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Protein immobilization on plasma-polymerized ethylenediamine-coated glass slides

Jinmo Kim,[a] Heonyong Park,[b] Donggeun Jung,[a] and Sunghoon Kim[c,*]

[a] *Department of Physics, Brain Korea 21 Physics Research Division, and Institute of Basic Science, Sungkyunkwan University, Suwon 440-746, Republic of Korea*

[b] *Department of Molecular Biology, Dankook University, Seoul 140-714, Republic of Korea*

[c] *National Creative Research Initiatives Center for ARS Network, College of Pharmacy, Seoul National University, San 56-1, Shillim-dong, Kwanak-ku, Seoul 151-742, Republic of Korea*

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Abstract

For protein chip construction, protein immobilization on the surface of the glass slide is essential. It was previously reported that glass slides are embedded with chemicals that contain primary amines and aldehydes for protein immobilization. We fabricated a plasma-polymerized ethylenediamine (PPEDA)-coated slide that exposed primary amines. For the plasma polymer deposition on the glass slide, the inductively coupled plasma (ICP) power was found to be a critical factor in sustaining a high density of amine on the surface of the PPEDA films. We prepared PPEDA-coated slides at three different ICP powers (3, 30, or 70 W). In the slide that was prepared at a low ICP power (3 W), we detected a high density of primary amine. Therefore, the fluorescein isothiocyanate-conjugated immunoglobulin G (IgG) was highly immobilized to the PPEDA-coated slide that was prepared at the low ICP power. For protein immobilization, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) was used as a cross-linker. The immobilization of the protein to the PPEDA-coated slide was carried out by consecutive incubations with 1 mg/ml EDC for 5 min and 0.1 mg/ml IgG for 1 h. This efficiently produced the functionally active protein-immobilized slide. Therefore, this work shows that the plasma technique can be applied to produce a high-quality glass surface for the immobilization of proteins and other materials.

Since a majority of cellular events are related to protein functions, the functional studies for proteins are most important in understanding their roles in the functional network of biological systems. Although various approaches have been developed and applied to identify the functions for individual proteins, few high-throughput methods for determining protein function have been established. Interestingly, high-throughput techniques were recently introduced for DNA works. DNA chips, as high-throughput tools for DNA analysis, were developed and utilized for several purposes, such as disease diagnosis or drug discovery and development [1,2].

As many cellular proteins have recently been discovered and identified, protein chips are now also required for efficient, functional studies of proteins. Since three-dimensional folding of proteins is very important to their biological activities, more care needs to be taken to create protein chips with the desired quality. Furthermore, because of the different chemical properties of DNA and protein, ways to construct the two biopolymer chips are applied differently. Covalent cross-linking immobilization of protein to solid slides has been reported for the construction of protein chips [3–5].

Recently, there has been considerable research on the immobilization of biopolymers to solid slides via functional groups that are contained in plasma polymer films that are deposited on solid slides [6,7]. In the deposition of plasma polymers, monomeric chemicals are transported into the deposition chamber, activated and/or decomposed into reactive species by plasma, and

* Corresponding author. Fax: +82-2-875-2621.
E-mail address: sungkim@snu.ac.kr (S. Kim).

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then condensed on the glass slides that form thin films of polymers. If the precursors that contain functional groups (such as amine and aldehyde) are used, then there could be a considerable number of functional groups on the surface of the plasma polymer films. These functional groups could be utilized to immobilize the proteins to the solid slides. The properties of plasma polymer thin films are considered to be different from those of conventionally chemical-synthesized polymer films. Plasma polymer thin films are pinhole free, mechanically and chemically stable, and strongly adherent to glass slides because of their highly cross-linked network structures [6–8]. In addition, plasma polymers can be deposited with a high thickness controllability and uniformity within relatively short periods of time. These are great advantages for constructing a protein chip. Due to their excellent uniformity, highly networked structure, and strong adhesion to the slides of plasma polymers, the slides that are coated with plasma polymers with functional groups can provide surfaces with highly uniform, dense, and reliable functional groups.

In this work, we report the immobilization of proteins on plasma polymer-coated glass slides. The plasma polymer that was studied in this work was formed from the ethylenediamine monomer and referred to as plasma-polymerized ethylenediamine (PPEDA).[1] Immobilization of the protein through the cross-linker on the PPEDA-coated slides was affected by the deposition conditions of PPEDA films. Also, the proteins that were immobilized on the PPEDA-coated slides were functionally active.

Materials and methods

Reagents

1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and fluorescein isothiocyanate (FITC)-conjugated mouse immunoglobulin G (IgG) were obtained from Pierce and dissolved in phosphate-buffered saline (PBS). A mouse anti-p18 antibody was prepared as described previously [9]. The Corning (Amino-Silane Coated Slides), Perkin–Elmer (Amino-Silane Coated Slides), and Cel-Associate (Silanated Slides) slides were used as the control slides. The PPEDA-coated glass slides were constructed in this work and are available upon request.

[1] *Abbreviations used*: EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; ICP, inductively coupled plasma; IgG, immunoglobulin G; PPEDA, plasma-polymerized ethylenediamine; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; r.f., radio frequency; SB, substrate bias; BSA, Bio-Imaging Analyzer System.

PPEDA deposition on glass slides

The PPEDA deposition chamber that was used in our work is diagrammatically shown in Fig. 1. Ethylenediamine that was used as the monomer was put in a stainless steel bubbler, which was heated to 50 °C for vaporization. The inert argon (Ar) gas carried the vaporized ethylenediamine monomers to the shower ring, where the vaporized monomers were dispersed into the deposition chamber. Inductively coupled plasma (ICP) was generated around the shower ring by the circular coil that was connected to a 13.56-MHz radio frequency (r.f.) generator through a matching box. Connecting the slide holder to another r.f generator provided the substrate bias (SB) power, which also generated plasma around the glass slide. The deposition chamber is made of stainless steel and has a cylindrical shape. The diameter and height of the deposition chamber were 30 and 28 cm, respectively. Walls of the deposition chamber were grounded. PPEDA films were deposited on the glass slide. Before being loaded into the deposition chamber, the glass slides were cleaned in trichloroethylene, acetone, and methanol and then washed with deionized water and dried. The base pressure of the deposition chamber was less than $\sim 10^{-6}$ Torr, when pumped with a turbo molecular pump. PPEDA films were deposited at the substrate temperature of ~ 27 °C with the deposition pressure of 30 mTorr, the deposition time of 2 min, and the Ar flow rate of 30 sccm. The ICP power affected the density of the amine (NH_2-) functional groups at the surface of the PPEDA films and was the main deposition parameter that was studied in this work. The ICP power varied from 3 to 70 W, and the SB power was fixed at 3 W.

Preparation of protein array and immobilization of immunoglobulin

We fabricated a handmade array for the protein chip by the attachment of the 7×3 circle (3 mm diameter)-punctured rubber mask to the top of a glass slide. Then, the EDC solution was incubated in the holes of the rubber mask for the indicated time points at room temperature. The unbound EDC was thoroughly washed more than four times with PBS. Then, the IgG-diluted solution was incubated for 1 h, washed with PBS or methanol, and dried for fluorescence detection.

Labeling with FITC

Since p18 is barely water soluble, the solubilized NusA, His-tagged p18 protein was constructed and purified as previously described [9]. The purified protein (0.1 mg/ml) was incubated with 200 μM FITC for 10 min at 0 °C in the dark for FITC labeling [10]. Then the reaction was dialyzed twice against cold PBS.

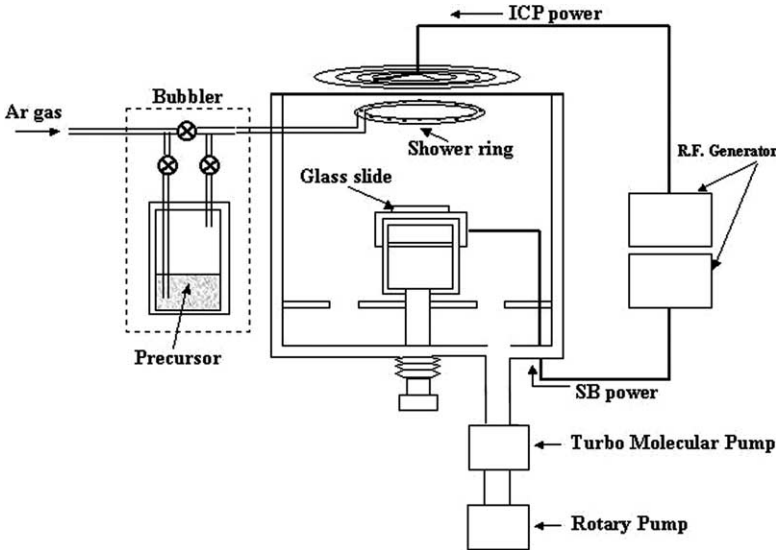

Fig. 1. Simplified diagram depicting the PPEDA deposition chamber. Ethylenediamine was used as the precursor, contained in a bubbler, and heated to 50 °C. Inert Ar gas was used to carry the vaporized ethylenediamine monomers into the deposition chamber. The main parameters of the deposition condition are ICP power, deposition pressure, and deposition time.

Fluorescence detection

FITC-conjugated proteins that were immobilized on the glass slide were detected by the Bio-Imaging Analyzer System (BAS; Fuji Photo Film) at excitation and emission of 490 and 520 nm, respectively.

Results and discussion

Preparation of PPEDA-coated glass slides

Three different types of PPEDA-coated glass slides were prepared and tested to select the slide that would be most appropriate for preparing protein chips. The slides were incubated with EDC for 1 h at the indicated concentration, reincubated with an excessive amount of FITC-conjugated IgG for another hour, and visualized by BAS. Among the three types of slides, the glass slide in the top panel of Fig. 2A was IgG-immobilized to the highest level, indicating that primary amines on this slide may be very densely exposed to react with EDC. The glass slide in the top panel was coated with PPEDA that was deposited at 3 W of the ICP power, it was concluded that 3 W of the ICP power is optimal for protein immobilization. A low amount of IgG was also immobilized even in the EDC-untreated samples (*see* the middle and bottom panels of Fig. 2A). To discover whether the weak binding of IgG in the EDC-untreated samples is a result of the nonspecific binding, the slides were thoroughly washed with methanol and rescanned. Interestingly, the binding signals that are shown in the top panel of Fig. 2A (slides prepared at 3 W ICP power) were unaffected by the methanol wash. However, the weak binding signals in the slides that are shown in the middle and bottom panels were removed by the methanol wash, indicating their nonspecific binding in the middle and bottom slides. In addition, we measured the number of amine groups at the top of the three different slides (Fig. 2B). The slide that was manufactured at the

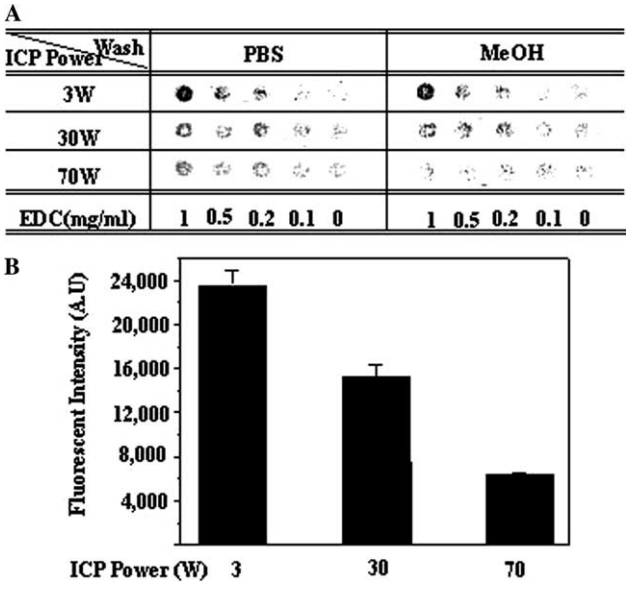

Fig. 2. PPEDA film deposition at 3 W of the ICP power was the best condition for IgG immobilization to the slide. (A) The three different glass slides on which PPEDA was deposited at 3, 30, or 70 W of ICP power were cross-linked to IgG with various amounts of EDC and then finally washed with PBS and methanol. After the slides were subsequently washed with PBS and methanol, the slides were visualized by BAS. (B) The three different glass slides on which PPEDA was deposited at 3, 30, or 70 W of ICP power were reacted with FITC and then washed and dried. Free amine groups in the glass slide were quantified by fluorescence (Ex: 490 nm, Em: 520 nm). The data in the bar graph were obtained from three independent experiments.

ICP power of 3 W contained the highest number of amines. Conclusively, in our experiment, the PPEDA film deposition that was obtained at 3 W of the ICP power is optimal for the construction of protein chips.

Immobilization condition of proteins to the slides

To covalently link proteins to the slides, we chose a cross-linker, EDC, which catalyzes a linkage between the primary amines and the carboxylates. The proteins displayed both primary amines in lysine and N-terminal amines and carboxylates in aspartic acid, glutamic acid, and C-terminal carboxylic acid. We first determined the amount of the IgG immobilization using the BAS. To begin the experiment, the slide was pretreated with an excessive amount of EDC for 1 h at room temperature and then incubated with various concentrations of IgG. The time course data of IgG immobilization showed that IgG immobilization was initiated within 10 min and the maximal efficiency was achieved after a 30-min incubation (Figs. 3A and D). For the dose curve, IgG immobilization was detected in the IgG solution at 0.1 to 1 mg/ml (Figs. 3B and D), which is comparable to the previous observation in which the immobilized protein was visualized at the concentration of 0.1 mg/ml IgG [5].

For the time course of the EDC treatment, the slides were treated with EDC at various time points, incubated with 0.1 mg/ml of IgG for 30 min, and then visualized by BAS after washing off the unbound IgG. In Fig. 3C and D, the 5-min incubation of EDC was sufficient to reach the plateau. This result indicates that the cross-linking of EDC to the PPEDA surface is much faster than the cross-linking execution for an aldehyde-coated slide, which required a 3-h incubation [3]. Therefore, this suggests that the current amine-coated slide may provide a better platform to covalently link proteins than does the conventional aldehyde-coated slide.

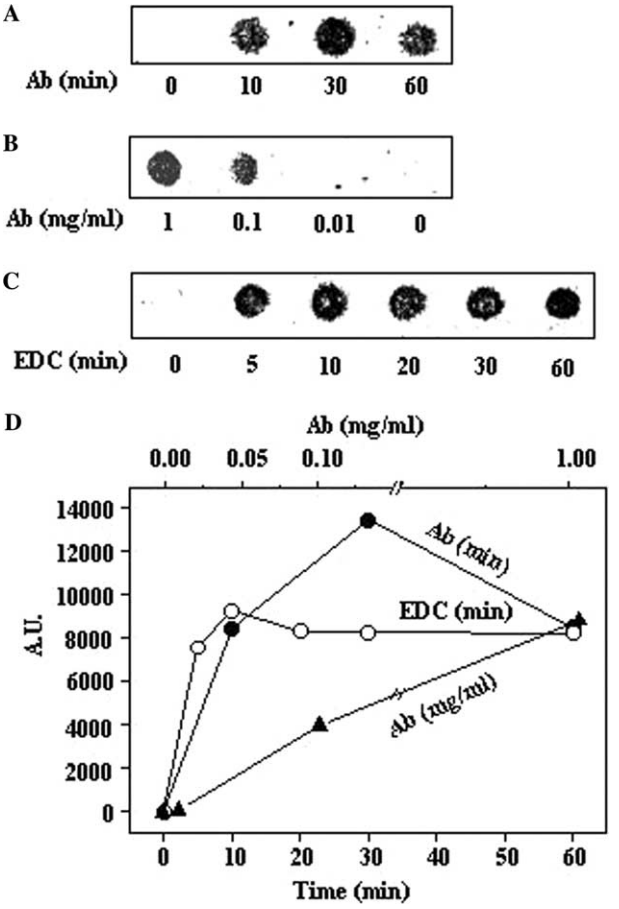

Fig. 3. The optimal condition for immobilization of IgG to the glass slides. Dose- or time-curve experiments were executed to obtain an efficient immobilization of the proteins. (A) EDC (1 mg/ml) was preincubated for 1 h, and 1 mg/ml of the FITC-conjugated antibodies was subsequently incubated at the various time points. (B) After preincubating with an excessive amount (1 mg/ml) of EDC for 1 h, the slide was incubated with the indicated concentration of IgG for 30 min. FITC-conjugated immobilized IgG was then detected. (C) The slide was incubated with 1 mg/ml of EDC for various time points, reincubated with 0.1 mg/ml of IgG for 30 min, and scanned. (D) The fluorescence intensity of each spot in A (closed circle), B (closed triangle), and C (open circle) was plotted after quantification.

Comparison of the PPEDA-coated slide with commercial slides

We compared the PPEDA-coated slide with three other commercial amine-exposed slides for protein cross-linking. In Fig. 4, the IgG binding property for the PPEDA-coated slide (Fig. 4A) is much better than that for the other commercial slides (Figs. 4B–D), when the immobilization was performed under the optimal condition that was determined here.

Immobilized protein is capable of binding its antibodies

To determine whether immobilized proteins are active, an antibody that is specific for a human protein (called p18) was immobilized on the PPEDA-coated slide in the previously mentioned optimal condition and then incubated with its secondary FITC-conjugated antibodies (Fig. 5A) or the FITC-conjugated p18 protein (Fig. 5B). The immobilized FITC-conjugated proteins were visualized when compared to other control samples. The signals were undetected from the spots to which anti-IκB and JNK antibodies were immobilized; however, the binding signal was detected when the FITC-conjugated anti-p18 antibody was used. This indicates that the protein that is immobilized on the surface of the PPEDA-coated slide is functionally active.

Conclusion

Immobilization of immunoglobulins through a cross-linker on the PPEDA-coated slide was affected by the

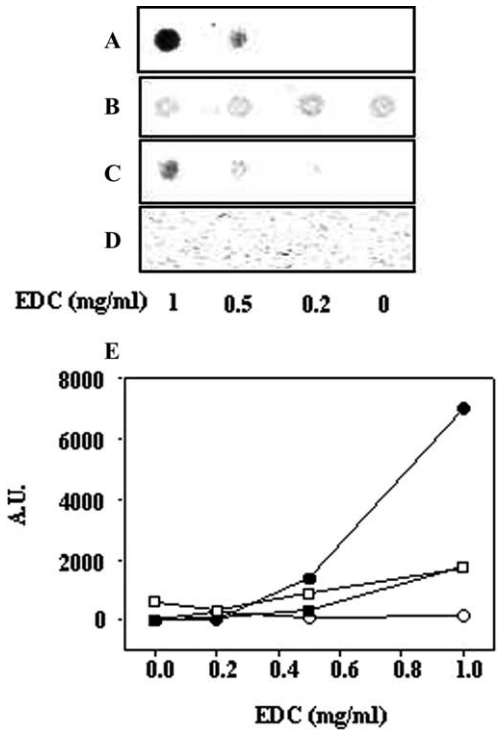

Fig. 4. The PPEDA-coated slide (A) is better qualified for the preparation of protein chips than commercial slides (B, C, and D). The slides were incubated with various amounts of EDC for 10 min and then reincubated with 0.1 mg/ml of FITC-conjugated IgG for 1 h. Immobilized IgG was detected with a BAS photoimager. The Amino-Silane Coated Slides (B, the Corning slide; C, the Perkin–Elmer slide; D, the Cel-Associate slide) were used as the control slides. (E) The fluorescence intensity of each spot was quantified by BAS and plotted as line graphs (closed circles, the PPEDA-coated slide; open squares, the Corning slide; closed squares, the Perkin–Elmer slide; open circles, the Cel-Associate slide).

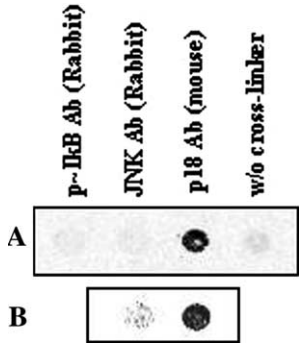

Fig. 5. The proteins that were immobilized on the PPEDA-coated slide are active. (A) No antibody, or the mouse anti-p18 antibody [9], or two different rabbit antibodies–anti-pIκB and anti-JNK antibodies (BD Pharmingen, USA)—were immobilized on the PPEDA-coated slide. Then 0.1 mg/ml of the FITC-conjugated anti-mouse antibody was incubated, washed as described previously, and visualized by BAS. (B) After the mouse anti-p18 antibody and the anti-JNK antibody were immobilized on the PPEDA-coated slide, then 0.1 mg/ml of the FITC-conjugated p18 was incubated and washed. The immobilized FITC-conjugated proteins were visualized by a BAS photoimager.

ICP power that was used during the deposition of the PPEDA films. With optimized deposition conditions, the immobilization of the protein on the PPEDA-coated glass slides was superior to those of the commercially available glass slides in terms of efficiency and time. In addition, the protein that was immobilized on the PPEDA-coated slide was recognized by its specific antibody. Therefore, this slide will be utilized as a very simplified tool for analyzing the protein–protein interaction and as a protein microarray to detect the differential expression of multiple proteins. All of these results suggest that the immobilization of proteins to the PPEDA-coated slide provide an excellent way to produce protein chips.

Acknowledgments

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