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# Immobilization of ultra-thin layer of monoclonal antibody on glass surface

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## ABSTRACT

When preparing an affinity column and a biosensor, it is desirable to immobilize a unimolecular layer of pure protein on a matrix. In this work, we tried to immobilize a monoclonal antibody on a surface of a glass test-tube as a model, to confirm the stability of this ultra-thin layer by an enzyme immunoassay, and to estimate the thickness of the layer on a slide glass by Fourier transform infrared reflection spectrometry. A new test-tube was washed and dried. The tube was filled with 5% 3-aminopropyltriethoxysilane. The 3-aminopropylsilylated surface was treated with glutaraldehyde and $5.6 \cdot 10^{-2}$ mg/ml solution of a normal mouse monoclonal antibody. The Schiff base between glutaraldehyde and the antibody was further reduced with $7.9 \cdot 10^{-3}$ % $NaBH_4$. The tube was washed with 0.05% Tween 20 to block non-specific binding. The antibody immobilized on the surface was measured by an enzyme immunoassay based on a reaction of anti-mouse immunoglobulin G labelled with alkaline phosphatase, with which *p*-nitrophenol was produced from *p*-nitrophenylphosphate as a substrate. Meanwhile, various amounts of the antibody were immobilized on slide glasses in the same manner. The antibody on each surface was measured by Fourier transform infrared reflection spectrometry. The antibody immobilized under the final conditions was detectable by the enzyme immunoassay, and stable at 4°C for ten days. The antibody on the slide glass was a unimolecular layer, as judged from the Fourier transform infrared spectra referred to –CONH– band semiquantitatively. Thus, we found the optimal conditions for immobilizing an ultra-thin layer of the monoclonal antibody on the glass surface. These conditions will be applicable to the surface of many matrices containing silanols.

## INTRODUCTION

Many biological proteins, such as enzymes and antibodies, have been immobilized on matrices of high molecular mass for affinity chromatography and biosensors [1,2]. It is critical for an affinity column and a biosensor to deposit a unimolecular layer of immobilized pure protein, which gives rise to efficient binding equilibrium with the corresponding ligand. Membranes of biosensors so far developed are neither sufficiently thin nor adequately chemically characterized. In

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this study, we tried to immobilize an ultra-thin layer of a monoclonal antibody on a glass surface as a model, to confirm the stability of this layer by an enzyme-linked immunosorbent assay (ELISA), and to estimate the thickness of the layer by Fourier transform infrared reflection (FT-IR-REF) spectrometry [3].

## EXPERIMENTAL

### *Materials*

3-Aminopropyltriethoxysilane (ATES) was obtained from Nakarai (Kyoto, Japan). *p*-Nitrophenylphosphate disodium salt and 25% glutaraldehyde were obtained from Wako (Osaka, Japan). Polyoxyethylene sorbitan monolaurate (Tween-20) was obtained from Kanto (Tokyo, Japan). Bovine serum albumin (BSA) of 98–99% purity was obtained from Sigma (St. Louis, MO, U.S.A.). Goat anti-mouse immunoglobulin G (IgG), labelled with alkaline phosphatase at 5 mg/ml in 50 m*M* Tris buffer (pH 8.0), was obtained from Tago (Burlingame, CA, U.S.A.). Purified gelatin A was donated by Fuji Photo Film (Tokyo, Japan). A purified monoclonal antibody of mouse normal IgG was donated by Yamasa Shoyu (Choshi, Japan).

### *Immobilization of antibody on glass surface of test-tube*

A glass test-tube (3 cm × 6 mm I.D.) was soaked in 0.05 *M* $H_2SO_4$ overnight. The tube was washed with water and acetone, then dried at room temperature. A monoclonal antibody was immobilized on the inner wall of the tube as shown in Fig. 1. The dried tube was filled with various solutions of ATES, which were stirred at 37°C for various times with a microplate stirrer. Best results were obtained with a 5% solution of ATES and a time of 30 min. The solution in the tube was decanted, and the tube was washed with acetone and water five times each. The tube was filled with 2.5% glutaraldehyde in 0.1 *M* phosphate-buffered (pH 7.3) 0.15 *M* saline (PBS) and left for 2 h in a water-bath. The solution was discarded and the tube was washed with PBS. A 50-$\mu$l volume of a $5.6 \cdot 10^{-4}$–$5.6 \cdot 10^{-2}$ mg/ml solution of the monoclonal antibody in PBS was added to the tube, which was left overnight in the water-bath at 37°C. The solution was decanted, and the tube was washed with PBS. The tube was filled with $7.9 \cdot 10^{-3}$% $NaBH_4$ in 0.1 *M* borate buffer (pH 7.3) and maintained at 37°C for 1 h. The tube was washed with 0.5 mg/ml Tween-20 in PBS (TPBS).

### *ELISA for antibody immobilized on the surface of test-tube*

BSA in TPBS (1%) was added to the test-tube containing the immobilized antibody and left at room temperature for 5 min. The solution was decanted and the tube was washed with a tube full of TPBS three times to block any remaining vacant surface areas. A 50-$\mu$l volume of a $1 \cdot 10^{-2}$ mg/ml solution of anti-mouse IgG labelled with alkaline phosphatase in TPBS, and 200 $\mu$l of 16 m*M* *p*-nitrophenylphosphate in 100 m*M* bicarbonate buffer with 1 m*M* $MgCl_2$ (pH 9.8) were

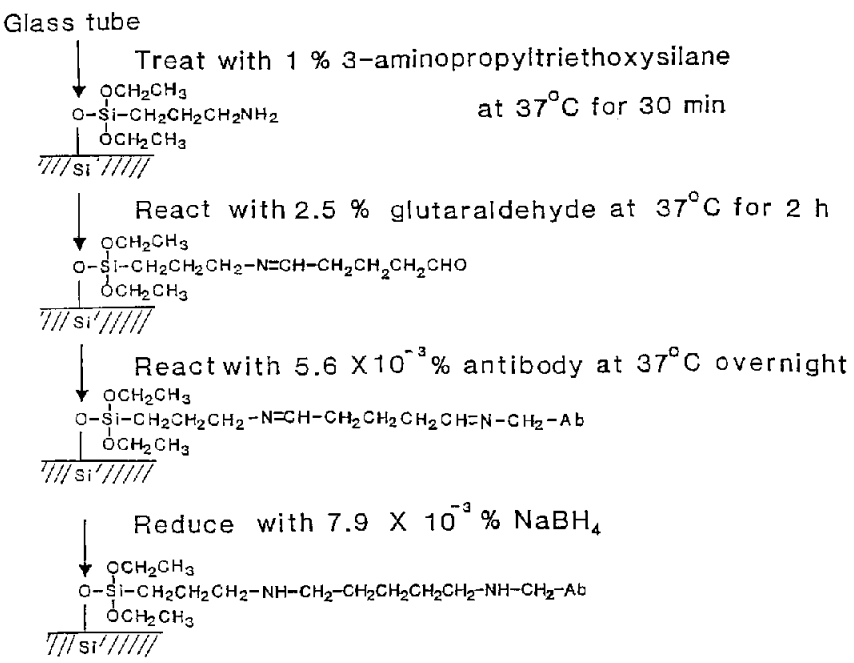



Fig. 1. Immobilization of antibody (Ab) to a glass test-tube.

added. The tube was left at 37°C for 30 min. The absorbance of the reaction solution at 405 nm was measured with a Bio-Rad enzyme immunoassay (EIA) reader. To measure stability of the immobilized antibody, the solution in the tube was discarded. The tube was washed with PBS three times, filled with 0.1% $NaN_3$ in PBS and stored in a refrigerator at 4°C for various periods.

The solution in the stored tube was discarded. The tube was washed with PBS, and 50 $\mu$l of the second antibody labelled with alkaline phosphatase solution were added. The tube was left at 37°C for 1 h. (The addition of the second antibody was sometimes omitted.) The tube was washed with TPBS and a further 200 $\mu$l of 16 m*M* *p*-nitrophenylphosphate were added. The tube was incubated at 37°C for 30 min. The absorbance was measured again. In this way, the stability test was repeated.

*Preparation of immobilized antibody on surface of slide glass*

A slide glass cut in half (26 mm × 3.8 mm) was immersed in 0.1 *M* $H_2SO_4$ overnight. The glass was washed with water and acetone and dried. A 5-$\mu$l volume of a $5.0 \cdot 10^{-3}$ mg/ml aqueous solution of the monoclonal antibody, containing 1.8% gelatin as a typical protein for amide bands, was spread over the whole area of the glass with an edge of a cover glass to form a thin layer. The coated glass was dried overnight. To make a quantitative measurement of the absor-

bance of the monoclonal antibody on the glass, 20 and 50 $\mu$l of a 500 $\mu$g/ml solution of the monoclonal antibody without the gelatin were spread over washed glasses. These glasses were dried over phosphorus pentoxide. For the covalent immobilization of the monoclonal antibody on the washed glass and a glassy carbon plate (9 cm × 9 cm), each plate was immersed upright in 5% ATES in acetone in a 100-ml beaker at 37°C for 30 min. The plate was washed successively with acetone and water, the immersed upright in 2.5% glutaraldehyde in a 100-ml beaker at 37°C for 2 h. The plate was washed with water and left to dry at room temperature. On one surface of the plate, 20 $\mu$l of a $4.3 \cdot 10^{-2}$ mg/ml solution of the monoclonal antibody in PBS was spread with an edge of a cover glass. The glass was left to dry at room temperature. The glass was washed successively with PBS and water. The plate was immersed in $7.9 \cdot 10^{-3}$% $NaBH_4$ in 10 m*M* borate buffer (pH 7.3) in a 100-ml beaker at 37°C for 1 h, then washed with water and dried over phosphorus pentoxide.

*FT-IR-REF spectrometry of layers on various matrices*

The glass coated with the antibody was dried in the sample chamber of a Digilab FTS-60 FT-IR spectrometer by passing a stream of dry air at a flow-rate of 20–30 l/min with a compressor for 20 min. The glass was irradiated with perpendicularly polarized light at an incident angle of 70°C. The light was reflected once. The resolution of the light was 4 $cm^{-1}$. Scans of the signal were accumulated 256 times and the spectrum was drawn with a Nova 3 computer in Fourier transform. The intensity of the reflected light from the samples ($\Delta R$), was divided by that ($R$) fom each reference glass to obtain a reflection ratio of $\Delta R/R$.

## RESULTS

The silanol groups of the glass surface of the test-tube were treated with 5% ATES at 37°C for various times. The reaction was rapid and the rate was constant for 60 min. The 30-min reaction was adopted for the final procedure. The effect of the concentration of ATES on the reaction was examined at 10 and 30 min. In the 10-min reaction, the immobilized amount increased in proportion to the concentration of ATES. In the 30-min reaction, the immobilized amount reached a plateau at 5% ATES, which was larger than the amount immobilized with 20% ATES during 10 min. The amount was dependent on the antibody concentration from $5 \cdot 10^{-4}$ to $5 \cdot 10^{-2}$ mg/ml. It was unnecessary to increase the concentration above $5.6 \cdot 10^{-2}$ mg/ml ($5.6 \cdot 10^{-3}$%) to form the monolayer of the antibody, judging from the FT-IR-REF spectrometry described later. Physical adsorption of the antibody on the surface occurred without the expected reaction of ATES. This adsorption was based on the hydrophobic interaction between the surface and the antibody. The hydrophobic area of the surface was decreased by the reaction of ATES, because the surface of the glass after the reaction became strongly hydrophilic. The physiological adsorption was suppressed by addition of

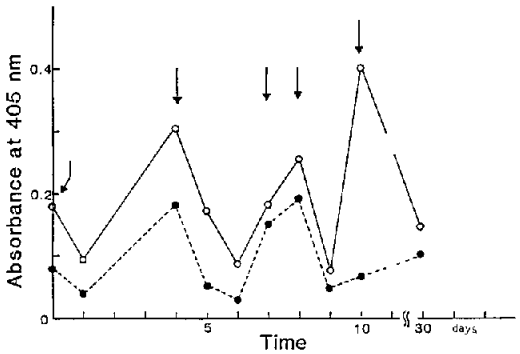



Fig. 2. Time courses of antibody immobilized with (○) and without (●) ATES and washed with PBS. The test-tube containing the immobilized antibody was stored with 0.1% $NaN_3$ at 4°C then washed with PBS. The antibody adsorbed was measured by ELISA. The arrows show when repeated additions of the second antibody labelled with alkaline phosphatase were made. Points without arrows mean that the second antibody was omitted.

a high concentration of another protein, such as BSA. The final concentration of the antibody was $5.6 \cdot 10^{-2}$ mg/ml ($5.6 \cdot 10^{-3}$%) for the immobilization.

The immobilized antibody was stable in the presence of $NaN_3$ as a bacteriostat at 4°C for up to ten days, as shown in Fig. 2. Thus, repeated use of the immobilized antibody was possible. Physiological adsorption of the antibody without ATES seems inevitable and should be subtracted as the blank, if necessary.

In order to measure the thickness of the layer of antibody on the surface, we tried to obtain FT-IR spectra of peptide bonds. We selected gelatin as a typical

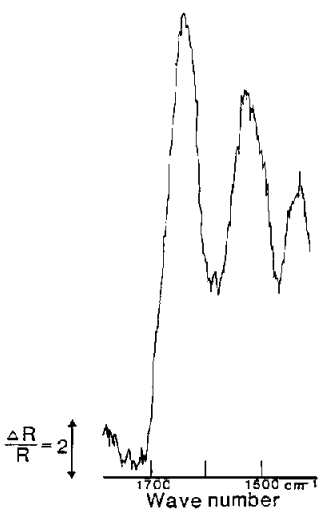
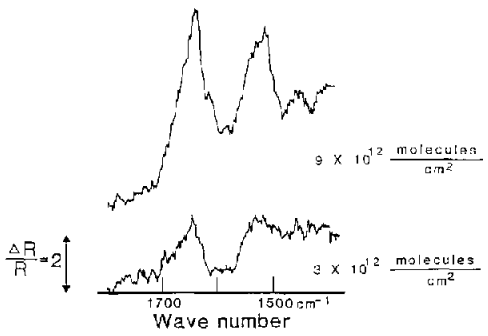



Fig. 3. FT-IR spectrum of gelatin layer of 1 μm thick on glass. A 1.8% gelatin solution was layered on the surface of the slide glass, dried and measured by an FT-IR spectrometer as described in the text.



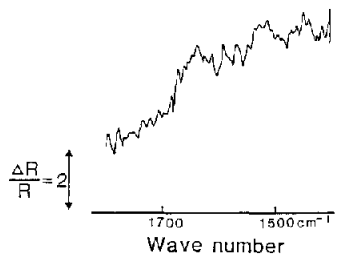




Fig. 4. FT-IR spectrum of antibody deposited on glass: 20 and 50 $\mu$l of the 500 $\mu$g/ml antibody solution were placed on slide glasses and dried. The spectra were measured as described in the text.

Fig. 5. FT-IR spectrum of antibody covalently immobilized on a slide glass: 20 $\mu$l of the $4.3 \cdot 10^{-2}$ mg/ml antibody solution were immobilized as described in the text.

protein, from which a thin layer was easily made. As shown in Fig. 3, we obtained a fine spectrum. The absorptions at 1645 and 1535 $cm^{-1}$ were assigned to the amide I band and the amide II band, respectively. A known amount of antibody was applied and dried on the slide glass, and FT-IR spectra were measured as shown in Fig. 4. The reflectivities were proportional to the amounts. The number of molecules on the glass was calculated as shown in Fig. 4. If the antibody molecule was roughly a sphere of 150 Å in diameter [4], the lower spectrum at 1645 $cm^{-1}$ corresponded to 1.5 layers, and the upper one to 4.5 layers. From this calibration of the absorption intensity, the layer of covalently immobilized antibody as shown in Fig. 5 was assumed to be unimolecular. The antibody immobilized on glassy carbon showed the same spectrum as shown in Fig. 5.

## DISCUSSION

Under moderate conditions, we were able to immobilize a monolayer of the monoclonal antibody. The layer was stable enough for repeated use for ten days. We succeeded in obtaining a fine FT-IR spectrum of the layer, although the method of the immobilization is conventional [1].

The optimal conditions for immobilizing a unimolecular layer of the monoclonal antibody are original. The reaction of ATES at 37°C for 30 min is very mild, compared with the lengthy reflux method previously used [1]. Finding the right concentration of the antibody is the secret to obtaining a unimolecular layer. The concentration of $4.3 \cdot 10^{-2}$ mg/ml will be a clue to help extend the technique to other proteins, such as enzymes.

The sensitivity of FT-IR-REF spectrometry seems as high as possible. This semiquantitative method will be applicable to the characterization of not only the monoclonal antibody, but also many other proteins, such as enzymes. For the

characterization of proteins immobilized on matrices, amino acid analysis is usually used [1]. The hydrolysis of the proteins before amino acid analysis is time-consuming and 100% recovery of all the amino acid residues is difficult because some residues are covalently coupled to the matrix. The analysis by the analyser is very accurate, but needs skilful technique and larger amounts of the sample [1]. In any case, the amino acid analysis is destructive.

Other methods have been described to characterize proteins immobilized on matrices. ELISA is the most popular technique for quantitative and specific determination of the proteins. $\beta$-Subunits of *Escherichia coli* tryptophan synthetase adsorbed on the well of a microplate were treated with the corresponding monoclonal antibodies. Denatured forms of the enzyme were discriminated by this ELISA [12]. Monoclonal antibodies to $\alpha$-mannosidase of *Dictyostellium discoidem* were screened by ELISA. The antibodies were found to be heterogeneous, because conformations of the antigens on a plastic surface were different from those in solution [13]. Myoglobin adsorbed on a polydimethyl siloxane surface lost specifically certain antigenic determinants to its monoclonal antibodies [14]. Radioimmunoassay is precise and the in principle most sensitive method. Fragment D of human fibrinogen was adsorbed on a polystyrene plate and immobilized on Sepharose beads. The antigenic properties on both supports were different from those in solution [15]. Circular dichroism is good for charaterization of $\alpha$-helix and $\beta$-structure of proteins, but not so sensitive. It was used to estimate indirectly the structural changes of proteins adsorbed on columns, which were eluted in eluents [16]. FT-IR-REF spectrometry is non-destructive, and the sample is a tiny surface to be focused on by the light. Since the monoclonal antibody is a pure protein of high molecular mass, it is possible to detect its unimolecular layer. Previously, a polyclonal antibody adsorbed on a polyethylene surface was measured by FT-IR-REF spectrometry [5,6]. The adsorption was due to the hydrophobic interaction of the antibody with the film [7,8]. In our immobilization, hydrophilic interaction is involved in the covalent bond formation.

The immobilization method will be useful for making immunosensors. The basic surfaces of sensors, such as glass electrodes, glassy carbon plates, and field effective transistors are abundant in silanol groups [2]. We prepared immunosensors using monoclonal antibodies to cAMP and human serum albumin. The immunosensors were specific to each hapten and antigen, but unstable for repeated use, because the antibodies were not covalently bound to the surface of the glass electrodes [9]. The present immobilization method is useful for making immunosorbents of enzyme-linked immunosorbent assay. It is also good for making enzyme reactors [2].

Affinity chromatography is an important technique in biotechnology. For the base matrices of columns, silica gel and porous glass are suitable for high-performance liquid chromatography. These matrices are abundant in silanol groups [1]. The present method is suitable for the preparation of affinity matrices. We have also developed a sheet for porous glass, called "new glass for thin-layer chromatography" [10]. This kind of affinity matrix is under investigation.

In the affinity matrices so far obtained, the immobilized proteins and enzymes were not confirmed to be present in a unimolecular layer. The proteins immobilized are assumed to overlap with each other. These thick layers are not efficient for the binding equilibrium with the ligands. The present layer will be superior in this respect, although the orientation of the antibody molecules could not be confirmed from the FT-IR spectra. If the layer is more than ten-fold, it is possible to estimate the orientation from $\alpha$-helix of 1654 $cm^{-1}$ or $\beta$-structure of 1639 $cm^{-1}$ of the amide band [5,6]. However, if carbohydrates of the antibody as described by Turková *et al.* [11] are coupled to the aminopropylsilylated surface, oriented immobilization of the unimolecular layer of the antibody is possible. Furthermore, it is interesting to study the functional binding capacity of the immobilized monoclonal antibody with many kinds of anti-mouse monoclonal antibody, according to the previous methods [12–16].

## CONCLUSION

A technique for forming a unimolecular layer, and semiquantitative FT-IR-REF spectrometry of the layer, have been described. Both methods will be important for studies of the surface modification of biosensors and affinity chromatography columns.

## ACKNOWLEDGEMENT

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