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Adsorption Characteristics of γ-Globulin on Carboxylated Microcapsules

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Poly(1,4-piperazinediylterephthaloyl) microcapsules having different degrees of carboxylation were prepared and the adsorption characteristics of bovine serum γ-globulin on these microcapsules were examined. The amount adsorbed was larger and the rate of adsorption was lower for the microcapsules of low surface negative charge than for those of high surface negative charge, and the effect of temperature upon the adsorption on the former was greater. These observations suggested the existence of differences in orientation and conformational changes of adsorbed γ-globulin depending on the surface negative charge of the microcapsules. In addition, complement was more extensively activated by γ-globulin adsorbed on the microcapsules of high surface negative charge than by that adsorbed on those of low surface negative charge, which indicates that molecular assembly of adsorbed γ-globulin is also an important determinant for biocompatibility of biomedical materials.

Keywords——microcapsule; surface charge; γ-globulin adsorption; complement activation

Introduction

In the development of such biomedical materials as artificial organs, artificial blood vessels, *etc.*, it is a prerequisite that the materials should not cause adverse effects on the living body. In this context, many efforts[1] have been made to prepare biocompatible materials. However, no satisfactory general understanding of the biocompatibility of artificial materials has so far been established because of the complexity of the living body.

We have prepared hemolysate-loaded microcapsules by making use of the microencapsulation technique[2] in the hope of utilizing them as artificial red blood cells, but it was found that rabbit thrombocytes easily adhere to these microcapsules and that introduction of anionic charges on the surface of the microcapsules fails to prevent thrombocyte adhesion.[3] On the other hand, rabbit thrombocytes were found to be more adhesive to the microcapsules of high surface negative charge than to those of low surface negative charge, although electrical repulsive forces between thrombocytes and the microcapsules would be expected.[4]

In our previous paper,[5] it was found by using carboxylated poly(1,4-piperazinediylterephthaloyl) microcapsules, that rabbit thrombocyte adhesion to the microcapsules is mediated by adsorbed complement systems through the classical activation pathway which is triggered by γ-globulin adsorption. It was suggested that adsorption of γ-globulin is greatly influenced by the surface negative charges of microcapsules. Therefore, in this paper, the dependence of the adsorption characteristics of γ-globulin on the degree of carboxylation of the microcapsule surface was examined, and the complement activation by adsorbed γ-globulin was also investigated, in view of its biological importance.

Experimental

Preparation of Carboxylated Microcapsules——Microcapsules of carboxylated poly(1,4-piperazinediyl-terephthaloyl) membrane were prepared by making use of the interfacial polycondensation reaction between terephthaloyl dichloride and diamines according to the procedure described earlier.[4] The diamines used were mixtures of L-lysine and piperazine in molar ratios of 0, 0.2 and 0.4 (M/M). The microcapsules obtained are designated hereafter as MC-1, -2 and -3, respectively. They were washed thoroughly on a centrifuge and finally dispersed in Tris-buffer solution (0.14 M NaCl + 0.014 M Tris–HCl, pH 7.4). Since the microcapsules were found to be nearly perfectly spherical in shape, the surface area of the microcapsules was calculated on the basis of their mean diameters, and each of the MC suspensions was adjusted to have identical total surface area (*ca.* 2×10^2 cm^2/ml).

Adsorption of γ-Globulin on the Microcapsules——Known amounts of bovine γ-globulin (Frac. II, Sigma) dissolved in the Tris–HCl buffer solution were added to each MC suspension, and each mixture was incubated for given periods of time at different temperatures. Then, it was immediately centrifuged to precipitate the MC and the amount of adsorbed γ-globulin was determined by measuring colorimetrically (A_{280}) the amount of the protein remaining in the supernatant. In addition, the MCs with γ-globulin adsorbed were incubated with fluorescein isothiocyanate-labelled protein A (FITC-protein A, from *S. aureus*, ZYMED) solution at 25 °C for 30 min. Then, the MCs were centrifuged off and the amount of FITC-protein A remaining in the supernatant was determined by fluorometry ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm).

Complement Activation by Adsorbed γ-Globulin——The γ-globulin-adsorbed MC suspension was added to fresh gunia pig serum, and the mixture was incubated at 25°C for 30 min. Then, the MC was centrifuged off, and the complement titer remaining in the supernatant was assayed according to a modification of Mayer's procedure,[6] the details of which were described earlier.[5]

Results and Discussion

The first column of Table I lists the electrophoretic mobilities of the MC measured with a microelectrophoresis apparatus (Rank Brothers Inc.). The data indicate that the number of carboxyl groups of L-lysine residues in the membrane increases with increase in the molar

TABLE I. Electrophoretic Mobility of Microcapsules

	Electrophoretic mobility (μm/s/V/cm)		Mean diameter (μm)
	Bare	γ-Globulin coated	
MC-1	−0.73±0.08	−0.23±0.07	8.20±3.51
MC-2	−1.28±0.13	−0.26±0.09	8.75±3.26
MC-3	−1.70±0.09	−0.31±0.11	9.15±2.86

Electrophoresis was carried out at 25±1 °C in medium of pH 7.4 and at an ionic strength of 0.01.

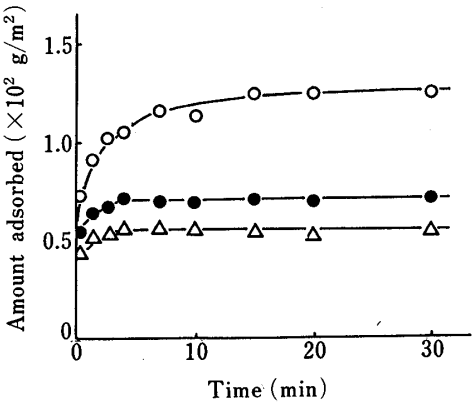

Fig. 1. Adsorption of γ-Globulin

○, MC-1; ●, MC-2; △, MC-3.
γ-Globulin solution (2.0 g/l) was added to MC suspension, and the mixture was incubated at 25±1 °C, (n=6).

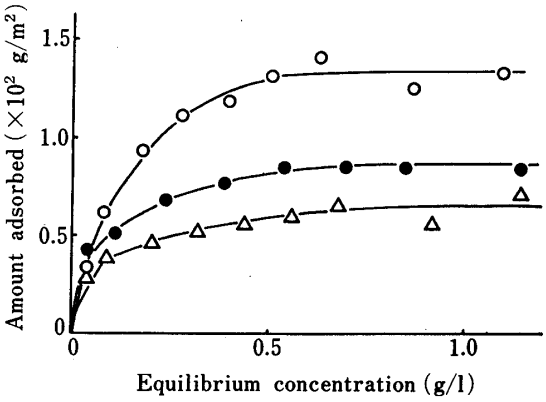

Fig. 2. Adsorption Isotherms of γ-Globulin

A mixture of γ-globulin solution and MC suspension was incubated for 30 min at 25±1 °C. Symbols used here are the same as in Fig. 1, (n=6).

ratio of the amino acid in the diamine mixtures, resulting in the differences in surface negative charge of the MC.

Figure 1 shows the adsorption profile of γ-globulin on each MC. Although adsorption was rapid and considerable amounts were adsorbed within a few minutes irrespective of the surface negative charges on the MCs, it took longer for weakly charged MC to reach saturation adsorption. Reversible adsorption or rearrangement[7] of adsorbed γ-globulin may take place on weakly charged MC until saturation adsorption is reached. On the other hand, saturation occurred more quickly with the highly charged MC. In general, polymer adsorption is more rapid than desorption because of the plurality of binding sites. Therefore, multiple binding sites between γ-globulin and the highly charged MC may restrict desorption or rearrangement.

The adsorption isotherms in Fig. 2 indicate that γ-globulin is adsorbed more on weakly charged MC than on highly charged MC. Considering that the saturated amounts of γ-globulin are similar to or less than the value for hexagonally close-packed adsorption (*ca.* 13.4 mg/m^2)[8] calculated from the hydrodynamic dimensions of γ-globulin, the adsorption of γ-globulin on the MC should be no more than a monolayer. In addition, since the electrophoretic mobilities of γ-globulin-adsorbed MCs gave decreased and nearly identical values (the second column of Table I), the MC surfaces are likely to be covered with γ-globulin fully enough to shield the original surface negative charges. Therefore, the differences in the saturated amount of adsorption would suggest that molecular deformation of adsorbed γ-globulin occurs, depending on the surface negative charge of the MC.

γ-Globulin is, as is well known, an anisotropic protein both configurationally and functionally. It was reported[9] that γ-globulin is adsorbed on a hydrophilic surface through electrostatic and/or hydrogen bondings, directing its Fc moiety outward, whereas it is adsorbed on a hydrophobic surface through hydrophobic interaction with its Fab moieties orienting outward. On the other hand, the surface of the MC may have both hydrophobic and hydrophilic portions, and the former would be relatively larger on the weakly charged MC than on the highly charged MC.

Figures 3 and 4 show the effects of temperature and pH of the medium on the adsorption of γ-globulin, respectively. It is clear from Fig. 3 that the amount of γ-globulin adsorbed is larger at high temperature than at low temperature, and this trend is more marked for the weakly charged MC than for the highly charged MC. This would suggest that the amount of

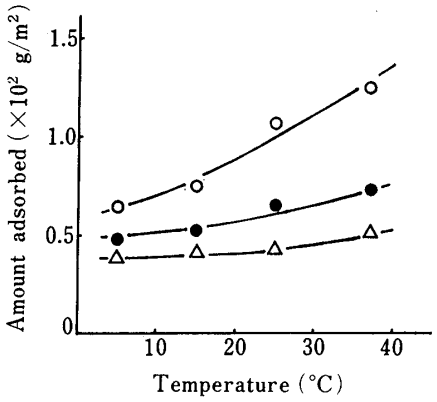

Fig. 3. Effect of Temperature on the Adsorption of γ-Globulin

A mixture of γ-globulin solution (2.0 g/l) and MC suspension was incubated for 30 min. The plots show the mean values of three separate runs. Symbols used here are the same as in Fig. 1.

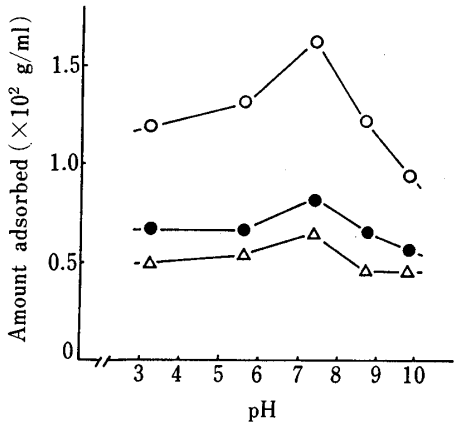

Fig. 4. Effect of pH on the Adsorption of γ-Globulin

A mixture of γ-globulin solution (2.0 g/l) and MC suspension was incubated for 30 min at 25±1 °C. The plots show the mean values of three separate runs. Symbols used here are the same as in Fig. 1.

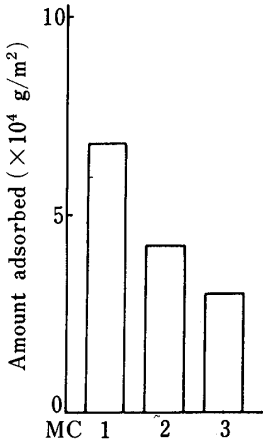

Fig. 5. Adsorption of Protein A on γ-Globulin-Coated MC

Data are the mean values of three separate runs.

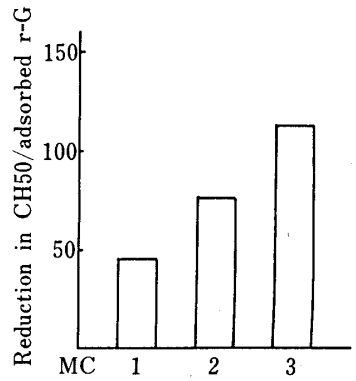

Fig. 6. Activation of Complement Caused by Adsorbed γ-Globulin

The ordinate represents the reduction in complement titer of fresh serum caused by unit amount of γ-globulin adsorbed on the MC. Data are the mean values of three separate runs.

γ-globulin hydrophobically adsorbed through the Fc moiety is larger for the weakly charged MC.

The isoelectric point (pI) of γ-globulin is assumed to be around pH 7, so that its net charge would be practically zero at pH 7.4, above and below which the protein is negatively and positively charged, respectively. It was reported[8] that γ-globulin shows a considerable "flattening" of conformation in solutions of pH below and above the pI because of intramolecular electrostatic repulsive forces, which would account for the lesser amounts of adsorption at pHs higher and lower than the pI shown in Fig. 4. In addition, it can be expected that γ-globulin would be subjected to strong electrostatic repulsive forces by the surface negative charges on the MC at high pHs. On the other hand, strong electrostatic attractive forces can be anticipated between the protein and the MC at low pHs. However, strong attractive forces may cause molecular deformation of the protein and, since more binding sites exist on the highly charged MC than on the weakly charged MC, such deformation could be more extensive for the former than the latter, which would result in the lesser amounts of adsorption for MC-3 than for MC-1. At pH 7.4, γ-globulin molecules are likely to take compact forms,[8] and they would be adsorbed to some extent through hydrophobic interaction, since electrostatic interactions between γ-globulin and MC seem to be less strong at this pH. The amount adsorbed was found to be more sensitive to the change in pH for weakly charged MC than for highly charged MC (Fig. 4), which suggests that hydrophobic interaction is more dominant in the former case than in the latter.

The observations described so far indicate that different adsorption patterns of γ-globulin exist depending on the surface negative charge of the MC. That is, under physiological conditions, the adsorption with Fab moieties is dominant on the highly negatively charged MC and there are many binding sites, so that conformational changes of γ-globulin can be expected. On the other hand, hydrophobic adsorption through the Fc moiety occurs on the weakly negatively charged MC.

In order to verify this assumption, the amounts of protein A adsorbed on γ-globulin-adsorbed MC were measured. Protein A is well known to be adsorbed selectively by the Fc moiety of γ-globulin.[10] The results are shown in Fig. 5; the amounts adsorbed were unexpectedly found to be larger with the weakly charged MC than with the highly charged MC. These results indicate that the amount of γ-globulin adsorbed through Fab moieties is

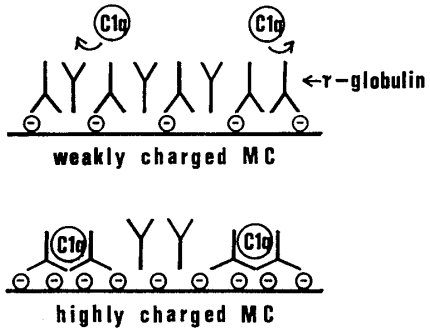

Fig. 7. Illustration of the Adsorption States of γ-Globulin

larger in the former than in the latter, which is contrary to our expectation.

Another set of experiments was done on complement activation by adsorbed γ-globulin. Complement activation through the classical pathway is known to be initiated with binding of subcomponent, Clq, of complement systems to the Fc moiety of γ-globulin.[11] However, Fig. 6 shows that complement is activated to a great extent by γ-globulin adsorbed on highly negatively charged MC, though the amount adsorbed of the protein with Fab moieties directed toward the surface seems to be smaller on the basis of the protein A adsorption (Fig. 5). This apparent discrepancy in the orientation of γ-globulin adsorbed can only be explained by the fact that complement activation is facilitated when the adsorbed γ-globulin undergoes considerable conformational changes and γ-globulin molecules are adsorbed in close proximity to each other.[12]

In conclusion, the adsorption characteristics of γ-globulin depending on the surface negative charge of the MC may be summarized as illustrated in Fig. 7. Adsorption of γ-globulin *via* its Fc moiety through hydrophobic interaction is dominant in the case of weakly charged MC. A considerable amount of the protein is also adsorbed with its Fab moieties directed to the MC surface, but conformational changes of the adsorbed molecules are less extensive and the molecules thus oriented in one direction are prevented from adjoining each other by the large amount of molecules oriented in the opposite direction, which would result in a low complement activation. On the other hand, γ-globulin is adsorbed on highly negatively charged MC *via* its Fab moieties through electrostatic and/or hydrogen bondings, though hydrophobic bonding between the surface and the Fc moiety cannot be excluded. Moreover, the adsorbed γ-globulin undergoes considerable conformational changes, presumably due to a high electric field near (and the many binding sites on) the surface of the MC, and it seems more probable that the molecules thus oriented are adsorbed closely to each other, which would account for the lesser amount of adsorption and higher activity of complement activation.

When the biocompatibility of biomedical materials is tested, the role of amounts and species of adsorbed proteins has always been emphasized. However, it must also be considered that orientation and conformational changes of adsorbed proteins are important determinants for the biocompatibility of the materials. Furthermore, the molecular array and assembly of adsorbed proteins also seem to be important; this aspect should be investigated in more detail.

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