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Stabilization of phospholipid polymer surface with three-dimensional nanometer-scaled structure for highly sensitive immunoassay

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ABSTRACT

A phospholipid polymer platform and an antibody as a bioaffinity ligand were used to construct a biointerface for a highly sensitive immunoassay. The platform had a nanometer-scaled particle deposition surface and it was constructed with poly[2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-*n*-butyl methacrylate (BMA)-*co*-*p*-nitrophenyloxycarbonyl poly(ethylene glycol) methacrylate (MEONP)] (PMBN) by an electrospray deposition (ESD) method. The PMBN surface could immobilize specific antibodies through covalent chemical bonding by the reaction between MEONP units and amino groups in the antibody. In addition, the PMBN could prevent nonspecific protein adsorption from an analyte. However, the nanometer-scaled structure of the PMBN lost its shape after immersion in an aqueous medium. To stabilize the nanometer-scaled structure in an aqueous medium, the PMBN was cross-linked with 1,4-butylenediamine and then heat-treated. These treatments effectively improved the stability of the nanometer-scaled structure, that is, the structure had a high porosity even after immersing in an aqueous medium. The stabilization affected the specific signal in the enzyme-linked immunosorbent assay (ELISA), that is, the specific signal in ELISA was enhanced.

1. Introduction

Immunoassays, especially enzyme-linked immunosorbent assay (ELISA), have been used widely in the fields of bioanalysis and clinical diagnosis. However, it is necessary to realize a highly reliable and extremely sensitive ELISA for quantifying a minute amount of biomolecules for accurate diagnosis and for understanding the pathophysiology of diseases. Considering the principle of ELISA, maintaining the bioaffinity of the antibody and the enzyme, capturing the target molecules, and suppressing undesired reactions on the substrate are important to enhance the specific signals and suppress the nonspecific ones. To enhance the specific signal, it is important to immobilize a large amount of antibodies on a substrate with high activity. In the design of a substrate for a high-sensitivity immunoassay, (1) a nano- or micro-ordered surface structure, (2) protein adsorption property, and (3) immobilization method of the antibody on the surface should be considered.

A nanometer-scaled structure having a large surface area is beneficial for the enhancement of the specific signal due to the increase in the amount of immobilized antibodies. Biosensing materials can be manufactured using a variety of nanofabrication techniques such as photolithography, thin-film growth/deposition, etching, and bonding. Nanoporous materials can be manufactured using a variety of techniques including self-assembly and templating (with or without calcinations) and lithography, as well as via many other nanofabrication techniques [1]. Among currently used micro-/nanofabrication methods, electrosprayed deposition (ESD) has been recognized as being one of the most promising methods [2]. The advantage of this method is that polymer nano-/microscaled structures ranging from spheres to fibers can be deposited [3]. When using an ESD substrate to support biomolecules, functional polymers can be electrosprayed to meet different requirements as supports. Furthermore, the high porosity of ESD substrates and their extremely high surface-area-to-volume ratios can provide large specific surface areas for highly efficient immobilization [4].

On the other hand, to reduce the nonspecific signals, nonspecific adsorption of analytes, labeled antibodies, and other proteins on a surface should be suppressed. For this purpose, protein-based blocking reagents such as bovine serum albumin and casein are commonly used in laboratories worldwide. However, protein-based blocking reagents denature easily, and the cross-reaction between detection reagents and blocking reagents remains one of the main causes of a high background and low signal-to-noise ratio.

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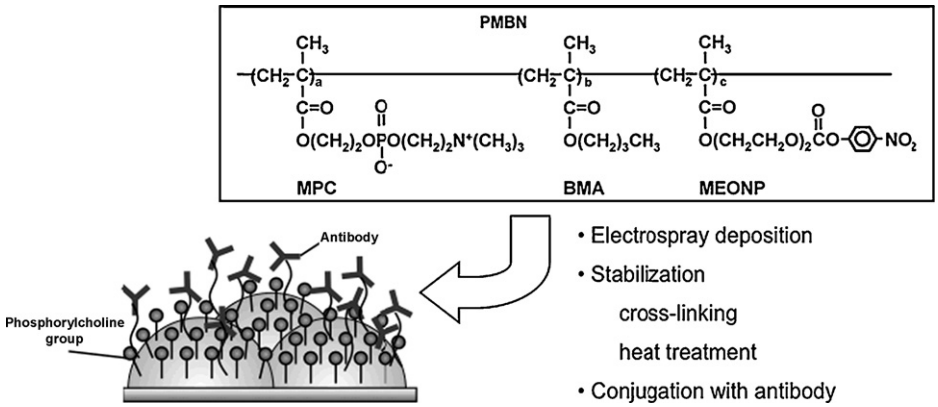

Fig. 1. Schematic illustration of nanometer-scaled structure of PMBN surface and chemical structure of PMBN.

Based on the concept of a protein-free blocking reagent, several types of artificial blocking reagents have been examined [5,6]. Water-soluble amphiphilic 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers have been reported as excellent blocking reagents for ELISA [7–9]. These polymers exhibit high resistivity to protein adsorption and effectively suppress the denaturation of biomolecules [10,11]. The plausible mechanism for the suppression of protein adsorption was discussed in terms of water structure on the surface [12,13]. The MPC polymer surface is hydrophilic, however, it interacts with water molecules very weakly. It means that the surface is covered with bulk-water like water molecules (free-water). Although the main force for adsorbing protein on the surface is based on the hydrophobic interaction, on the MPC polymer surface, the hydrophobic interaction between protein and surface hardly occur due to the water structure.

In our previous study, we developed a solid biointerface for ELISA by integrating a water-insoluble MPC polymer, namely, poly[MPC-*co*-*n*-butyl methacrylate (BMA)-*co*-*p*-nitrophenyloxycarbonyl poly(ethylene glycol) methacrylate (MEONP)]: PMBN [14,15] platform and antibodies as a bioaffinity ligand. The characteristics of MEONP units are suitable for immobilizing biomolecules under very mild conditions, and the biomolecules on the surface exhibited very high activity [16]. Thus, the PMBN surface suppressed the nonspecific signals and maintained the residual activity of the immobilized antibodies after long-term storage [17].

While electrosprayed nanostructure has a high potential such as extremely high surface-area-to-volume ratios, the stability of nanostructure in solution is poor due to its metastable phase [18,19]. To stabilize an electrosprayed structure, several treatments such as cross-linking, heating, and blending have been carried out [20–22]. For applications in immunoassays, an electrosprayed structure should be stabilized to obtain the advantages offered by the nanostructure. Furthermore, the effects of stabilization and the shape of the nanostructure on the surface reaction in the immunoassay should be studied with a view to enhance the sensitivity. We investigated the improvement of the stability of PMBN nanometer-scaled structures in an aqueous medium by cross-linking the PMBN layer and carrying out heat-treatment. The effects of this improvement on the sensitivity of ELISA were described.

2. Experimental

2.1. Synthesis of phospholipid polymer

PMBN was synthesized from the corresponding monomers by a conventional radical polymerization in ethanol using 2,2′-azobisisobutyronitrile (AIBN) as an initiator. After the polymerization, the reaction mixture was poured into a mixture of chloroform and diethylether (2:8) as a solvent to precipitate the polymer. Each monomer unit composition in PMBN was determined by ^{1}H NMR (JEOL α-300, Tokyo, Japan). The molecular weight of PMBN was evaluated by gel-permeation chromatography (GPC) (OHpak SB-804 HQ column, Shodex, Tokyo, Japan) with poly(ethylene oxide) (PEO) standards in a mixture of water and methanol (3:7). The chemical structure of the PMBN is shown in Fig. 1.

2.2. Preparation of nanometer-scaled structured polymer platform

To create a conductive substrate for ESD, gold (Au) was sputtered on a slide glass (Matsunami Glass Ind., Ltd., Osaka, Japan) using a sputtering device (SCOTT-C3, Ulvac, Kanagawa, Japan). 1,4-Butylenediamine (Wako Pure Chemistry Osaka, Japan), a diamine compound that can react with the MEONP unit in PMBN, was used as a cross-linker, and it was added to the PMBN ethanol solution. The values of the functional groups, i.e., number of amino groups in 1,4-butylenediamine/number of MEONP units, were varied as 0, 0.25, 0.50, 0.75, 1.00, and 1.50. The final concentration of the mixed ethanol solution was adjusted to 5.0 wt%. After the solution was kept at room temperature for a certain period of time, it was sprayed on the Au surface using an ESD device (esprayer ES-1000, Fuence, Tokyo, Japan) while maintaining a voltage of 20 kV between the polymer solution and the Au surface. The sprayed substrate was heated at 60 °C for 10 h. A schematic illustration of the nanometer-scaled structure of the PMBN surface is shown in Fig. 1.

To evaluate the stability of the nanometer-scaled structure of PMBN in an aqueous medium, the substrate was immersed in water at 25 °C for 10 h. Then, the substrate was freeze-dried. The morphologies of the surfaces were observed using a scanning electron microscope (SM-200 scanning microscope, Topcon Co., Tokyo, Japan) at an acceleration voltage of 15 keV.

2.3. Quantification of reacted active ester group in PMBN

To investigate the rate of the cross-linking reaction between PMBN and 1,4-butylenediamine, the following experiments were carried out. A desired amount of 1,4-butylenediamine was added to the PMBN ethanol solution and the final concentration of the mixed ethanol solution was adjusted to 5.0 wt%. After the mixed solution was kept at room temperature to allow the condensation reaction to occur, the solution was diluted with ethanol to 0.010 wt% and the absorbance of the solution was measured using an UV/vis spectrophotometer (V-560, JASCO, Tokyo, Japan).