Transactions of the Materials Research Society of Japan 31[3] 641-644 (2006)

Non-biofouling Surface Prepared by Living Radical Graft Polymerization of 2-Methacryloyloxyethyl Phosphorylcholine for Micro-biochip

James Sibarani, Tomohiro Konno, Madoka Takai, Kazuhiko Ishihara*
Department of Material Engineering, School of Engineering and Center for Nanobio Integration, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8656, Japan
Fax: 81-3-5841-8647, e-mail: *ishihara@mpc.t.u-tokyo.ac.jp

Surface modifications with highly biocompatible polymers have been researched widely to inhibit blood coagulation on the medical devices or to prevent non-specific biological interactions at the interface of biosensors. These non-biofouling surfaces became much important in the nano-bioengineering fields. In this study, to obtain higher S/N ratio in sensing process using microfluidics, new surface was prepared. It is grafting with 2-methacryloyloxyethyl phosphorylcholine(MPC) using photosensitive macroiniferter comprised of 2-ethylhexyl methacrylate (EHMA) and vinylbenzyl-*N,N*-diethyldithiocarbamate (VBDC). The poly(EHMA-co-VBDC) was coated on the substrate and the polymerization of MPC was initiated by photoirradiation. The density and length of the grafting were varied via composition of VBDC and photoirradiation time, respectively. The modified surfaces were characterized by water contact angle measurement, X-ray photoelectron spectroscopy and ellipsometry. The surface became hydrophilic significantly after grafting the MPC polymer. This is due to the phospholipid polar group in MPC units, which were located at the surface. The protein adsorption resistance of these modified surfaces was investigated using human plasma proteins. The modified surfaces excellently prevent protein adsorption. Abundant of free water layer on hydrophilic surface and the mobility of the graft polymer chain and/or polar groups at the surface are the reasons of the preventing of protein adsorption.

Key words: Non-Biofouling Surface, MPC Polymer, Grafting, Photoiniferter

1. INTRODUCTION

Microfluidic devices have been widely used and extensively developed for many applications in handling biological entities such as DNA separation [1-2], immunoassay [3-4], cell sorting [5], biosensors [6-8], and enzymatic assay [9] since some advances offered by miniaturization including high throughput of the analysis, low-cost fabrication, multiplex functionality, and portability. Therefore, the non-biofouling property of the base microchip surface must be fulfilled for obtaining a good performance of the devices as could be evaluated from the signal to noise ratio (S/N). In fact, since non-specific protein adsorption and denaturation of antibody in solid-phase immunoassay for instance, the ratio of signal to noise is very low. Detection limits of the assay will depend on the ability to control the non-specific adsorption of the analyte and secondary antibody [10]. Using protein blocking buffers did not effectively alleviate this problem. Surface modification of the base microchip surface with biocompatible polymers offers a good solution for obtaining the non-biofouling surfaces.

Biomembrane-like surface modifications with highly biocompatible 2-methacryloyloxyehyl phosphorylcholine (MPC) polymers have been researched intensively to inhibit blood coagulation on medical devices or to prevent non-specific biological interaction at the interface of biosensors [11-18]. Such surfaces have been prepared by a number of methods including coating and grafting through radical polymerization initiated by different approaches. Surface-initiated grafting polymerization of MPC using atom-transfer radical polymerization (ATRP) to prepare uniform coverage of surfaces has been intensively studied [19-22] and the effect of graft density and chain length on protein repulsion also has been reported [20-22]. Although ATRP in microchannel has been carried out previously [23], we are aware that it is very difficult to make patterning and selective modification inside the microchannel.

Living radical polymerization based on dithiocarbmate as photoinifereter (initiator, chain transfer and terminator) which allows surface modification with micron-order regional precision and the possibility to construct a well-defined, patternable, and layer-by-layer integrated molecular system on surfaces has been proposed by Otsu *et al* [24] and Nakayama *et al.* [25]. With this method, it will be easy to make selective modification and layer-by-layer construction in microchannel.

In this study, we prepare non-biofouling MPC-grafted surface on polymer substrate by photochemical process using dithiocarbamate as photoiniferter. By the same way, patterned- reactive layer to bind with specific biomolecules over the non-biofouling surfaces will be constructed for highly sensitive micro-biochip.

2. EXPERIMENTAL

2.1 Materials

Sodium-*N,N*-diethyldithiocarbamate trihydrate, (Wako Pure Chemical Industries, Ltd, Japan), 4-vinylbenzyl chloride (Acros Organics, USA), Sodium Sulfate, 2-ethylhexyl methacrylate (EHMA), 2,2`-azobis (isobutyronitrile) (AIBN), and all analytical grade solvents are purchased from Kanto Chemical (Japan). Fluorescent isothiocyanate labeled albumin (FITC-albumin) was obtained from Sigma Chemical Co (St. Louis, USA). 10X Dulbecco's Phosphate Buffered Saline (PBS) (without calcium chloride and magnesium chloride) was purchased from Invitrogen Corporation. All solutions were prepared with water purified with a Millpore (Progard^{TM} 2 Silver, Japan) system. PET plate was purchased from Iwaki (Japan)

2.2 Macroiniferter Synthesis

The photoreactive monomer vinylbenzyl *N,N*-diethyl-dithiocarbamate (VBDC) was prepared from 4-vinylbenzyl chloride and sodium *N,N*-diethyldithiocarbamate trihydrate as described in [3]. The macrophotoreactive copolymers were prepared by conventional radical copolymerization of VBDC and EHMA using AIBN as initiator in tetrahydrofuran (THF) at 60°C for 8 hours at argon atmosphere. The concentration of VBDC was varied as can be seen in Table I. Total concentration of the monomers and [Monomer]/[Initiator] ratio is 1 M and 500, respectively. After reaction, the copolymers were obtained by dropwisely pour the solution into large amount of methanol-ethanol mixtures (8:2 by volume). For further purification, the obtained copolymers were dissolved again in THF and dropped into large amount of methanol-ethanol mixtures. The copolymers were dried in vacuum and analyzed with gel permeation chromatography (GPC) and ^{1}H-NMR.

2.3. Coating Process

PET plates (50 x 20 x 1 mm) were firstly ultrasonically cleaned for 2 minutes with hexane and coated by dipping for 10 seconds into 0.25% solution of macrophoto-iniferter in toluene and kept in the toluene atmosphere and dark place for 1 night.

2.4. Surface Photo-Graft MPC polymerization

Each iniferter-coated plate was vertically mounted on pyrex test tubes containing 25 ml aqueous solution of 0.3M MPC. Before, the MPC aqueous solution was degassed under vacuum for 1 hour. The solution was bubbled again in the tubes with Argon for 5 minutes before sealed. The plate was then irradiated from a 10 cm distance (intensity 41,000 Lx) with a Hg-Xe lamp (UVL-400HA, RICO, Japan). The temperature of polymerization was maintained at room temperature. The poly(MPC)-grafted plates were washed with a 20 minutes sonication in water and dipping in ethanol before drying under vacuum.

2.5. Surface Characterizations

The surface composition was measured by X-ray photoelectron spectroscopy (XPS) on a Kratos Analytical (AXIS HSi 165 and ULTRA, Manchester, England) with Mg Kα X-rays. The data were collected at takeoff angle of 90°. Water static contact angle was measured by using FACE Automatic Contact Angle Meter CA-W (Kyowa Interface Science. Co. Ltd, Japan). The thickness of macroiniferter-coating layer on PET and the grafted-MPC layer were measured by ellipsometer (NL-MIE, Nippon Laser & Electronics Lab., Nagoya, Japan).

2.6 Determination of Molecular Weight of Grafted Poly(MPC)

To determine the molecular weight of the poly(MPC) layer grafted on the surface, the total amount of the iniferter coated on the PET surface was determined by using UV spectroscopy. Breifly, the iniferter coated on the surface was detached by sonication for 30 minutes in chloroform. A sequence of standard solutions of 4-vinylbenzyl *N,N* diethyldithiocarbamate in chloroform was prepared to make standard curve and the absorbance of the solutions was recorded at 261 nm. The molecular weight was calculated from the weight of the grafted poly(MPC) on the surface (w) by using formula as below:

$$Mw = w/([Sample] \times V_{sample})$$

2.7 Protein Adsorption

Untreated PET plate and some of the MPC-grafted plate (1 cm x 1 cm) were cut and contacted with FITC-albumin solution 0.45 g/dL (BSA) in PBS, pH 7.1, at 37°C for 60 minutes. After that, the plates were rinsed twice with 500 ml fresh PBS by stirring method (300 rpm for 5 minutes). The plates were dried under vacuum and the fluorescence intensity was observed with a confocal fluorescent microscope (Axioskop 2 plus) connected with a camera (Keyence VB-7010).

3. RESULTS AND DISCUSION

Surface modification for obtaining non-biofouling surfaces was conducted by grafting biocompatible MPC on the substrate using photosensitive macroiniferter comprised of EHMA and VBDC. Firstly, this macroiniferter was coated on PET substrate and the thickness of the coated layer was 25.6 nm as measured by ellipsometer. Surface-initiated graft polymerization (`grafting from`) was conducted by irradiating the macroiniferter-coated plate in an aqueous MPC solution. The density of the inifeter and chain length of the grafted poly(MPC) were varied by changing the concentration of BVDC on the macrophotoiniferter and irradiation time, respectively.

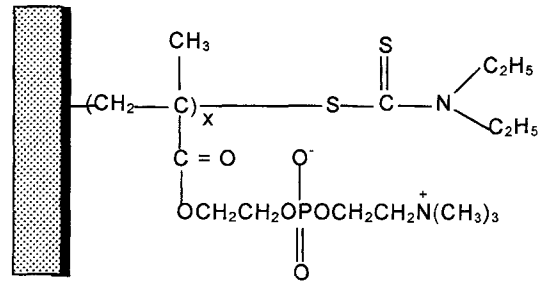

Figure 1: Schematic illustration of poly(MPC)-grafted surface

The schematic structure of the MPC-grafted surface is shown in Figure 1.

3.1. Surface composition

The confirmation of grafted surfaces was conducted by measuring the atomic surface composition before and after grafting using XPS. The peak of P_{2p} (133 eV) that

Table I: Characterization of Poly(EHMA-co-VBDC) (PEV)

Iniferter	EHMA/VBDC		M_w[b]	M_w/M_n[b]
	in feed	in copolymer[a]		
PEV10	90/10	92/8	1.0×10^5	1.55
PEV20	80/20	81/19	1.0×10^5	1.84
PEV30	70/30	68/32	9.0×10^4	1.93
PEV40	60/40	63/37	8.5×10^4	1.98

[M] total = 1M; [M]/[I] = 500; Polymerization time = 8 hours
[a]Determined by 1H-NMR; [b]Determined by GPC, PSt Standard

specifically belongs to MPC did appeared after grafting as can be seen in Figure 2. The value of P_{2p}/C_{1s} ratio increases by grafting time and density of the iniferter on the surface especially in the lower grafting time. The highest value of the ratio (0.07) which is closed to the theoretical value (0.09) indicates that the poly(MPC) was highly oriented on the surfaces (Figure 3).

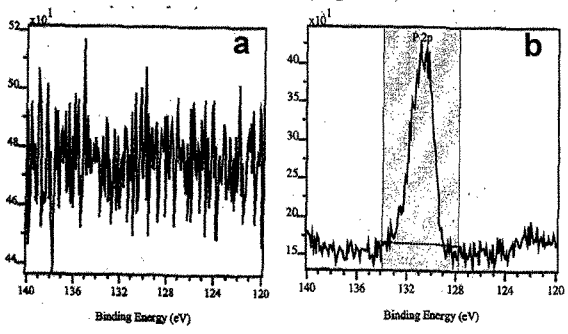

Figure 2: XPS spectra of P2p before (a) and after grafting (b)

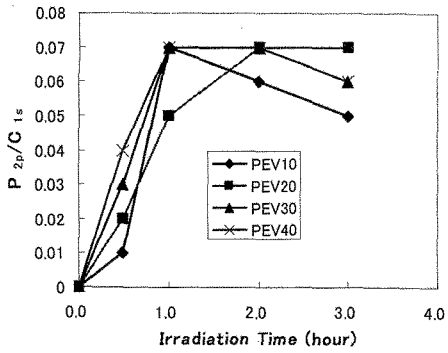

Figure 3: P2p/C1s ratio at variation of irradiation time and photoiniferter density

3.2. Surface water contact angle

The hydrophilic property of the surfaces that is believed plays an important role in protein repelling mechanism was evaluated by static water contact angle as shown in Figure 4. The hydrophilicity of the grafted surfaces is highly improved that can be seen from the

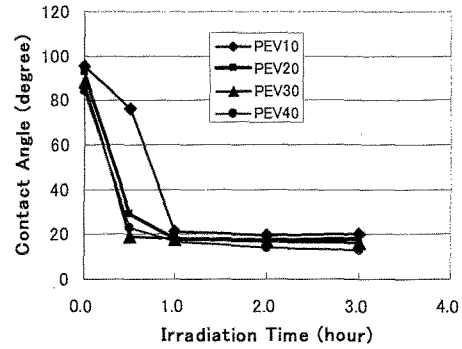

Figure 4: Static water contact angle of MPC-grafted surface as function of irradiation time and photoiniferter density.

decreasing of the water contact angle from more than 80° to less than 30° after 30 minutes irradiation indicating that po(MPC) well-oriented on the surface and covers the surface. Good covering also can be achieved by increasing the density of the macroiniferter. After the poly(MPC) covers the surface, the increasing of the chain length only slightly decreases the water contact angle or became plateau due to the complete covering of the surface by poly(MPC). The same phenomenon was also reported by Iwata et al. [20] and Feng et al. [21] for poly(MPC)-grafted silicon wafer prepared by atomic-transfer radical polymerization (ATRP) that when the thickness was higher than 2.9 nm, the water contact angle remained constant. Hydrophilic property of the this surface is very important for obtaining good protein repelling or non-biofouling surface as protein will contact the surface reversibly without significant conformational change [26].

3.3 Molecular weight and thickness of poly(MPC)

Assumed that all the iniferter available on the macroiniferter-coated surface will proceed the formation of poly(MPC) chain growth under UV irradiation, the molecular weight of the poly(MPC) can be calculated by measuring the weight of the grafted poly(MPC) and the total amount of the photoiniferter on the surfaces. The molecular weight of poly(MPC) increases by increasing the grafting time as shown in Figure 5. The thickness of the grafted poly(MPC) measured by elllipsometer also increases as grafting time as shown in Figure 6. The thickness of the grafted layer will be depended on the photoiniferter density as reported by some other research

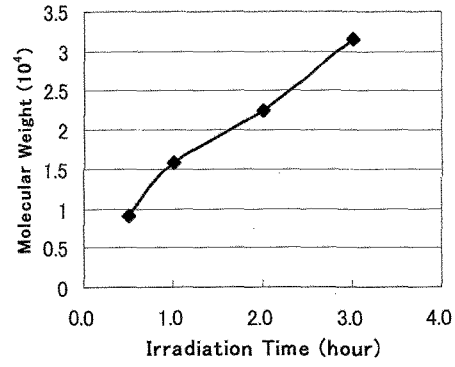

Figure 5: Molecular weight of poly(MPC) on PEV40-coated PET as function of irradiation time

group [19-21]. Jones et al. [19] reported the influence of initiator density on controlled growth of polymer brushes in aqueous solution at room temperature. They found an almost linear relationship between initiator density and the growth rate of the polymer brush.

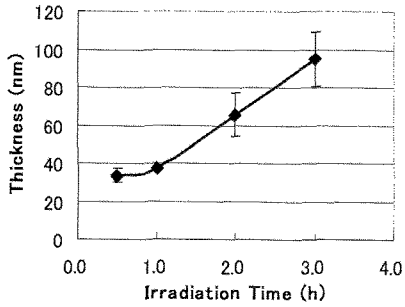

Figure 6: Thickness of poly(MPC) layer on PEV40-coated PET as function of irradiation time.

3.4 Protein Adsorption

The evaluation of protein repelling property, the MPC-grafted surfaces and PET plate as control were contacted with FITC-labeled Albumin at pH 7.1. Albumin which has an isoelectric point of 4.7 is negatively charge at this condition. Fluorescence intensity was measured by a fluorescence microscope completed with a camera. The fluorescence intensity significantly decreases on the MPC-modified surfaces compared to PET bare one as can be seen in Figure 7.

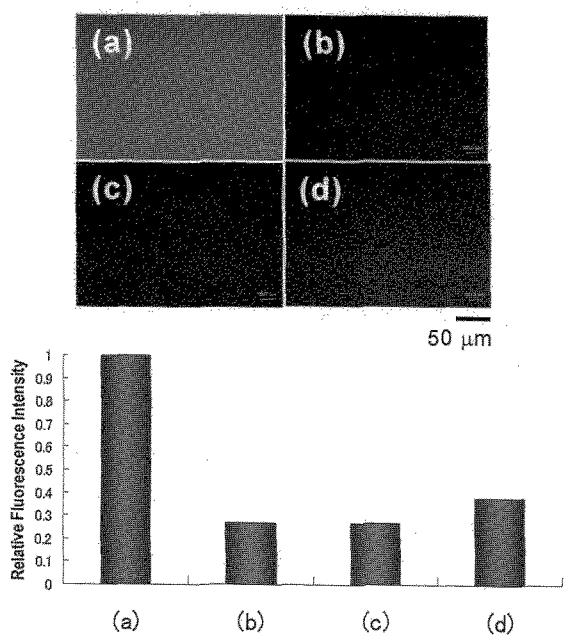

Figure 7: Fluorescence intensity profile of FITC-labeled BSA (Top) of bare PET plate (a), PEV10-g-MPC_{1h} (b), PEV40-g-MPC_{1h} (c) and PEV40-g-MPC_{3h} (d). Fluorescence intensity of those surfaces (Bottom)

As expected, all the MPC-grafted surfaces excellently reduce protein adsorption (up to ~72%) due to the hydrophilic property of the surfaces as the PC groups are highly hydrated and take up large quantities of free water and that protein contacted without changing the native conformation while the bare PET surface induced significant changes in conformation [26]. Besides, protein adsorption to the surface is believed to be resisted by the entropic movement of the highly mobile poly(MPC) chain on the surface [27]. The quantitative amount measurement of the total protein adsorbed on the surfaces is still ongoing.

4. CONCLUSION

Preparation of a non-biofouling surface that became so important in nano-biotechnology fields was successfully conducted by living radical polymerization of MPC onto PET substrate using photoiniferter. All the modified surfaces show an excellent preventing of protein adsorption that hopefully would lead to the enhancing of sensitivity of micro-biochip.

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(Received December 10, 2005;Accepted June 13, 2006)