Plasma-based Surface Functionalization of Polystyrene Substrate for Cell Culture Application

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Abstract: The method to modify the surface of polystyrene (PS) substrate for stable and versatile biomolecule immobilization was developed, which consists of two steps: 1) surface treatment by argon plasma irradiation to introduce hydroxyl group on PS and 2) covalent immobilization of vinylmethylether-maleic acid copolymer (VEMAC) through a coupling reaction between hydroxyl group on plasma-irradiated PS and carboxyl group of VEMAC. For cell culture application, the cell adhesive GRGDS peptide was conjugated with VEMAC immobilized on PS substrate. The cell culture experiments using NIH3T3 (mouse embryonic fibroblast) indicated that the GRGDS conjugated with VEMAC was specifically recognized by cell-surface integrin and promoted the adhesion and proliferation.

Keywords: plasma surface treatment, vinylmethylether-maleic acid copolymer, polystyrene, biomolecule immobilization, cell adhesion

1. Introduction

The covalent immobilization of biomolecules onto a polymer substrate has been of great interest for the development of advanced biomaterials. [1] For example, the polymer surface with a bioactive molecule such as ligand for distinct biological activities is useful for the functional cell culture substrate to study the ligand-cell surface receptor interaction. [2] Polystyrene (PS) is commonly employed for cell culture substrate due to the excellent optical properties as well as mechanical properties. However, PS surface is chemically inert so that the surface is less suitable for the immobilization of bioactive molecules. The plasma surface treatment is an effective method for modifying polymeric materials without altering their bulk properties and has widely been used to improve surface wettability of hydrophobic polymer substrate. [3] On the other hand, the hydrophobic recovery is commonly observed on the plasma-irradiated polymer surface, which is a matter of great concern in the practical use. [4]

In this contribution, we report a plasma-based method to introduce a large amount of carboxyl groups on chemically inert PS surface. This method involves the immobilization of vinylmethylether-maleic acid copolymer (VEMAC) on PS substrate through a coupling reaction between hydroxyl group on plasma-irradiated PS and carboxyl group of VEMAC. For cell culture application, cell adhesive peptide was conjugate with VEMAC immobilized on PS substrate and the effect on the cell adhesion and proliferation were examined using mouse embryonic fibroblast (NIH3T3) as a model anchorage-dependent cell.

2. Experimental

2.1. Plasma irradiation

A commercial polystyrene (PS) dish (35 mm in diameter) cleaned by ultrasonication in methanol and dried in vacuo at room temperature, which was used as a non-treated PS. Ar plasma irradiation to the non-treated PS was carried out using the essentially same apparatus as that reported earlier. [5] The plasma state was generated by the use of radio-frequency (rf) discharge of inductive coupling with five loop antenna at 13.56 MHz with the prescribed power. Flow volume (50 ml/min) and pressure (0.5
Torr) of argon gas were controlled by flow meter and evacuating speed. The sample was placed in the reaction chamber (230 mm long, 45 mm in diameter) to ensure homogeneous exposure to plasma gas. After plasma irradiation, the sample was immediately taken out from the reaction chamber to expose the surface to air.

2.2. Surface functionalization

VEMAC was prepared by hydrolysis of maleic anhydride moiety of commercial vinylether-maleic anhydride copolymer (VEMA) (Fig. 1). The immobilization of VEMAC on plasma-irradiated PS was carried out within 2 h after plasma irradiation to minimize the effects of aging processes of the plasma-modified PS surface. VEMAC was dissolved in 50 mM phosphate buffer solution (PBS) (pH 5.8), and then 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sNHS) were added into the VEMAC solution. The final concentration of VEMAC, EDC and sNHS in the solution was 2% (w/v), 0.2M and 0.1M, respectively. The reaction mixture was stirred for 1h to activate carboxyl groups of VEMAC to form sNHS ester. Then, the solution was poured onto the plasma-irradiated PS dish and incubated at 25 °C for 2 h with gently agitation to immobilize VEMAC on PS. After washing the treated PS with distilled water, to conjugate cell adhesive GRGDS peptide with the VEMAC immobilized on PS dish, 1 mg/mL of GRGDS peptide in PBS (pH 7.8) was poured onto the dish. On the other hand, to prepare VEMAC-immobilized PS (VEMAC/PS), sNHS ester groups of VEMAC immobilized on PS were hydrolyzed with 10mM NaOH. Figure 2 shows the reaction scheme for the preparation of VEMAC/ PS and GRGDS-conjugated VEMAC/PS (GRGDS-VEMAC/PS). For the control experiment, non-adhesive GRGES peptide conjugated VEMAC/PS (GRGES-VEMAC/PS) was prepared by the same method as described above.

2.3 Surface characterization

The density of carboxyl group on PS surface was determined according to the method reported by Sano et al., based on the assumption that Toluidine Blue O (TBO) was complexed to equivalent moles of carboxyl group on solid surface. [6] The measurement of X-ray photoelectron spectroscopy (XPS) was carried out for surface structural analysis of the PS samples using ESCA-3400 (Kratos Analytical Ltd., Japan).

2.4. Cell culture

GRGDS-VEMAC/PS was sterilized by UV irradiation before use. NIH3T3, mouse embryonic fibroblast cell line, was used as a model anchorage-dependent cell for cell adhesion test. NIH3T3 was routinely cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10 % calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C under the humidified atmosphere.

![Fig. 1 Structures of VEMA and VEMAC.](image)

![Fig. 2 Schematic illustration for preparation of VEMAC/PS and GRGDS-VEMAC/PS.](image)
of 5 % CO₂. After trypsin treatment, the cell suspension of the prescribed concentration was prepared with serum-free DMEM and was seeded into each dish. At a given time, the behavior of cell adhesion was observed with phase contrast microscope for the evaluation of cell adhesion properties. The number of cells adhered on each dish was determined by the Cell Proliferation Reagent for WST-1 assay (Dojindo Laboratories, Japan).

### 3. Results and discussion

XPS was applied to characterize the modified surfaces to obtain the information on chemical structure. Table 1 shows the atomic concentration of non-treated PS, Ar plasma-irradiated PS and VEMAC with sNHS ester-immobilized on PS (VEMAC-sNHS/PS) surfaces, which are calculated from XPS wide scan spectra. The N\(_{1s}\) and S\(_{2p}\) signals derived from sNHS ester group appeared after immobilizing VEMAC with sNHS ester. Figure 3 shows the narrow scan spectra of C\(_{1s}\) of each sample and Table 2 summarizes the results of deconvolution analysis of C\(_{1s}\) peak shown in Fig. 3. These results indicate that the VEMAC is successfully immobilized on PS substrate and a large amount of carboxyl group is introduced, because the C\(_{1s}\) peak at 290.3 eV assigned to O−C=O bond was observed in VEMAC/PS surface.

In this method, since VEMAC is immobilized on PS substrate via hydroxyl groups introduced by oxidation of plasma-induced surface radicals of PS, it is likely that the surface density of carboxyl group on VEMAC/PS depends on the plasma conditions. Figure 4 shows the effects of rf power and plasma duration for the plasma surface treatment on the density of carboxyl group on VEMAC/PS. As shown in Fig. 4A, when the plasma duration was fixed at 30s, the observed surface density of carboxyl group was not significantly affected by rf power but the highest density was obtained at 40W of rf power. On the other hand, it can be seen in Fig. 4B that the highest density of carboxyl group on VEMAC/PS was observed at 30s-plasma irradiation when the rf power was fixed at 40W under our experimental setup. These results suggest that the density of carboxyl group on VEMAC/PS can be controlled by plasma conditions.

For cell culture application, the cell adhesive GRGDS peptide was conjugated with VEMAC immobilized on PS substrate and the cell adhesion behavior of the surface was examined. Arginine-Glycine-Aspartic acid (RGD) sequence in GRGDS has been identified as a minimal sequence recognized by cell surface integrins in many extracellular matrix (ECM) proteins. [7] The density of GRGDS immobilized on VEMAC/PS was 1.7 ± 0.2 µg/cm\(^2\), which was estimated by quantifying the unreacted peptide in the immobilization process. Figure 5 shows the morphologies of NIH3T3 on GRGDS-

![Fig. 3 C\(_{1s}\) narrow scan spectra of (A) non-treated PS, (B) Ar plasma-irradiated PS and (C) VEMAC/PS. Plasma conditions: 40W, 30s.](image)

![Fig. 4 Effects of plasma irradiation on the density of carboxyl group on PS/VEMAC dish. The error bars represent the mean ± S.D. (n=3) A: Effect of rf power (plasma duration: 30s) B: Effect of plasma duration (rf power: 40 W)](image)
VEMAC/PS at 24 hours of cell culture with serum-free medium, together with those on non-cell adhesive GRGES-conjugated VEMAC/PS (GRGES-VEMAC/PS) for the comparison purpose. The degree of cell spreading was greater on the surface presenting GRGDS than on GRGES. The shape of most cells adhered on the former was spindle-like whereas cells attached to the latter mostly remained in a rounded shape. These results mean that the GRGDS conjugated with VEMAC is specifically recognized by cell surface and induces cell adhesion, considering the cells are cultured in serum-free medium. To evaluate the effect of GRGDS conjugated with VEMAC on PS on the cell proliferation, the number of NIH3T3 adhered on the surface was compared with those on collagen (type I)-coated PS (BD BioCoat™), GRGES-VEMAC/PS, VEMAC/PS and tissue culture PS (TCPS) dish. The collagen is a component of ECM proteins and the collagen-coated surfaces work as a scaffold for many kinds of anchorage-dependent cell. Figure 6 shows the number of cells adhered on each dish at 2 days in culture with serum-free medium after seeding at 1.0×10⁵ cells/dish. As is apparent from Fig. 6, the cell proliferation was observed only on GRGDS-VEMAC/PS, indicating that cell adhesion to GRGDS conjugated with VEMAC stimulates cell proliferation on PS substrate.

4. Conclusion

We have presented the method to fabricate a versatile platform for immobilizing bioactive molecules on chemically inert PS substrate by immobilizing VEMAC onto plasma-irradiated PS. The peptide conjugated with VEMAC on PS is specifically recognized by cell surface of NIH3T3 and stimulates cell proliferation. The present method is useful to introduce a large amount of carboxyl group onto chemically inert polymer substrate to immobilize biomolecules with high activity for the development of polymeric biomaterials.

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References