaces were prepared on silicon wafer.

The morphology of each polymer grafted surfaces in air condition is shown in Fig. 4. The RMS values which means the surface roughness, of unmodified Si, PMPC50, PPEGMA50, and PHEMA50 were 0.18 nm, 0.27 nm, 0.29 nm, and 0.28 nm respectively. The RMS values of all polymer surfaces were less than 0.60 nm. Compared with previous report [15], these values were very low. This indicated that polymerization by

Fig. 4 AFM 3D images of (a) unmodified Si, (b) PMPC50, (c) PPEGMA50, and (d) PHEMA50 in air.

SI-ATRP proceeded uniformly.

3.2 Adsorption force measurement between BSA and polymer brush surfaces

Force curve measurement by AFM was performed in contact mode in PBS using BSA-immobilized cantilever. In each force measurement, 100 approaching/retracting f-d curves were collected, and the histogram was made [16]. Force curves and histograms of BrC10DMCS, PMPC10, PPEGMA10, and PHEMA10 were shown in Fig. 5. Each force curve differed in form, and the histograms had different peak in each surface. The PMPC could maintain low adsorption force for protein, consistent with the previous report that PMPC has the high biocompatibility [1]. Then the adsorption force was plotted as a function of the polymer layer thickness (Fig. 6). Adsorption force depended on both chemical structure and thickness of the polymer brush layer. It indicated clear tendency to decrease in adsorption force with an increase in the thickness in each polymer case.

The relationship between hydrophilicity on the surface and the adsorption force is shown in Fig. 7. The protein adsorption force as a function of $\cos(180^\circ - \beta)$, where β is air bubble contact angle in water. The increase in $\cos(180^\circ - \beta)$ means the increase in the hydrophilicity. The increase in the hydrophilicity led to the decrease in the adsorption force as a whole. However, adsorption

Fig. 5 Force curve and histogram of the each surface.

Fig. 6 Adsorption force as a function of thickness of the polymer layer.

force showed difference at the surface with almost the same contact angle. The hydrophilicity of the surface is important to reduce protein adsorption, as generally believing, but not a direct parameter to affect the protein adsorption force. The vertical morphology of polymer brush surface in aqueous medium would affect the protein adsorption force in thick layer areas. As the longer polymer chains reduced the adsorption force effectively, it is considered that mobility of the polymer chains related to hydration with water may be other parameter to determine the adsorption force of protein. More experiments to indicate the direct explanations for protein adsorption on the brush surface are now under going in our laboratory.

4. CONCLUSIONS

Well-defined hydrophilic polymer brush surface was prepared with controlling the polymer chain length to clarify significant parameters of the surface for determining protein adsorption.

Hydrophilic polymer brush surface tended to suppress the protein adsorption, but different adsorption force observed even the surface with almost the same hydrophilicity. Considering the thick polymer layers reduced the protein adsorption force, it was suggested that the vertical morphology of the polymer brush layer related to water hydration was the other important factor on generation of the protein adsorption force.

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Fig. 7 The relationship between adsorption force and hydrophilicity.

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