Stabilization of Bioconjugated Phospholipid Polymer
Nanometer Scaled Structures for Highly-sensitive Immunoassay

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Biointerface for highly sensitive immunoassay was constructed by molecular integration between a phospholipid polymer platform and an antibody as a bioaffinity ligand. The phospholipid polymer platform with nanometer-scaled particle deposition surface was constructed with poly[2-methacyryloyloxyethyl phosphorylcholine (MPC)-co-n-butyl methacrylate (BMA)-co-n-nitrophenyloxycarbonyl poly(ethylene glycol) methacrylate (MEONP)] (PMBN) by an electrospray deposition (ESD) method. The PMBN surface, which can conjugate the antibodies, prevented the nonspecific protein adsorption. However, the nanometer-scaled structure of PMBN lost their shape after contact with an aqueous medium. To stabilize the nanometer-scaled structure in an aqueous medium, the PMBN was cross-linked with 1,4-butylenediamine and then heating was applied. Both cross-linking of PMBN and heating were very effective for improving the water stability of the nanometer-scaled structure, that is, remaining high porosity even after immersing in water. The specific signal in the enzyme-linked immunosorbent assay (ELISA) was enhanced after improvement of water stability of the PMBN platform. We concluded that the stabilization of the nanometer-scaled structure of the platform against water was effective to obtain highly sensitive immunoassay.

Key words: MPC polymer, biointerface, biosensor, electrospray deposition, cross-linking

1. INTRODUCTION

Immunoassays, especially enzyme-linked immuno-sorbent assay (ELISA), have been the widely used method in the fields of biochemistry and clinical diagnosis. However, it is necessary to achieve highly reliable and extremely sensitive ELISA for quantifying a minute amount biomolecule for accurate diagnosis and for understanding the pathophysiology of diseases. On the other hand, microchip-based ELISA system is a promising interdisciplinary tool for the next generation clinical diagnosis [1-3]. In a microchip-based ELISA, the sensitivity is also required because the significant decrease in sample volume results in low specific signals. To obtain a highly sensitive immunoassay, both enhancement of specific signal and suppression of nonspecific signal should be achieved.

To enhance the specific signal, it is important to immobilize a large amount of antibodies on a solid surface with its high activity. Many kinds of protein immobilization techniques were reported [4] to enhance the sensitivity in immunoassay. Nanometer-scaled structure, which has large surface area, is beneficial for enhancement of specific signal due to increase in the amount of immobilized antibodies.

On the other hand, to reduce the nonspecific signal, nonspecific adsorption of analyte, labeled antibody and other protein on a surface should be suppressed. To suppress this, protein-based blocking reagents such as albumin are commonly used in laboratories worldwide. However, protein-based blocking reagents denature easily and cross-reaction between detection reagents and blocking reagents persist as one of the main causes of a high background and low signal-to-noise ratio. Based on the concept of protein-free blocking reagent, several types of artificial blocking reagents have been studied and reported [5,6]. The 2-methacyryloyloxyethyl phosphorylcholine (MPC) polymer, which is based on the structure of cell membranes with phosphorylcholine groups, is reported as an excellent blocking reagent for ELISA [7]. It shows a high resistivity to protein adsorption and effectively decreases the denaturation of biomolecules [8-10].

In our previous study, we developed a solid biointerface for ELISA by integration between the MPC polymer, namely, poly[MPC-co-n-butyl methacrylate (BMA) -co-p-nitrophenyloxycarbonyl poly(ethylene glycol) methacrylate (MEONP)]: PMBN (Fig. 1) platform and antibodies as a bioaffinity ligand [11]. The PMBN platform was prepared with a nanometer scaled structure by the electrospray deposition (ESD) method. The MEONP unit contains active ester groups for the conjugation of antibodies via the oxyethylene chain. The PMBN comprises units to prevent nonspecific adsorption as well as to conjugate antibodies in a single polymer chain. The PMBN surface reduced the nonspecific signal and maintained a residual activity of immobilized antibodies after long-term storage [12].

The ESD method can produce nano-micrometer scaled structure films of polymers, proteins, and DNA in forms ranging from particles to fibers by just impressing the
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PMBN was determined by 1H-NMR (JEOL JNM-NR30, solvent. The ratio of monomer unit composition in polymerization, the reaction mixture was precipitated with poly(ethylene oxide) (PEO) standards in a mixture (OHpak SB-804 HQ column, Shodex, Tokyo, Japan) with poly(ethylene oxide) (PEO) standards in a mixture of water and methanol (3:7).

2. EXPERIMENTAL
2.1 Synthesis of the phospholipid polymer
PMBN was prepared using MPC, BMA, and MEONP [15,16]. PMBN was synthesized by conventional radical polymerization of MPC, BMA, and MEONP by using α,α′-azobisisobutyronitrile (AIBN) as an initiator. After polymerization, the reaction mixture was precipitated using a mixture of chloroform and diethyl ether (2:8) as a solvent. The ratio of monomer unit composition in PMBN was determined by 1H-NMR (JEOL JNM-NR30, 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).

2.2 Preparation of nanometer scaled structured polymer platform and evaluation of stability in water
To make a conductive substrate for ESD, Au was sputtered on a slide glass (Matsunami Glass Ind., Ltd., Osaka, Japan) using sputtering device (SCOTT-C3, Ulvac Kiko Inc., Kanagawa, Japan). A diamine compound as a cross-linker, 1,4-butylendiamine (Wako Pure Chem. Ind., Ltd., Osaka, Japan), which can react with the active ester unit in PMBN were added in the PMBN ethanol solution. The mole ratio of amino group to active ester group in PMBN was 1 to 2. The final concentration of the mixed ethanol solution was adjusted to 5 wt%. This solution was sprayed to the Au surface by the ESD device (esprayer ES-1000, fuence, Tokyo, Japan) impressing a voltage at 20 kV between polymer solution and Au surface. The sprayed substrate was heated at 60 °C for 10 h. To evaluate the water stability of nanometer scaled structure, 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 the reacted active ester group in PMBN
MEONP unit in PMBN has an active ester group which can react with amino group. When active ester group in MEONP react with amino group, p-nitrophenol is released as a leaving group. The conversion is estimated by the released p-nitrophenol which has absorbance peak at 405 nm.

The PMBN sprayed substrates prepared by ESD with or without heating were immersed in a phthalate buffer (pH 4). The hydrolysis of the active ester group is accelerated under alkaline conditions producing the p-nitrophenol but the hydrolysis is ignorable under acidic condition below pH 4. After 5 minutes, the substrates were removed from phthalate buffer and concentration of p-nitrophenol in phthalate buffer was determined (C1) by measuring the absorbance at 405 nm using a multilabel counter (Wallac ARVOxs1420, Perkin Elmer). Then, these substrates were immersed in 0.1 M sodium hydroxide (pH 13). After 15 minutes, the substrates were removed and concentration of p-nitrophenol in sodium hydroxide solution was determined (C2) by measuring the absorbance at 405 nm. C1 is the amount of p-nitrophenol released from already reacted active ester group in PMBN before immersing buffer. C2 is the amount of the p-nitrophenol released from residual active ester group in PMBN. The ratio of reacted active ester group by diamine or heating was calculated as C1 divided by total active ester group (C1+C2).

2.4 Protocol of ELISA with PMBN platform
ELISA was carried out with PMBN surfaces as following protocol. Separated micro-well which each volume was 300 L (ProPlate, Grace Bio-Labs, Inc. OR, USA) was attached on the Au sputtered slide glass which surface was already coated by PMBN using ESD. To compare with ESD sprayed surface, polystyrene plate which surface was already coated by PMBN using ESD was not stable in water. Nanometer scaled structures prepared by ESD with hydrophilic polymer was often lost their shape by immersing water without cross-linking or blend with other polymer [14]. In this report, we investigated the effects of improvement of water stability of the nanometer scaled structures on specific and nonspecific signals in ELISA to achieve the high sensitivity. To improve the water stability, PMBN was cross-linked by diamine compound and nanometer scaled structure was heated to change the polymer conformation.

Fig. 2 Schematic illustration of the nanometer scaled structure of PMBN surface.
buffer solution (pH 8) was pipetted into the well and allowed to react with the active ester group of PMBN for 10 h at 25 ºC. After incubation, the wells were washed three times with phosphate buffered saline (PBS, pH 7.1). To perform the antigen-antibody reaction, 100 L of human TSH (Biogenesis Ltd., England, UK) in PBS solution was pipetted into the wells. After the incubation for 2 h at 25 ºC, the wells were washed three times with PBS solution. After that, 100 L of 1 g/mL horseradish peroxidase (HRP) labeled hTSH IgG (Leinco Technologies, Inc., St. Louis, USA) in PBS solution containing 1 wt% bovine serum albumin (BSA) (Sigma-Aldrich, Corp., St. Louis, USA) was pipetted into the wells. After incubation for 1 h at 25 ºC, the wells were washed five times with PBS solution. Then, 100 L of tetramethylbenzidine solution (SUMILON peroxidase chromogenic substrate T, Sumitomo Bakelite Co., Ltd., Tokyo Japan) was pipetted into the wells as a substrate for HRP and incubated for 20 min at 25 °C. After incubation, 100 L of H2SO4 solution was added into the wells and the absorbance at 450 nm and 620 nm were measured using a multilabel counter.

To compare with the conventional method, BSA was used as a blocking reagent by pipetting 1 wt% BSA solution after immobilizing antibodies on the bare polystyrene plate. The antigen-antibody reaction was carried out using this surface by same protocol as mentioned above.

3. RESULTS AND DISCUSSION

3.1 Morphology and water stability of nanometer scaled structured PMBN surfaces

The monomer unit compositions in the PMBN were MPC: BMA: MEONP = 22:65:13. The weight average molecular weight was 3.0 x 10^5.

The PMBN solutions with or without diamine were sprayed by ESD device. Then, these surfaces were heated and contacted with water for 10 h. SEM images of these surfaces are shown in Fig. 3. The nanoparticle-shaped polymer deposition was observed on the surface sprayed by ESD (Fig. 3(a)). However, the non-heated nanometer scaled structure of PMBN without diamine lost their shape drastically (Fig. 3(a-w)). It was due to the hydrophilicity of PMBN, high degree of swelling and conformational instability.

In the case of heated nanometer scaled structure of PMBN without diamine (Fig. 3(b)), the nanometer scaled structure was changed by heating. The nanoparticle-shaped polymer was connected and construct larger scale polymer network because mobility of polymer chain was increased at a high temperature. The nanoparticle-shaped polymer deposition was observed on the surface sprayed by ESD (Fig. 3(a)). The water stability of this surface was improved (Fig. 3(b-w)) compared with non-heated nanometer scaled structure without diamine. The amino group reacted with active ester group in PMBN and the polymer chains were cross-linked. The water stability of nanometer scaled structure was improved by the cross-linking, but its nanometer scaled structure was changed by immersing water.

In the case of the heated nanometer scaled structure of PMBN with diamine was not changed after immersing water (Fig. 3(d), (d-w)). This surface showed the best water stability and completely remained its initial shape which has high porosity.

From these results, it was found that the water stability of PMBN nanometer scaled structure was improved by heating or cross-linking. Both heated and cross-linked nanometer scaled structure was not changed its shape at all after immersing in water.

3.2 The ratio of reacted active ester group in PMBN by diamine and heating

The ratio of reaction between active ester group and amino group was determined by detecting the released p-nitrophenol. The results are shown in Fig. 4. From this result, the ratio of reacted active ester group in PMBN was increased about 30 % by adding the 1,4-butylendiamine. It indicates that the active ester group in PMBN reacted with amino group and PMBN was cross-linked by diamine. Considering that the mole ratio of amino group to ester group was 1 to 2 in the solution before ESD, 60 % of amino group reacted with active ester group.

The active ester group was hydrolyzed with water in the atmosphere about 10 % only by heating. The heated nanometer scaled structure cross-linked with diamine showed the highest ratio of reacted active ester group, 65 %, but residual 35 % of active ester group was much higher amount than amount of antibodies immobilized on the PMBN platform by theoretical calculation.
3.3 Effects of water stability of PMBN platform on sensitivity in ELISA

ELISA with PMBN coated surfaces was carried out to evaluate the specific and nonspecific signals. The results are shown in Fig. 5. The both non-cross-linked and non-heated surface showed small specific signal compared with background level. This surface can immobilize the large amount of antibodies, but the large amount of immobilized antibodies could not enhance the specific signal. It is considered that immobilized antibodies were buried by polymer because nanometer scaled structure was lost by swelling in water as shown in Fig. 3(a-w). Therefore, antigen could not access and react with immobilized antibodies, and scarcely showed a specific signal.

In the case of either heated or cross-linked surface which nanometer scaled structures were relatively stable compared with both non-cross-linked and non-heated surface showed higher specific signal. The shape of nanometer scaled structure after immersing in water affected the specific signal.

Both cross-linked and heated PMBN surface which has high water stability shows large specific signal. Because this nanometer scaled structure has high water stability remaining its high porosity of nanometer scaled structure after immersing in water, the antigens can access the immobilized antibodies without any obstacle caused by swelled polymer. Therefore, the immobilized antibodies can react with antigen and showed high specific signal. It was also confirmed that the residual amino groups did not affect the assay because of its background level was not changed compared with non-cross-linked nanometer scaled structure. The sensitivity was enhanced by using both cross-linked and heated PMBN surface compared with PMBN dip coating and BSA blocking. From these results, the PMBN nanometer scaled structure, which has high porosity and high water stability shows the high sensitivity in the immunoassay.

4. CONCLUSION

The PMBN platform with nanoparticle-shaped surface was constructed by the ESD method. Though the nanometer scaled structure was lost after immersing in water for 10 h, we could improve the water stability by cross-linking and heating of PMBN platform. The specific signal in the ELISA was enhanced with stabilized PMBN platform. Thus, the nanometer scaled structure of PMBN realized highly sensitive immunoassay.

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References


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