Adsorption of anti-C-Reactive Protein Monoclonal Antibody and Its F(ab')2 fragment on Plasma-Polymerized Styrene, Allylamine and Acrylic Acid Coated with Quartz Crystal Microbalance

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Styrene, allylamine, and acrylic acid were polymerized on a quartz crystal microbalance (QCM) by plasma polymerization technique under 100 W, 100 P, and a settled period of each. Coating amounts of plasma polymerized films (pp-films) were determined by QCM technique. Adsorption amounts of anti-C-reactive protein monoclonal IgG antibody (CRP antibody) and its F(ab')2 fragment of IgG antibody (CRP-F(ab')2 antibody) on three plasma-polymerized films and on a gold QCM surface as a reference were also determined by QCM technique. Immunoreaction amounts between antigen (CRP) and antibodies adsorbed QCM on pp-films were determined by QCM. Adsorption amount of CRP antibodies or CRP-F(ab')2 antibodies for pp-films and gold were almost similar levels in the range of 50 to 100 Hz per area. Immunoreaction amounts of CRP depended on the combination of pp-films and antibodies. The largest dose-responses of immunoreaction were obtained for pp-acrylic acid for CRP antibodies, and pp-allylamine for CRP-F(ab')2 antibodies. These results imply that molecular orientation of antibodies adsorbed on pp-films may be performed in different manners.

Keywords: plasma polymerization allylamine and acrylic acid, quartz crystal microbalance (QCM), C-reactive protein (CRP), anti-CRP monoclonal IgG antibody, F(ab')2 fragment of anti-CRP monoclonal IgG antibody

1. Introduction

Pneumonia is the main cause of death of aged people in Japan. The C-reactive protein (CRP) is well-known as a good marker protein to diagnose pneumonia in human blood. Increased CRP of human blood is also an indicator for viral and bacterial infection [1]. Sensing devices for CRP in blood must be developed. Therefore, study of a conventional CRP detection method should seek to produce highly sensitive, rapid, cost-effective, portable, and simple processing.

Immunosensor is one candidate method for conventional CRP detection. Immunosensor advantages include high sensitivity, high selectivity, and low cost. However, this method uses label analysis for transduction of antigen-antibody complex formation [2, 3]. Immunosensors are another candidate for conventional CRP detection. Quartz crystal microbalance (QCM) sensors are currently one of the most exciting technologies to emerge from analytical science. Immunosensors using QCM without non-label analysis for immunoreaction have been reported in recent years [4, 5]. The QCM method is a conventional technique with short detection time and cost effectiveness. Therefore, this study uses the QCM method in all procedures for mass change detection.

It is important to immobilize an antibody on a solid phase surface without inactivating the antibody. One immobilization method is a chemical process. It is somewhat troublesome because several chemical treatments cause antibody denaturation. Another method is a physical process in which the antibody is spontaneously adsorbed on
a solid phase surface. The surface coating material must be selected so that a great quantity of antibody is adsorbed without antibody denaturation [6-8].

Plasma polymerization has been used to form ultra-thin films in a variety of substrates. Plasma polymerization film adheres firmly to a substratum and is highly resistant to chemical and physical treatment [9-14]. Since plasma polymerization allows easy modification of material surfaces, we can expect to increase the amount of antibody adsorption on a solid phase surface using this technique. In previous studies, we found that a plasma-polymerized (pp-) allylamine film adsorbed considerable amounts of F(ab')2 fragments of anti-human IgG antibody, lost relatively small amounts via desorption, and hardly exchanged F(ab')2 fragments of anti-human IgG antibody for other proteins once bound [15-20]. We selected allylamine, styrene and acrylic acid as monomers for plasma polymerization because styrene is well investigated as an antibody immobilized material and acrylic acid has a carboxylic group for physical immobilization of antibodies.

This paper focuses on the relationship between adsorption amount of antibodies on pp-films coated QCM and the immunoreaction amount of CRP for antibodies-adsorbed QCM coated pp-films. We found that adsorption amounts of CRP antibody or CRP-F(ab')2 antibody for pp-films and gold were almost similar levels in the range of 50 to 100 Hz per QCM electrode area. Immunoreaction amounts of CRP depended on the combination of pp-film and antibody. The largest dose-response of immunoreaction for each antibody was obtained at pp-acrylic acid for CRP antibody, and at pp-allylamine for CRP-F(ab')2 antibody, respectively. These results imply that molecular orientation of antibodies adsorbed on pp-films may be affected by the immunoreaction mechanism.

2. Experimental
2.1. Materials and method
The monomers styrene, allylamine, and acrylic acid used in this experiment were all of guaranteed grade and were purchased from Wako Chemical Co., Inc. (Tokyo, Japan); they were used without further purification. However, monomers were thoroughly degassed by repeated freeze-pump-thawing under vacuum to remove trapped atmospheric gases prior to use. Plasma polymerization equipment, model BP-1 Mark II, from Samco International Co., (Kyoto, Japan) was used to plasma-polymerize monomers onto QCMs [12-14]. Water was prepared by Milli-Q (Millipore Ltd., Tokyo, Japan) with specific resistance in excess of 18 MΩ cm⁻¹.

The C-reactive protein (CRP, 1 mg/mL) and Anti-CRP monoclonal IgG antibody (CRP antibody, 10.6 mg/mL) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan); F(ab')2 fragments of anti-CRP IgG antibody (CRP F(ab')2 antibody, 5 mg/mL) were obtained from Sekisui Chem. Co.. Other chemicals used were of guaranteed reagent grade and were purchased from Wako Pure Chemical Co., (Osaka, Japan).

2.2. Plasma polymerization of monomers
Deposition amounts of pp-films were measured by QCM technique during the polymerization process. A QCM with gold electrodes on both surfaces (AT-cut of 9 MHz: 8 x 8 x 0.15 mm) was purchased from Nihon Dempa Kogyo Co., Ltd. (Tokyo, Japan). The pp-films were deposited on both sides of the QCM. Eight QCMs were placed on the lower electrode (diameter 100 mm) for glow discharge. Position settings of eight QCMs on the lower electrode are denoted as shown in Fig. 1(b) in ref [21]. Prior to plasma polymerization, both sides of eight QCMs were treated by plasma sputtering for 60 s under 100 W of RF power and 100 Pa of He pressure.

Vapor pressure of monomers and RF power of glow discharge are two of the most important controllable parameters in plasma polymerization. In this study, polymerization conditions were always maintained at vapor pressure of 100 Pa and RF power of 100 W. Our studies showed that a good film-state of the polymer was obtained under these conditions. Monomers were vaporized at a constant temperature of liquid that was maintained by an oil bath. Monomer vapor was then allowed to flow into the plasma reactor between two parallel electrodes that were 6 cm apart. A needle valve was used to control pressure inside the reactor. Plasma polymerization was performed at 60 s for styrene, 30 s for allylamine, and 30 s for acrylic acid, respectively. We obtained good film-state of polymers under these conditions. The apparatus and procedure for determination of the deposition rate have been described previously [21]. Oscillating frequency of the QCM was measured by incorporating the QCM in a transistor-transistor logic electronic circuit described in ref. 22 (circuit II). A universal counter was used to read the frequency; data were stored in a microcomputer. The relationship between pp-film weight and frequency shift is expressed in ng/Hz units. When an AT-cut quartz crystal with fundamental oscillation frequency of 9 MHz is used, adsorption...

2.3. Immunoreaction of antigen (CRP) for antibodies-adsorbed QCM coated with pp-films

Three main processes using antibodies-adsorbed QCM for CRP detection are shown in Figure 1. Frequency shifts (ΔF) of QCM are calculated at each step. Overall experimental procedure is as follows: (1) plasma-polymer is coated onto gold surfaces of QCMs and antibody adsorption pretreatment is done. Untreated QCMs (eight pieces) were measured for oscillation frequency (F1) after He sputter treatment; then, oscillation frequency was measured under N2 gas (1.0 L/min) at 25± 0.1°C. For physical adsorption of CRP antibody, gold electrode surfaces on QCMs were coated with pp-films. Deposition rates of pp-films onto the QCM were measured directly by the oscillating frequency shift (data not shown). We controlled QCM frequency shift at around 2,000 Hz for each pp-film. All QCM-coated pp-films were immediately soaked in 100 mL of pure water under stirring at 5 min after plasma-polymerization and then washed twice in 100 mL of pure water under stirring for 5 min each. The QCMs were dried in N2 flow (1.0 L/min); then they were measured for frequency shift. After 60 min in the drying step, frequency shift indicated a steady state, implying that applied water on QCM was completely removed by N2 gas flow after 60 min. For other processes, adequate drying times were the same as in the washing process. Coated amounts of QCMs with pp-films were measured by oscillation frequency (F2). Amount of pp-film on QCMs was calculated from ΔF1=F1-F2.

(2) Adsorption of CRP antibody or CRP F(ab')2 antibody on QCMs: 400 µL of CRP antibody solution (PBS buffer, pH7.4) was applied from 10 µg/mL to 50 µg/mL of ranges of concentration at the pp-film coated QCM for 16 h at 4°C. Oscillating frequencies of antibody-adsorbed QCMs were measured after washing twice with 10 mL of pure water and drying treatment (F3). Three QCM pieces were used. Amounts of adsorbed CRP antibody on QCMs were calculated from ΔF2=ΔF1-F3.

(3) Detection of immuno-reaction: To detect the CRP dose-response, we used two concentrations of CRP (10 and 100 µg/mL). Volume of the CRP solution was 400 µL (PBS buffer, pH7.4). Oscillating frequencies of immunoreacted QCMs were measured after washing twice with 10 mL of pure water and drying treatment (F4). It used three pieces of QCMs. The amount of immunoreaction on QCM for CRP is calculated from ΔF3=ΔF2-F4.

![Fig. 1. Experimental procedure of immunoreaction between antigen (CRP) and antibody (CRP antibody or CRP-F(ab')2 antibody) adsorbed on pp-films coated QCMs is shown. Each reaction step is described in detail in the text. All experiments for determination of the coated amount of pp-films, adsorption amount of antibody of pp-film coated QCMs, and immunoreaction amounts of antibody adsorbed on pp-films coated QCM were carried out at 25 ± 0.1°C under flow of N2 gas at 1.0 L/min on QCMs.](image)

3. Results and Discussions

3.1. Adsorption of antibody on pp-film coated QCMs

Adsorption amounts of CRP antibody on physical process for pp-films are shown in Figs. 2(a)-(d). Adsorption of CRP antibody on reference QCM (a) showed large values with great variation on antibody concentration (80 to 130 Hz per area of QCM electrodes). Adsorption of CRP antibody on pp-styrene coated QCM (b) indicated small and flat values in the concentration range of antibodies (around 50 Hz per area of QCM electrodes). Adsorption of CRP antibody on pp-acrylic acid
Fig. 2. Adsorption amounts of antibodies on pp-films were measured on pp-film coated QCMs: (a) without pp-film (only gold electrodes), (b) with pp-styrene, (c) with pp-acrylic acid, (d) with pp-allylamine, (e) without pp-film (only gold electrodes), (f) with pp-styrene, (g) with pp-acrylic acid, and (h) with pp-allylamine.
Fig. 3. Dose-responses of immunoreaction for CRP were measured by antibodies adsorbed on pp-film coated QCMs: (a) without pp-film (only gold electrodes), (b) with pp-styrene, (c) with pp-acrylic acid, (d) with pp-allylamine, (e) without pp-film (only gold electrodes), (f) with pp-styrene, (g) with pp-acrylic acid, and (h) with pp-allylamine.
coated QCM (c) increased with increasing antibody concentration (50 to 80 Hz per area of QCM electrodes). Adsorption of CRP antibody on pp-allylamine coated QCM (d) showed small and flat values in the concentration range of antibodies (70 to 80 Hz per area of QCM electrodes).

Adsorption amounts of CRP-\(F(ab')_2\) antibodies on physical process for pp-film are shown in Figs. 2(e)-(h). Adsorption of CRP-\(F(ab')_2\) antibodies on reference QCM (e) showed large values of antibody concentration (50 to 130 Hz per area of QCM electrodes). Adsorption of CRP-\(F(ab')_2\) antibody on pp-styrene coated QCM (f) indicated scattered values in the concentration range of antibodies (around 70 Hz per area of QCM electrodes). Adsorption of CRP-\(F(ab')_2\) antibodies on pp-acrylic acid coated QCM (g) increased with increasing antibody concentration (50 to 90 Hz per area of QCM electrodes). Adsorption of CRP-\(F(ab')_2\) antibodies on pp-allylamine coated QCM (h) showed small values in the concentration range of antibodies (30 to 80 Hz per area of QCM electrodes).

3.2. Immunoreaction of CRP for antibodies adsorbed QCM coated with pp-films

Figure 3 (a)-(d) shows the relationship between dose-response of CRP antibody adsorbed on pp-films coated with QCM and concentration of CRP antibody. Immunoreaction using two kinds of CRP concentration (10 and 100 \(\mu\)g/mL) was performed. Dose-response of reference QCM (a) showed small values on all antibody concentrations. Dose-response of pp-styrene coated QCM (b) indicated large values with large variation for all concentration ranges of antibodies. Dose-response of pp-acrylic acid coated QCM (c) increased with increasing antibody concentration. Dose-response of pp-allylamine coated QCM (d) showed small values on all concentration ranges of antibodies.

Figures 3 (e)-(h) show the relationship between dose-response of immunoreaction using two kinds of CRP concentration (10 and 100 \(\mu\)g/mL) with pp-film coated QCM immobilized CRP-\(F(ab')_2\) antibodies and concentration of CRP-\(F(ab')_2\) antibodies. Dose-response of reference QCM (e) indicated unstable values in all antibody concentration ranges. Dose-response of pp-styrene coated QCM (f) decreased with increasing antibody concentration. Dose-response of pp-acyrlic acid coated QCM (g) decreased with increasing antibody concentration. Dose-response of pp-allylamine coated QCM (h) increased with increasing antibody concentration.

Favorable dose-responses for immunosensors were only obtained through a combination of pp-acrylic acid coated QCM and CRP antibodies, and pp-allylamine coated QCM and CRP-\(F(ab')_2\) antibodies. These results imply that molecular orientation of antibodies adsorbed on pp-films may affect immunoreaction behavior [25].

What are major factors affecting the amount of adsorption on antibodies and dose-response of immunoreaction on pp-films? We can infer two: one is different chemical structure on pp-film surfaces; another is different molecular orientation of antibodies on pp-film surfaces. The relationship between chemical structure of pp-film surfaces and antibody adsorption amounts and dose-response of immunoreaction will be reported elsewhere. We assumed that molecular orientation of CRP antibody on gold surfaces was random because small dose-response was obtained for CRP. We also assumed hinge regions of antibodies orientated to the side of the detection solution. Molecular orientation of CRP antibodies on pp-acrylic acid and CRP-\(F(ab')_2\) antibodies on pp-allylamine, yielded reasonable dose-response.

In clinical analysis, CRP of 1 mg/dL must usually be monitored. It is expected that this QCM sensor is sufficiently sensitive to analyze CRP concentration in human serum. We are now progressing with experiments to fabricate CRP immunosensors using pp-allylamine and pp-acrylic acid films; these results will be reported soon.

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References
6. B. D. Ratner and D. G. Castner, *Surface