Transactions of the Materials Research Society of Japan 32[2] 587-590 (2007)

Nanostructured Biointerface Using Phospholipid Polymer for Highly Sensitive Immunoassays

Kazuki Nishizawa, Tomohiro Konno, Madoka Takai and Kazuhiko Ishihara

Department of Materials 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: nishizawa@mpc.t.u-tokyo.ac.jp

To develop a highly sensitive immunoassay, both enhancement of specific signals and reduction of nonspecific signals should be achieved. In this study, we investigated a novel biointerface for highly sensitive immunoassays by integrating a phospholipid polymer with a nanoscale surface modification process known as the electrospray deposition (ESD) method. The surface prepared by ESD has a nanosphere-shaped polymer structure and therefore the specific signals from analytes in the enzyme immunoassay were significantly enhanced due to an increase in the surface area. The sprayed phospholipid polymer prevented nonspecific protein adsorption effectively lowering the nonspecific signals which cause the high background or noise level. Furthermore, the phospholipid polymer has active ester groups for conjugation of antibodies, and the stability of the antibodies conjugated to the polymer surface was improved drastically. The nanosphere-shaped phospholipid polymer surface can be used to yield a highly sensitive, stable, and reliable assay.

Key words: MPC polymer, biointerface, immunoassay, nanostructure

1. INTRODUCTION

Immunoassays are currently an indispensable analytical method in the fields of biochemistry and clinical diagnosis. Among several immunoassays, enzyme-linked immunosorbent assay (ELISA) has been the most widely used method [1, 2] because it can be easily applied to high sensitivity and reliability analysis. Recently, miniaturized biosensors for microdiagnostic devices have been designed to allow bedside monitoring of chemicals and biomolecules [3, 4]. The microchip-based ELISA system is a promising interdisciplinary tool for the next generation clinical diagnosis [5]. Compared with the conventional ELISA carried out in a polystyrene microtiter plate, the microchip-based ELISA enhances the reaction efficiency. In addition, it has other advantages such as a short assay time, low sample consumption, and assay automation. However, the significant decrease in sample volume results in low specific signals. Furthermore, in the microscale environment, the high surface area/volume ratio magnifies the influence of nonspecific binding of an analyte or a labeled antibody to the solid surface. These factors are the cause of low specific signal and high noise level. To develop a highly sensitive microchip-based ELISA system, both enhancement of specific signals and reduction of nonspecific signals should be achieved.

The following factors are important for the enhancement of specific signals: (1) increase the amount of immobilized antibodies on a solid surface, (2) enhance the antigen-antibody reaction efficiency, and (3) maintain the activity of immobilized antibodies on the solid surface. Microbeads-based assay [6] is beneficial for increasing the amount of immobilized antibodies. The use of biotinylated antibodies and streptavidin are well-known methods for amplifying signals to enhance the antigen-antibody reaction efficiency. It was reported that when the primary antibody is supported by some ligand via a spacer, the reaction efficiency is enhanced due to the high mobility of the antibody [7]. Although the denaturation of the primary antibody on the solid phase occurs easily, particularly in heterogeneous immunoassays such as ELISA, only few studies that report the long-term stability of the primary antibody have focused on its denaturation. Poly(oxyethylene)- [8] and poly(2-methacryloyloxyethyl phosphorylcholine) (MPC)- [9] brushed layers have been reported as the materials used to reduce nonspecific signals. Among these, the MPC polymer, which is based on the surface structure of cell membranes with phosphorylcholine (PC) groups, is well known as an excellent blocking reagent for ELISA [10]. It shows a high resistivity to protein adsorption and effectively decreases the denaturation of biomolecules because of high number of free water-binding sites in the PC group [11].

In this study, we developed a new solid biointerface with a high sensitivity by integrating a novel MPC polymer, namely, poly [MPC *-co-* *n*-butyl methacrylate (BMA) *-co-* *p*-nitrophenyloxycarbonyl poly(ethylene glycol) methacrylate (MEONP)]: PMBN (Fig. 1) with a nanoscale surface modification process known as the electrospray deposition (ESD) method [12]. The MEONP unit has active ester groups for the conjugation of antibodies via an oxyethylene chain. The PMBN has units to prevent nonspecific adsorption as well as to conjugate antibodies. Thus, this polymer is expected to serve as a suitable material for the construction of a

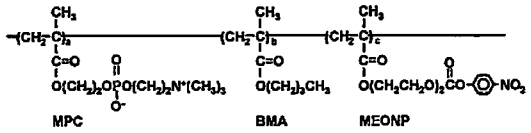

Fig. 1. Chemical structure of PMBN.

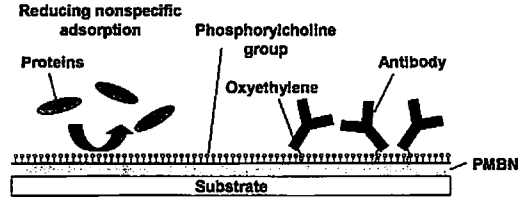

Fig. 2. Schematic illustration of the PMBN surface Antibodies were immobilized via an oxyethylene chain.

biointerface in heterogeneous ELISA that does not require a blocking step. Furthermore, the chemical binding via the oxyethylene chain can enhance the efficiency of antigen-antibody reaction. Schematic illustration of the nanosphere-shaped PMBN surface is showed in Fig. 2.

Similar to the nanoscale surface fabrication process, the ESD method is one of the simple techniques used for increasing the surface reaction area. Recent studies have explored the utility of this method for applications such as high performance filters [13], sensors [14], and scaffolds in tissue engineering [15]. This is because the ESD method can produce nanomicroscaled structure films of polymers, proteins, and DNA in forms ranging from spheres to fibers. The shape of the polymer was controlled by varying the polymer concentration and the electric field applied to the substrate and capillary tip filled with the polymer solution. We developed the highly sensitive biointerface which can conjugate a large amount of biomolecules using the ESD method.

2. EXPERIMENTAL

2.1 Synthesis of bioconjugated phospholipid polymer

The bioconjugate phospholipid polymer (PMBN) was prepared from MPC, BMA, and MEONP. MPC and MEONP were synthesized as previously reported in detail [16-18]. BMA purchased from Nacalai tesque, Inc. (Kyoto, Japan). The PMBN was synthesized by a conventional radical polymerization of MPC, BMA, and MEONP using α,α'-azobisisobutyronitrile (AIBN) as an initiator. After the polymerization, the reaction mixture was precipitated using chloroform:diethylether (2 : 8) as solvent. The ratio of monomer unit composition in the PMBN was determined by 1H-NMR(JEOL JNM-NR30, Tokyo, Japan).

2.2 Immunoassay with bioconjugated phospholipid polymer

To confirm the effect of compositions of PMBN on the ELSIA test, PMBN with various compositions were used in this assay. The well of 96-well polystyrene microtiter plate (Nunc Maxisorp F8, Nunc, New York, U.S.A.) was dipped with 0.2 wt% ethanol solution of each PMBN. After drying of the well, 150 μL of 10 μg/mL the anti-[human Thyroid Stimulating Hormone (TSH)] mouse IgG (Bioclone Australia Pty Ltd., Sydney, Australia) in phosphate buffer solution (pH 8) was pipetted into the well of the microtiter plate and allowed to react with the active ester of PMBN for 24 h at 25 °C. After incubation, the wells were washed three times with phosphate buffered saline (PBS, pH 7.1). To perform the antigen-antibody reaction, 150 μL of human TSH(Biogenesis Ltd., England, UK) in PBS solution was pipetted into the wells. After incubation for 2 h at 25 °C, the wells were washed six times with PBS solution containing 0.1wt% Tween20, and then 150 μL of 0.03 μg/mL anti-TSH IgG biotinylated (Bioclone Australia Pty Ltd., Sydney, Australia) 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 °C, the wells were washed six times with PBS solution containing 0.1wt% Tween20, and then 150 μL of 0.16 μg/mL streptavidin-horseradish peroxidase(HRP) (Zymed Laboratories Inc., CA., U.S.A.) in PBS solution containing 1 wt% BSA was pipetted into the wells. After incubation for 10 min at 25 °C, the wells were washed six times with PBS solution containing 0.1wt% Tween20, and then 100 μL 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 μL of H_2SO_4 solution was added into the wells and the absorbance at 450 nm and 620 nm were measured using a multilabel counter (Wallac ARVOsx1420, Perkin Elmer).

2.3 Preparation of nanostructured polymer surface

To make conductive substrate for ESD, Au was sputtered on a polyimide sheet (thickness 75 μm, Kapton, Du pont-Toray Co., Ltd., Tokyo, Japan) using sputtering device (SCOTT-C3, Ulvac Kiko Inc., Kanagawa, Japan). 5 wt% ethanol solution of PMBN was sprayed to the Au/polyimide sheet by the ESD device (esprayer ES-1000, fuence) at a voltage of 20 kV. To compare with ESD sprayed surface in the immunoassay, another Au/polyimide sheet was dipped with a 0.2 wt% ethanol solution of PMBN and dried.

2.4 Immunoassay with nanostructured polymer surface

The sputtered Au/polyimide sheets coated with PMBN by ESD or dip-coating were cut and placed at the bottom of a 96-well polystyrene microtiter plate. This plate was previously coated with an MPC-based polymer to prevent nonspecific binding of biomolecules. This MPC-based polymer was poly(MPC-co-BMA) (PMB30) [19], which monomer composition is MPC:BMA = 30:70. 150 μL of 10 μg/mL the anti-TSH mouse IgG in phosphate buffer solution (pH 8) was pipetted into the well of the microtiter plate and allowed to react with the active ester of PMBN for 24 h at 25 °C. After incubation, the wells were washed three times with PBS. To compare with the conventional method, bovine serum albumin (BSA) was used as a blocking reagent by pipetting 1wt% BSA solution after immobilizing antibodies on the bare polyimide sheet. The antigen-antibody reaction was carried out using these surfaces by same protocol as mentioned above.

3. RESULTS AND DISCUSSION

3.1 Immunoassay using PMBN with various compositions

Fig. 3 shows the results in the ELISA test using PMBN with various compositions. From this result, the MPC unit composition affects background. The increase of MPC unit composition decreases the background levels. But the PMBN which has only 20 % of MPC unit composition reduces the background level effectively. The increase of MEONP unit composition enhances the specific signal. Because of the increase of binding sites for primary antibodies, the amount of immobilized antibodies was increased and enhances the specific signal. From these results, bioconjugated phospholipid polymer was successfully synthesized, and it was confirmed that MEONP unit can conjugate antibodies and enhance the specific signal and MPC unit reduces the background level preventing the nonspecific adsorption of proteins.

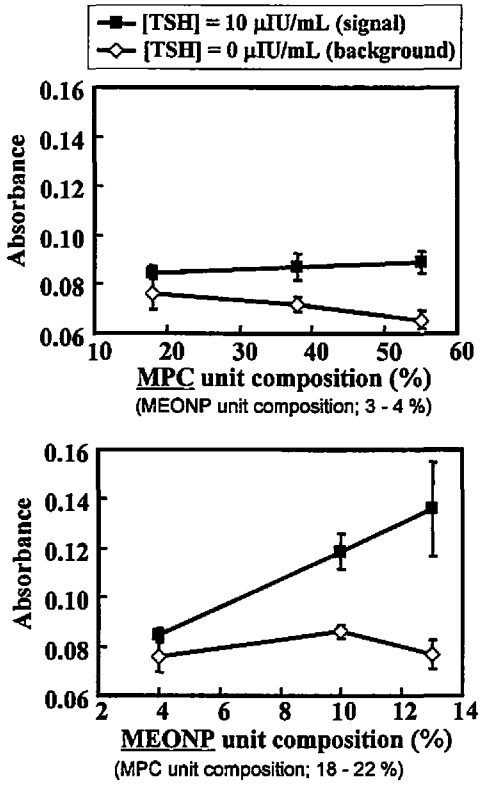

Fig. 3. Absorbance values(450 nm – 620 nm) in the ELISA test using various PMBN. Changes in the composition of PMBN.

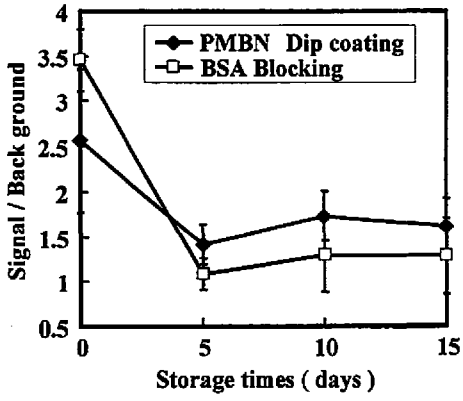

Fig. 4. Long term stability of the immobilized antibodies on various surfaces. Absorbance values (450 nm – 620 nm) in the ELISA test was measured after the immobilized antibodies were stored at 37 °C under dry conditions. Signal/Backgraound was calculated as (Absorbance values of TSH = 10 μIU/mL) / (Absorbance values of TSH = 0 μIU/mL).

3.2 Long term stability of the immobilized antibodies

In order to confirm the stability of the antibodies immobilized on solid surfaces, the wells were incubated under dry conditions at 37 °C for several days after primary antibodies were immobilized, and the assay was then carried out. The results are shown in Fig. 4. The surfaces coated with BSA as a blocking reagent showed a low stability of immobilized antibodies. Its specific signal was decreased drastically in 5 days. This indicates that the physical adsorption of the antibody to the polystyrene plate easily induces the denaturation of the antibody. Compared with the BSA blocking, the surface that was dip-coated with PMBN showed the relatively small change in the S/B ratio. PMBN surface had maintained the activity of the immobilized antibodies after 15 days. This indicates that the PMBN surface provides a stable condition for the antibodies due to the presence of the MPC unit and chemical binding to the polymer via the oxyethylene chain. The long-term stability of immobilized antibodies is necessary for heterogeneous immunoassays when used in diagnosis. Therefore, the PMBN surface has great advantages with regard to practical use in immunoassays.

3.3 Immunoassay with nanostructured polymer

Fig. 5 shows the results of the immunoassay with the nanostructured surface sprayed by the ESD method. In the Figure, the result with dip-coated surface is also displayed. With regard to specific signals, the PMBN surface prepared by dip-coating had a very low absorbance, whereas the absorbance of that sprayed by ESD was considerably higher. The specific signal of the PMBN surface sprayed by ESD was 4 times of that in the case of dip coating. This indicates that a large amount of antibodies were immobilized on the nanostructured surface because of the increase in the surface area. In the assay using the PMBN surface sprayed by ESD, the Signal/Background (S/B) ratio was significantly enhanced to 15 (Table I). The main factors responsible for this were the enhancement of specific signals and the reduction of background signals, which occurs due to the nonspecific binding of the analyte or enzyme. If the PMBN surfaces prepared by dip-coating and electrospraying are similar, the background signal level increases proportionally with an increase in the surface area. The enhancement of the S/B ratio cannot be solely explained by the increase in the surface area available for immobilization of the antibodies. It is suggested that the reason for the enhancement of the S/B ratio is related to the change in the conformation of the polymer during spraying and swelling; however, the mechanism is still under

consideration. In order to confirm the cause of increasing S/B ration we observed the surface morphology. Fig. 6 shows the images of surface sprayed with a PMBN by the ESD device and dip-coated with a PMBN. A surface dip-coated with PMBN is very smooth, while the nanosphere-shaped polymer network was observed on the surface sprayed by ESD. The highly uneven surface contributes to an increase in the surface area; this is believed to cause a drastic increase in specific signals.

4. CONCLUSION

The bioconjugated phospholipid polymer, PMBN, was successfully synthesized. MEONP unit can conjugate antibodies and enhance the specific signal, and MPC unit reduces the background level preventing the nonspecific adsorption of proteins. Additionally, the stability of the antibodies immobilized on the PMBN via the oxyethylene chain was increased effectively.

By using ESD method for surface modification, nanosphere-shaped polymer surface was constructed. The nanosphere-shaped PMBN surface sprayed by ESD which has high surface area showed a high S/B ratio. Based on these results, the nanosphere-shaped phospholipid polymer biointerface can be used to yield a highly sensitive, stable, and reliable assay. Furthermore, coating with PMBN by ESD can also apply to microchips on which a conductive substrate has been formed by micro patterning. This surface will also enable the assay microchip to become extremely sensitive.

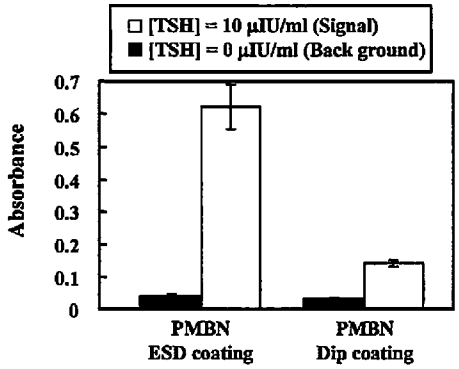

Fig. 5. Absorbance values in the ELISA. Absorbance values was calculated by subtracting the absorbance at 620 nm form absorbance at 450 nm.

Table I. Signal/Background(S/B) ratio in the ELISA test with various solid surfaces. S/B ratio was calculated as (Absorbance values of [TSH] = 10 μIU/mL)/(Absorbance values of [TSH] = 0 μIU/mL) in the ELISA test.

Sample	S/B ratio
BSA Blocking	9.1
PMBN Dip coating	4.3
PMBN ESD coating	15.3

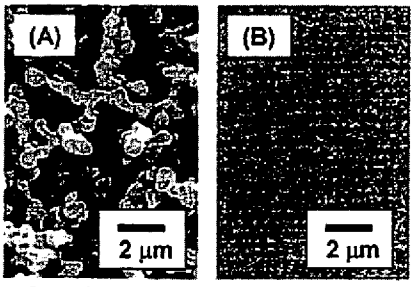

Fig. 6. Scanning electron microscope images of the PMBN surfaces (A) ESD coating at 20 kV and (B) dip-coating on sputtered Au/polyimide sheets.

Acknowledgement
Support in part by Grant for the 21st Century COE Program "Human-Friendly Materials based on Chemistry" from MEXT of Japan.

References

[1] A. G. Farr, P. K. Nakane, *J. Immunol. Method.* **47,** 129 (1981).
[2] L. Orci, *Diabetologia* **28,** 528 (1985).
[3] D. R. Reyes, D. Iossifidis, P. A. Auroux, A. Manz, *Anal. Chem.* **74,** 2623 (2002).
[4] P. A. Auroux, D. Iossifidis, D. R. Reyes, A. Manz, *Anal. Chem.* **74,** 2637 (2002).
[5] P. Arenkov, A. Kukhtin, A. Germmell, S. Voloshchuk, V. Chupeeva, A. Mirzabekov, *Anal. Biochem.* **278,** 123 (2000).
[6] S. Kiichi, M. Yamanaka, T. Hagino, M. Tokeshi, H. Kimura, T. Kitamori, *Lab. Chip* **4,** 570 (2004).
[7] J. Yakovleva, R. Davidsson, A. Lobanova, M. Bengtsson, S. Eremin, T. Laurell, J. Emneus, *Anal. Chem.* **74,** 2994 (2002).
[8] K. Uchida, H. Otsuka, M. Kaneko, K. Kataoka, Y. Nagasaki, *Anal. Chem.* **77,** 1075 (2005).
[9] W. Feng, S. Zhu, K. Ishihara, J. L. Brash, *Langmuir* **21,** 5980 (2005).
[10] S. Sakaki, Y. Iwasaki, N. Nakabayashi, K. Ishihara, *Polym. J.* **32,** 637 (2000).
[11] K. Ishihara, H. Nomura, T. Mihara, K. Kurita, Y. Iwasaki, N. Nakabayashi, *J. Biomed. Mater. Res.* **39,** 323 (1998).
[12] J. Doshi, J. Reneker, D. H. Reneker, *J. Electrostat.* **35,** 151 (1996).
[13] P. Gibson, H. Schreuder-Gibson, D. Rivin, *Colloids surf. A* **469,** 187 (2001).
[14] X. Wang, C. Drew, S-H. Lee, K. J. Senecal, J. Kumar, L. A. Samuelson, *Nano Lett.* **2,** 1273 (2002).
[15] W-J. Li, C. T. Laurencin, E. J. Caterson, R. S. Tuan, F. K. Ko, *J. Biomed. Mater. Res.* **60,** 613 (2002).
[16] K. Ishihara, T. Ueda, N. Nakabayashi, *Polym. J.* **22,** 355 (1990).
[17] T. Konno, J. Watanabe, K. Ishihara, *Biomacromolecules* **5,** 342 (2004).
[18] J. Park, S. Kurosawa, J. Watanabe, K. Ishihara, *Anal. Chem.* **76,** 2649 (2004).
[19] K. Ishihara, Y. Iwasaki, N. Nakabayashi, *Polym. J.* **31,** 1231 (1999).

(Received December 9, 2006;Accepted January 22, 2007)