



# Effect of the distribution of adsorbed proteins on cellular adhesion behaviors using surfaces of nanoscale phase-reversed amphiphilic block copolymers



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## ABSTRACT

In order to create suitable biocompatible materials for various tissue engineering applications, it is important to be able to understand protein adsorption and cell adhesion behaviors on the material's surfaces. It is known that the nanoscale distribution of adsorbed proteins affects cell adhesion behaviors. However, how nanoscale structures affect cell adhesion behaviors is still unclear. Therefore, in this study, we investigate the effect of the distribution of adsorbed proteins by the phase reversal of amphiphilic block copolymers composed of protein-non-adsorptive poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) and protein-adsorptive poly(3-methacryloyloxy propyltris(trimethylsilyloxy) silane) (PMPTSSi) on cell adhesion behaviors. The nanodomain structures of phase-separated block copolymers were successfully confirmed using transmission electron microscopy and atomic force microscopy. Surfaces that had PMPC dot-like domains ( $23 \pm 4$  nm) and ones that had PMPTSSi dot-like domains ( $25 \pm 6$  nm) were made. From protein adsorption and L929 cell adhesion measurements, it was found that even on surfaces with equal quantities of protein adsorption, the number of cells on surfaces with PMPC dot-like domains was larger than those with PMPTSSi dot-like domains. This suggests that the simple phase-reversal of the distribution of adsorbed proteins can be used to affect cell adhesion behaviors for designing biomaterial surfaces for tissue engineering applications.

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## 1. Introduction

It is generally known that protein adsorption and subsequent cell adhesion occur at the interface between biological systems and materials of medical devices. For several medical devices, such as the hollow fibers of artificial dialysis and artificial hearts, material surfaces to which cells cannot adhere are needed because cell adhesion can promote platelet adhesion and thrombosis [1]. In other cases, however, cell adhesion may be desired; for example, the precise orchestration of cell adhesion onto tissue scaffolds is critical in tissue engineering [2]. When the material surface comes into contact with body fluids, proteins adsorb onto the surface, then cells adhere to these protein scaffolds [3,4]. Therefore, the

study and understanding of the interaction between materials and proteins are crucial in designing biointerfaces that are suitable for different applications.

One way to understand the protein adsorption and cell adhesion behaviors on material surfaces is to use nano- and microstructured surfaces created through various nanoscale fabrication methods, such as nanolithography [5,6] and nanoimprinting [7]. Recently, it was shown that the distance between RGD peptides affects cell adhesion behaviors using the nanoscale surface patterns prepared by polymer grafting Au nanoparticles [5,8]. When the distance was more than 73 nm, cells did not adhere to the surface. On the other hand, cells adhered when the distance was less than 58 nm. It was suggested that the clustering of integrins, the adhesion molecules on the cell membrane that bind RGD peptides, was prevented when the distance between the integrins was larger than 58 nm, at which distance the cells could not form stable

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adhesion sites. From these results, it can be concluded that the condition of adsorbed proteins is critical for cell adhesion [9–14].

Block copolymers consisting of hydrophobic and hydrophilic units have been used to make phase-separated patterns on the scale of tens of nanometers to control and analyze the condition of adsorbed proteins [15–17]. In this system, hydrophobic moieties promote protein adsorption and cell adhesion, while the hydrophilic moieties suppress them; therefore, the precise control of hydrophilic/hydrophobic moieties is crucial in controlling cell adhesion behaviors [15,16,18,19]. For example, Okano and others [20] reported that amphiphilic block copolymers consisting of hydrophilic poly(2-hydroxyethyl methacrylate) and hydrophobic polystyrene form lamellar structures with sizes of about 20 nm. This block copolymer has high blood compatibility because the patterned adsorption of plasma protein on the block copolymer surfaces regulates membrane proteins and suppresses the activation of platelets. Because of this property, this block copolymer has been used for artificial blood vessels. In another example, block copolymers composed of poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) and poly(dimethylsiloxane) (PDMS) were used to create phase-separated surfaces with different hydrophobic domain sizes (140–170 nm) to which proteins adsorbed, and it was observed that a higher number of cells adhered to the surfaces with larger domain sizes [21]. As demonstrated in these reports, it is clear that the distribution of adsorbed proteins influences cellular behaviors, but exactly how it influences cell adhesion is not fully understood yet. Therefore, we researched into the relationship between the distribution of adsorbed proteins and cell adhesion behaviors in more detail.

In our study, we investigated the effects of the distribution of adsorbed proteins using amphiphilic block copolymers. For the fabrication of surfaces that differ in the distribution of adsorbed proteins, we used amphiphilic block copolymers with PMPC as the hydrophilic moiety and protein-adsorptive poly(3-methacryloyloxy propyltris(trimethylsilyloxy) silane) (PMPTSSi) as the hydrophobic moiety. PMPC has the ability to suppress protein adsorption [22–24] and PMPTSSi has the ability to promote it [25]. We fabricated surfaces that differed in the distribution of adsorbed proteins by phase reversal of the phase-separated surfaces and analyzed the differences in cell adhesion.

## 2. Materials and methods

### 2.1. Reagents

MPC was purchased from NOF Co. (Tokyo, Japan). MPTSSi was purchased from Shin-Etsu Chemical Co. (Tokyo, Japan). 4-Cyano(pentanoic acid)dithiobenzoate (CPD) was purchased from Strem Chemicals Inc. (Massachusetts, USA). 2,2'-Azobisisobutyronitrile (AIBN) was purchased from Kanto Chemical Co. (Tokyo, Japan). 2% osmium(VIII) oxide was purchased from Wako Pure Chemical Industries (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered

saline (PBS), Alexa Fluor 594 phalloidin and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Invitrogen (Carlsbad, CA, USA). Bovine serum albumin and fluorescein-4-isothiocyanate-labeled albumin were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Triton X-100 was purchased from Amersham Biosciences (New Jersey, USA). All the other reagents and solvents were commercially available in extra-pure grade and were used as purchased from Wako Pure Chemicals (Osaka, Japan).

### 2.2. Polymer synthesis

Poly(MPC-*block*-MPTSSi) was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization using CPD as the charge transfer agent, AIBN as the initiator, and MPTSSi and MPC as the monomers. The different types of synthesized copolymers are summarized in Table 1. Poly(MPC-*random*-MPTSSi) (M-*r*-M-1) was synthesized by free radical polymerization as previously reported [26]. The diblock poly(MPC-*block*-MPTSSi) (M-*b*-M-1) was synthesized with the following conditions. For the initial PMPC synthesis, 7.5 mmol of MPC, 0.063 mmol of CPD, 0.075 mmol of AIBN and 15 ml of ethanol were placed in a test tube, degassed by Ar bubbling for 15 min and then sealed. The test tube was placed in a 60 °C oil bath for 1 day. After polymerization, reprecipitation was conducted using chloroform and diethyl ether and the precipitate was dried under reduced pressure to obtain PMPC. For the subsequent PMPTSSi polymerization onto the PMPC, 0.0315 mmol of PMPC, 1.65 mmol of MPTSSi, 0.075 mmol of AIBN and 15 ml of a mixture of toluene and ethanol (toluene/ethanol = 7/3) were placed in a test tube, degassed by Ar bubbling for 15 min and then sealed. The test tube was placed in a 60 °C oil bath and stirred for 1 day. After polymerization, reprecipitation was conducted using acetone to obtain M-*b*-M-1. The triblock poly(MPTSSi-*block*-MPC-*block*-MPTSSi) (M-*b*-M-2) was synthesized by RAFT polymerization using the CPD as the charge transfer agent, AIBN as the initiator, and MPTSSi and MPC as the monomers. For the initial PMPTSSi synthesis, 12 mmol of MPTSSi, 0.32 mmol of CPD, 0.04 mmol of AIBN and 15 ml of toluene were placed in a test tube, degassed by Ar bubbling for 15 min and then sealed. The test tube was placed in a 60 °C oil bath and stirred for 35 h. After polymerization, reprecipitation was conducted using methanol to obtain PMPTSSi. For the subsequent PMPC polymerization onto the PMPTSSi, 0.0615 mmol of PMPTSSi, 7.5 mmol of MPC, 0.015 mmol of AIBN and 15 ml of a mixture of toluene and ethanol (toluene/ethanol = 4/6) were placed in a test tube, degassed by Ar bubbling for 15 min and then sealed. The test tube was placed in a 60 °C oil bath and stirred for 25 h. After polymerization, reprecipitation was conducted using hexane to obtain the product. The product was then dissolved in water and dialyzed. For the last PMPTSSi polymerization onto the diblock copolymer, 0.027 mmol of poly(MPTSSi-*block*-MPC), 0.828 mmol of MPTSSi, 0.015 mmol of AIBN and 15 ml of a mixture of toluene and ethanol (toluene/ethanol = 5/5) were placed in a test tube, degassed by Ar bubbling for 15 min and then sealed. The test tube was placed in a 60 °C oil

**Table 1**  
Molecular analysis of the synthesized copolymers with <sup>1</sup>H-NMR and GPC.

Polymer	Abbrev.	In copolymer <sup>*</sup> (mole fraction)		Molecular weight, Mn (Da)	Mw/Mn	Solvent used for film-forming
		MPC	MPTSSi			
Block copolymer	M- <i>b</i> -M-1	0.70	0.30	$9.1 \times 10^{4**}$	1.3**	Toluene
	M- <i>b</i> -M-2	0.74	0.26	$5.4 \times 10^{4**}$	2.0**	Ethanol
Random copolymer	M- <i>r</i> -M-1	0.70	0.30	$7.3 \times 10^{4**}$	3.7**	Ethanol

<sup>\*</sup> Determined by <sup>1</sup>H-NMR measurement.

<sup>\*\*</sup> Determined by GPC measurement.

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