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Immobilization of Antibodies and Enzyme-Labeled Antibodies by Radiation Polymerization

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Abstract

Immobilization of antibodies and enzyme-labeled antibodies by radiation polymerization at low temperatures was studied. The antibody activity of antibody was not affected by irradiation at an irradiation dose of below 8 MR and low temperatures. Immobilization of peroxidase-labeled anti-rabbit IgG goat IgG, anti-peroxidase, peroxidase, and anti-alpha-fetoprotein was carried out with hydrophilic and hydrophobic monomers. The activity of the immobilized enzyme-labeled antibody membranes varied with the thickness of the membranes and increased with decreasing membrane thickness. The activity of the immobilized antibody particles was varied by particle size. Immobilized anti-alpha-fetoprotein particles and membranes can be used for the assay of alpha-fetoprotein by the antigen–antibody reaction, such as a solid-phase sandwich method with high sensitivity.

Index Entries: Immobilization of antibodies, by radiation polymerization; antibody immobilization, by radiation polymerization; radiation polymerization, in antibody immobilization.

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Introduction

Studies of the interactions of antigens with antibodies have yielded information on the mechanism of the immune response as well as on numerous currently applied or potential clinical applications, such as diagnostic tests. The diagnostic tests requiring high sensitivity have been carried out by immunochemical methods, such as radioimmunoassay or enzymeimmunoassay. Recently, various assay kits containing immunoreagents have been developed. That is, diagnostic assays are sold as kits with immunoreagents in which a specific antibody is immobilized to polymeric supports by a chemical bonding method. Polymeric supports, such as polymeric immunomicrospheres, to which molecules of biochemical interest are covalently bound have been widely used as solid supports for affinity chromatography, for immobilization of enzymes, etc. (*1*, *2*).

We studied the immobilization of biological substances such as enzymes (*3*, *4*), microbial cells (*5–7*), and so on by radiation polymerization at low temperatures. The immobilization method using radiation polymerization creates a physical trapping of substances that become attached to the surface of the polymeric supports. In this work, the immobilization of antibodies and enzyme-labeled antibodies was studied by radiation polymerization of both hyrophilic and hydrophobic monomers at low temperatures.

Materials and Methods

Anti-alpha-fetoprotein rabbit sera were produced in rabbits by immunization of emulsions of human alpha-fetoprotein with equal volumes of complete Freund's adjuvant. Human alpha-fetoprotein was prepared by affinity chromatography on an anti-alpha-fetoprotein Sepharose 4B column, followed by gel-filtration on Sephacry S-300 column from the human plasma of a primary hepatoma patient (*8*). For the preparation of enzyme-labeled antibodies, anti-alpha-fetoprotein serum was purified by ammonium sulfate fractionation, followed by the ion-exchange chromatography on DEAE cellulose. The purified IgG fraction from anti-alpha-fetoprotein sera was coupled with peroxidase according to the method of Wilson and Nakane (*9*). Standard human alpha-fetoprotein preparation included using the Dinabott alpha-fetoprotein kit as a standard. Peroxidase, anti-peroxidase, and peroxidase-labeled anti-rabbit IgG goat IgG were obtained from Sigma and Japan Immuno Research Laboratory. Hydroxyethyl methacrylate (HEMA), neopentylglycol dimethacrylate (NPG), and trimethylolpropane triacrylate (A-TMPT) as monomer were obtained from Mitsubishi Gas Chemicals Co., Ltd.

The immobilization procedure was as follows. Antibodies or enzyme-labeled antibodies in a solution contianing 0.01*M* phosphate buffer saline (PBS), pH 7.2, were mixed with monomer in a glass tube vessel (20 cm in length and 0.8 cm in diameter). Immediately after shaking, the vessel was frozen at −78°C and irradiated by γ-rays from a ^{60}Co radiation source. After irradiation, the immobilized composites obtained using hydrophilic monomer (HEMA) were cut to thin membrane form using a microtome at room temperature. The immobilized composites

obtained using hydrophobic monomers (A-TMPT and NPG) gave small particle form (10–100 μm in diameter).

Enzyme immunoassay in alpha-fetoprotein was performed by the solid-phase sandwich method. Two hundred μL of human serum or the standard alpha-fetoprotein solution (from 10 to 320 ng/mL dissolved in 0.01*M* PBS solution containing 0.3% BSA) and immobilized anti-alpha-fetoprotein were added and then allowed to incubate. After the first incubation, the incubation solution was removed and immobilized antigen–antibody complex was washed with the PBS solution three times. Then, 50 μL of peroxidase-labeled anti-alpha-fetoprotein (anti-AFP POX) and 200 μL of the PBS solution were added and reacted at 37°C for 1 h. After the second reaction, the immobilized substances were washed three times with the PBS solution to remove the unreacted anti-AFP POX. The enzyme (peroxidase) reaction was carried out with 0.03% H_2O_2 solution and orthophenylenediamine, and terminated by the addition of 1.0*N* HCl solution. After the reaction, the optical density of the solution at 492 nm was measured with a spectrophotometer. The antibody activity of the antibodies was measured by Ouchterlony method.

Results and Discussion

Effect of Irradiation on Antibody

The effect of irradiation on antibody was studied using anti-peroxidase as a model of antibody. The relation between irradiation dose and relative antibody activity is shown in Fig. 1. The antibody activity was not affected by irradiation doses of below 8 MR, but decreased with increasing irradiation dose above 8 MR. The effect of irradiation temperature on the antibody activity at 1 MR is shown in Fig. 2. The

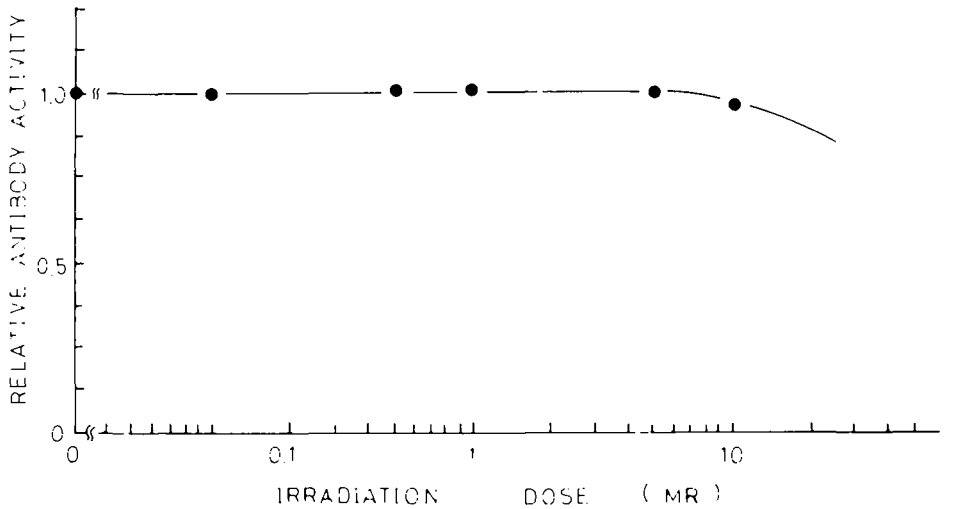

Fig. 1. Relation between relative antibody activity and irradiation dose. Irradiation dose, −78°C.

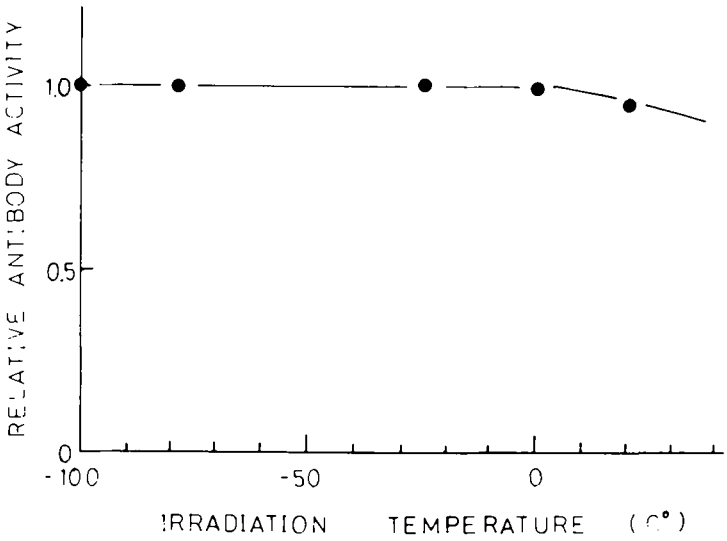

Fig. 2. Relation between relative antibody activity and irradiation temperature. Irradiation dose, 1 MR.

denaturation of the antibody by irradiation could be retarded under the condition of low temperature. It was found that the irradiation antibody was hardly denatured at the low temperature of −78°C and low irradiation doses of below 1 MR used in the polymerization as well as other biocatalysts of enzymes and microbial cells (*3–5*).

Immobilization of Peroxidase-Labeled Anti-Rabbit IgG Goat IgG Using Hydrophilic Monomer

Immobilization of peroxidase-labeled anti-rabbit IgG goat IgG (anti-rabbit IgG goat IgG POX) using hydrophilic HEMA monomer was carried out in various monomer concentrations. The variation of the enzymatic activity of immobilized anti-rabbit IgG goat IgG POX membranes with repeated batch enzyme reactions is shown in Fig. 3, in which the thickness of the immobilized membranes is 1.0 mm. The enzymatic activity of the immobilized membranes obtained from low monomer concentrations was slightly decreased with repeated reactions at the initial stages owing to the leakage of anti-rabbit IgG goat IgG POX, but had constant values at the later stages. The enzymatic activity at the later stages varied with the monomer concentration and increased with decreasing the monomer concentration. This decrease of the enzymatic activity at high monomer concentrations was caused by the increase of anti-rabbit IgG goat IgG POX occluded in the polymer matrix, in which the diffusion of the substrate is retarded. This retardation of the diffusion depended on the membrane thickness and the monomer concentration. The relation between the enzymatic activity and the membrane thickenss of immobilized anti-rabbit IgG goat IgG POX membranes is shown in Fig. 4, in which the enzymatic activity decreased with increasing the membrane thickness. The immobilized anti-rabbit IgG goat IgG POX membranes with thin membrane thickness obtained from low monomer concentrations had high enzymatic activity owing to the relaxation or opening of the active site of anti-rabbit IgG POX. The polymer

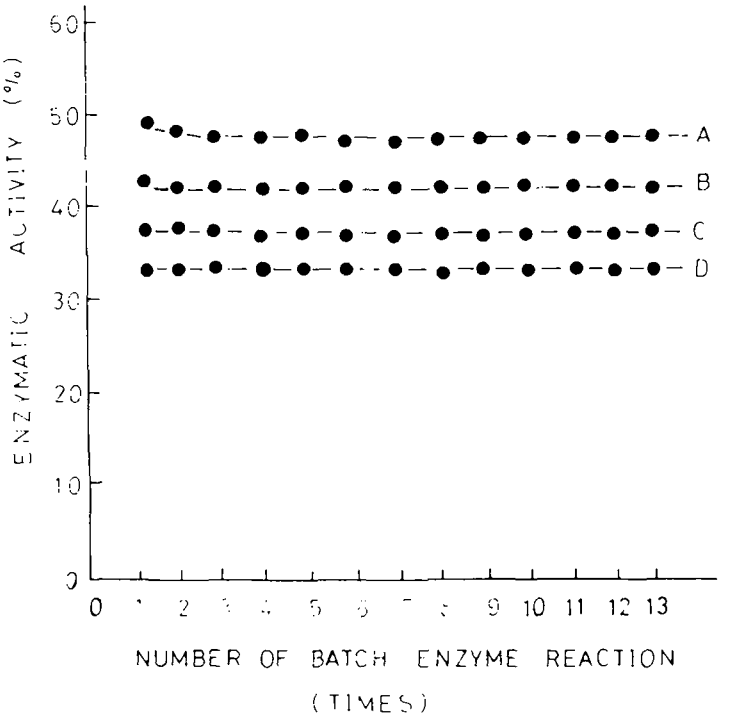

Fig. 3. Variation of the enzymatic activity of immobilized anti-rabbit IgG goat IgG POX membranes with repeated batch enzyme reactions. Batch enzyme reaction: 37°C, 1 h.; thickness of the membranes, 1 mm; HEMA concentration: A, 10%; B, 30%; C, 50%; D, 70%.

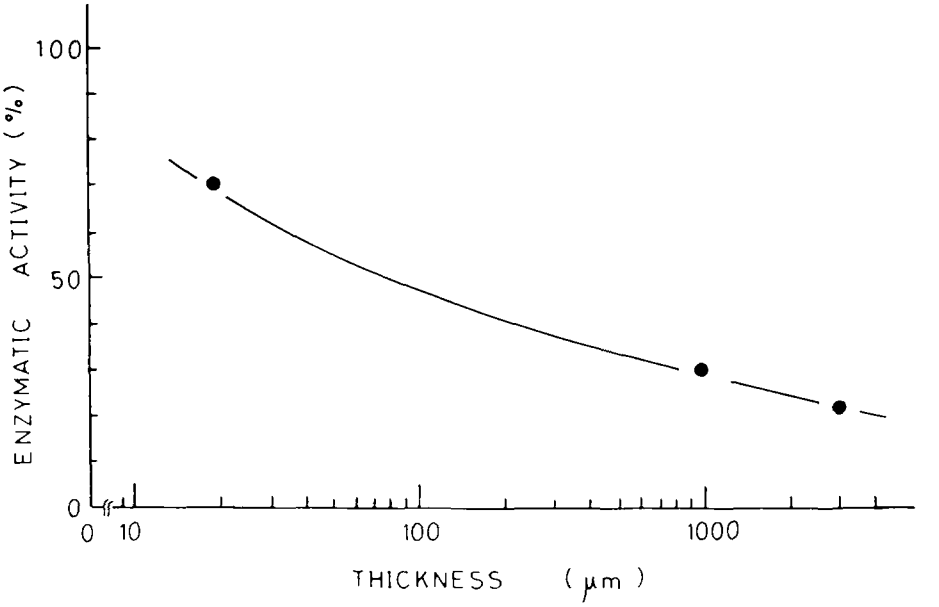

Fig. 4. Effect of the thickness of immobilized anti-rabbit IgG goat IgG POX membranes on the enzymatic activity. HEMA concentration, 50%.

matrix obtained by radiation polymerization of hydrophilic HEMA monomer at low temperatures had a porous structure. Such porous immobilized membranes would be useful as immunoreagents.

Immobilization of Peroxidase Using Hydrophilic Monomer

To compare the difference between the enzymatic activity of immobilized enzyme and enzyme-labeled antibody, the immobilization of peroxidase was carried out under the same immobilization conditions as for the immobilization of anti-rabbit IgG goat IgG POX (shown in Fig. 3). The relation between enzymatic activity and repeated batch enzyme reaction in immobilized peroxidase membranes is shown in Fig. 5. The decrease in the enzymatic activities with repeated reactions at the initial stages was large and also the enzymatic activities at the later stages were lower compared to those of immobilized anti-rabbit IgG goat IgG POX membranes. Since the molecular weight of the enzyme is smaller than that of the enzyme-labeled antibody, these results suggest that the initial decrease in the enzymatic activity is likely the result of some leakage of the enzyme from the polymer matrix. It was found that the leakage of the enzyme did not take place at monomer concentrations greater than 50% and in thin membrane preparations (10–20 μm in thickness).

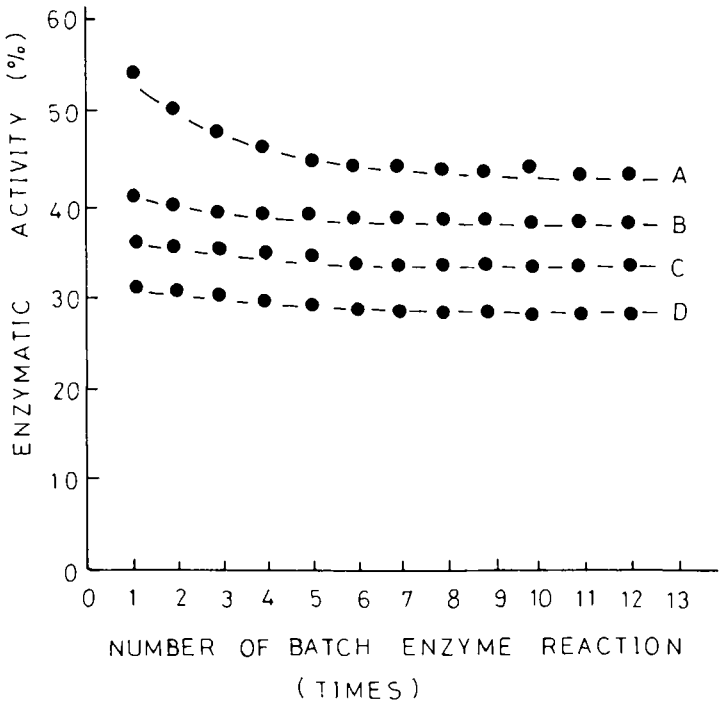

Fig. 5. Variation of the enzymatic activity of immobilized peroxidase membranes with repeated batch enzyme reactions. Batch enzyme reaction, 37°C, 1 h; thickness of the membranes, 1 mm; HEMA concentration: A, 10%; B, 30%; C, 50%; D, 70%.

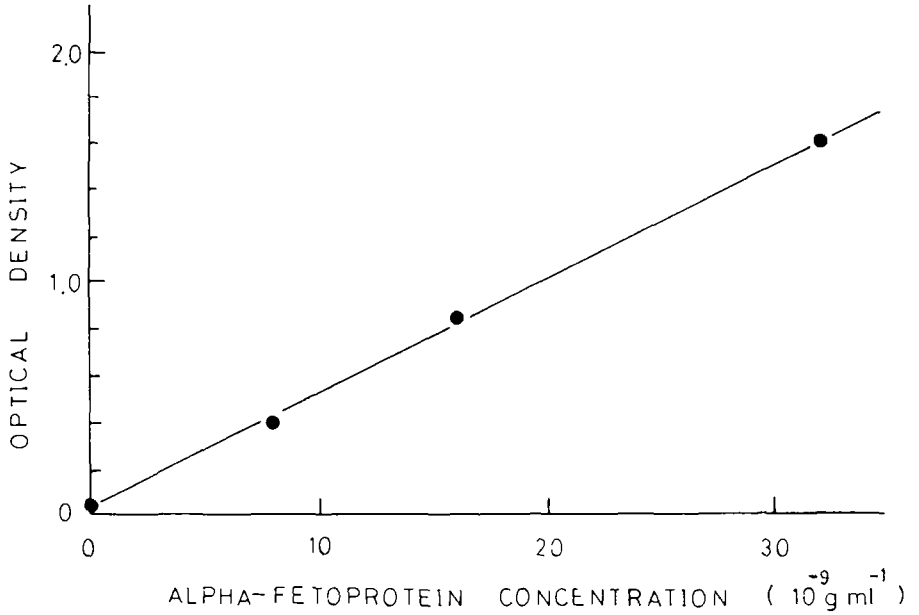

Fig. 6. Standard curve of immobilized anti-alpha-fetoprotein membranes for alpha-fetoprotein. Thickness of the membranes, 20 μm; HEMA concentration, 50%.

Immobilization of Anti-Alpha-Fetoprotein Using Hydrophilic Monomer

Immobilized anti-alpha-fetoprotein membranes were prepared using hydrophilic HEMA monomer. The immunochemical reaction of the immobilized antibody with antigen was examined. The relation between the optical density of the solution after the enzyme reaction and the concentration of alpha-fetoprotein is shown in Fig. 6, which is the standard curve of alpha-fetoprotein assay in solid-phase sandwich method. A good linear relationship was obtained using immobilized anti-alpha-fetoprotein membranes in alpha-fetoprotein concentration range of 0–30 $\times$ 10^{-9} g/mL, showing that the reaction of alpha-fetoprotein with immobilized anti-alpha-fetoprotein is efficient.

Immobilization of Anti-Peroxidase Using Hydrophobic Monomer

Anti-peroxidase was immobilized using hydrophobic monomer such as A-TMPT monomer, and the antibody-antigen reaction was examined. The relation between the optical density of the solution after the enzyme reaction and the monomer concentration is shown in Fig. 7, together with the result of the variation of the particle size by changing the monomer concentration. Immobilized anti-peroxidase obtained by radiation polymerization of hydrophobic monomer took small particle form, on which anti-peroxidase was trapped on the particle surface. The optical density of the solution increased with increasing the monomer concentration. The particle size of immobilized anti-peroxidase increased with increasing the monomer concentration. This increaes of the optical density of the solution by the increase of the particle size would result from the increase of the quantity of

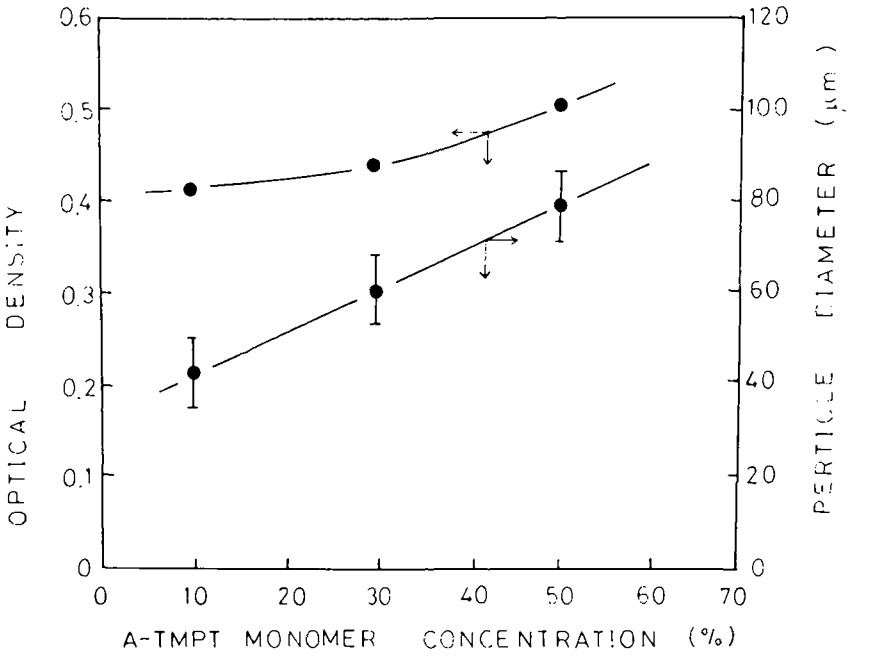

Fig. 7. Effect of the monomer concentration on the antibody activity of immobilized anti-peroxidase particles. Monomer, A-TMPT.

antigen–antibody complexes on the surface of the immobilized particles. Thus, the trapping yield of protein on the particles appeared to be independent of the surface area. In the present method of protein immobilization, the suspension state of the mixture solution containing proteins, hydrophobic monomer, and buffer solution plays an important role in the trapping of proteins. The trapping ability of proteins relates to the magnitude of the interaction between protein molecules and the surface energy of suspended monomer particle phase. It is believed that a localizing effect or partition effect of proteins on the surface exists in the situation of the trapping.

Immobilization of Anti-Rabbit IgG Goat IgG POX Using Hydrophobic Monomer

The immobilization of anti-rabbit IgG goat IgG POX was carried out using hydrophobic NPG monomer, for comparison with that using hydrophilic monomer. The enzymatic activity of immobilized anti-rabbit IgG goat IgG POX particles increased with increasing monomer concentration, resulting in an increase of the particle size, as shown in Fig. 8. The particle size dependency of the enzymatic activity is reasonable in comparison with the result in Fig. 7. The enzymatic activity of the immobilized particles was comparable or was slightly lower, compared with that of the immobilized membranes, owing to the difference of their trapping efficiencies.

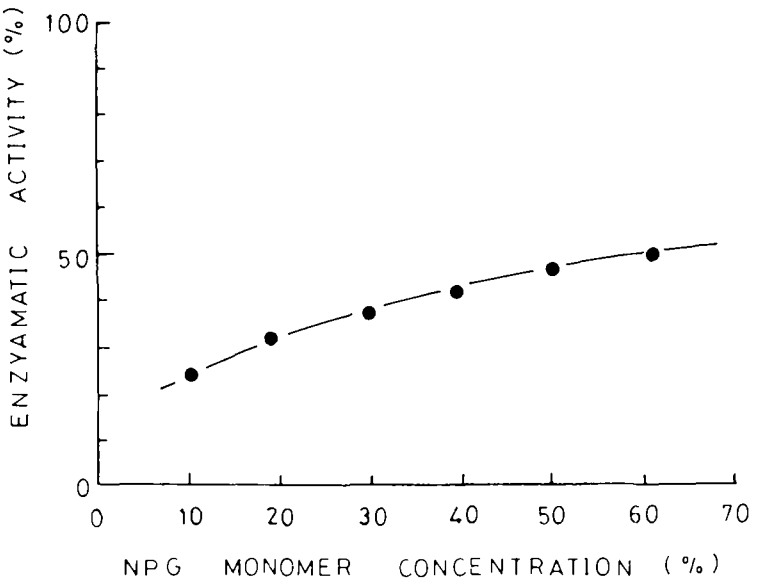

Fig. 8. Effect of the monomer concentration on the enzymatic activity of immobilized anti-rabbit IgG goat IgG POX particles. Monomer, NPG.

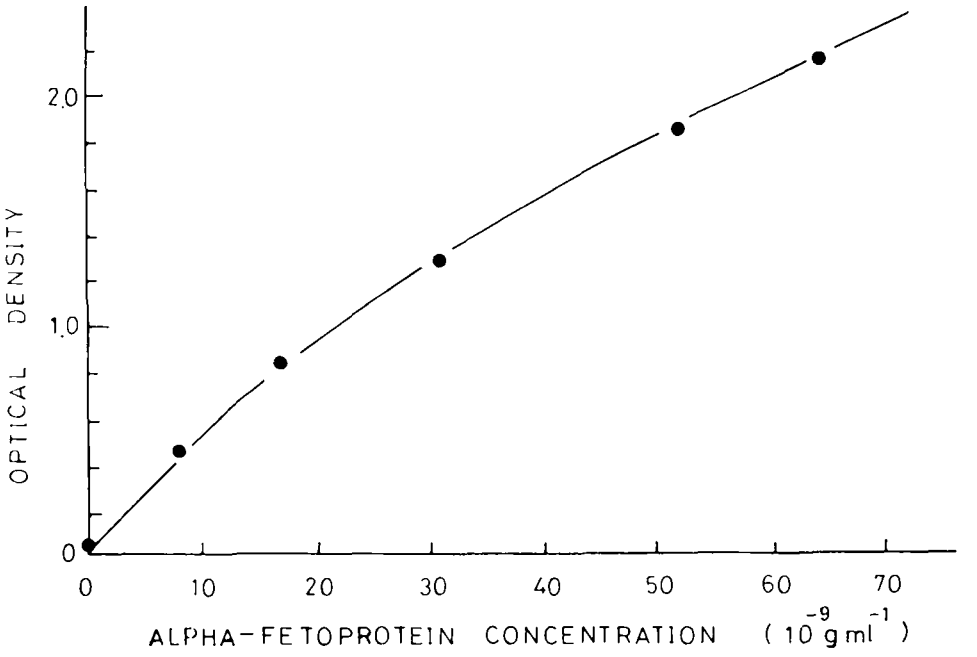

Fig. 9. Standard curve of immobilized anti-alpha-fetoprotein particles for alpha-fetoprotein. Size of particles, 10–30 μmϕ; monomer: NPG, 50%.

Immobilization of Anti-Alpha-Fetoprotein Using Hydrophobic Monomer

Immobilized anti-alpha-fetoprotein particles were prepared using hydrophobic NPG monomer, and the antigen–antibody reaction was studied by solid-phase sandwich method. The relation between the optical density of the solution and the concentration of alpha-fetoprotein is shown in Fig. 9. The quantity of alpha-fetoprotein reacted with the immobilized anti-alpha-fetoprotein was measured by the enzyme reaction after the second reaction. According to this result, it was found that the solid-phase sandwich reactions involving the first and second reactions took place efficiently on the surface of the particles. For such a solid-phase sandwich reaction requiring a broader reaction space, the immobilized particles would be favored over those immobilized membranes involving reactions occurring within porous membranes. However, in the experiments using the enzyme-labeled antibody, the trapping efficiency on the immobilization of the antibody in the immobilized membranes was higher than that in the immobilized particles. Since the concentration of alpha-fetoprotein in the serum of normal and abnormal persons (patient of primary cancer of liver) is about 10–20 $\times$ 10^{-9} g/mL, it was found that the immobilized anti-alpha-fetoprotein particles and membranes can be practically utilized for the diagnostic assay of alpha-fetoprotein.

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