Synthesis and properties of polymeric latex particles and their conjugates with human immunoglobulin G

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Polymeric latex particles of uniform size in the range of 750 to 10 000 Å in diameter were prepared by emulsion copolymerization of methyl methacrylate, methacrylic acid, 2-hydroxyethyl methacrylate and ethylene glycol dimethacrylate, using sodium dodecylbenzene sulphonate as an emulsifier and ammonium persulphate as an initiator. With a fixed composition of monomers the carboxyl groups on the latex particles ranged from 5×10^3 to 180×10^3 per particle, but the average number per unit surface area remained within a limited variation. Human immunoglobulin G (IgG) was covalently bonded to the latex particles by either the carbodiimide or the cyanogen bromide method, taking advantage of carboxyl and hydroxyl groups, respectively. The resulting conjugates exhibited a specific agglutination with rabbit anti-human IgG and sera from rheumatoid arthritis (*RA*) patients. The immunolatex particles were found to be superior to those derived from nylon-6, which had been used in our previous work, and are expected to offer useful means in various immunological studies.

INTRODUCTION

Polystyrene latex particles have been used as immunoadsorbents for purification of antigens or antibodies[1] or as the carriers for medical diagnostic tests[2,3]. However, since the bonding between latex particles and proteins is based on weak adsorption, the proteins partly dissociate from the latex particles to interfere with specific antigen–antibody reactions. If the latex particles have groups which can react chemically with proteins, the bonding should be firm enough to prevent non-specific reactions. Besides, if such particles were synthesized, they would be useful, for example, for the elucidation of immune mechanisms and even for immunological therapy to destroy tumors. For these motives, we had previously examined the possibility of using a methacrylic latex and nylon particles[4,5]. However, both have been found unsuccessful due to particle aggregation, non-specific adsorptions and so forth[6].

Based on similar ideas, Rembaum *et al.*[7–9] have recently prepared similar latex particles having hydroxyl, carboxyl and other functional groups on their surface by emulsion copolymerization or Co γ radiation in the presence of a crosslinking agent. Conjugates of these latex particles with antibodies exhibit specific agglutination by corresponding antigens. Thus, we have also prepared the same latex particles having carboxyl and hydroxyl groups by modifying their method and then, as a first step in application of the latex particles in immunological studies, antigen–latex conjugates using human immunoglobulin G (IgG). In this paper our results on the synthesis and properties of the latex particles and their conjugates with human IgG will be discussed in comparison with our earlier results[4,5] with nylon particles.

EXPERIMENTAL

Materials

Methyl methacrylate (MMA), methacrylic acid (MAA), ethylene glycol dimethacrylate (EGD), 1,7-diaminoheptane (DAH), and ϵ-aminocaproic acid (ϵ-ACA) were purchased from Tokyo Kasei Kogyo Co. Ltd. 2-Hydroxyethyl methacrylate (HEMA) was obtained from Yoneyama Chemical Industries Ltd., sodium dodecylbenzenesulphonate (SDBS) from Wako Pure Chemical Industries Ltd., ammonium persulphate (AP) from Koso Chemical Co. Ltd., and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) from Sigma Chemical Co.

MMA, MAA, EGD and HEMA were purified by fractional distillation, b.p. 64°C/155 mmHg for MMA, 55°C/7 mmHg for MAA, 105°C/9.5 mmHg for EGD and 92°C/0.6 mmHg for HEMA. Water was purified by distillation after ion-exchange.

Human IgG was isolated and purified from normal human serum with DEAE-Sephadex A50 following the batch procedure of Baumstark *et al.*[10] and the corresponding antisera were prepared by injecting it into rabbits with complete Freund's adjuvant.

Sera were collected from patients with classical rheumatoid arthritis (*RA*) at Kami-Itabashi Hospital.

Synthesis of latex particles

Method I. A 1 l, four-necked round-bottomed flask equipped with a mechanical stirrer, a reflux condenser, a thermometer and a gas inlet tube was thoroughly filled with pure nitrogen gas. The chemicals in *Table 1* in the indicated amounts were placed in the flask under a gentle stream of

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Table 1 Standard recipe for the preparation of latex particles

Compound	Quantity	
	g	wt %
MMA	18.24	9.1
MAA	3.2	1.6
HEMA	9.6	4.8
EGD	0.96	0.48
H_2O	168	83.9
SDBS	0.194	0.097
AP	0.020	0.010

nitrogen. The mixture was stirred for 1 h at room temperature while nitrogen gas was gently bubbled through and then heated gradually on an oil bath to a temperature of 70°C. The reaction was allowed to proceed for ~15 min at this temperature until rapid reflux subsided. Then the mixture was heated gradually to 96°C and refluxing was continued with stirring for 1 h. The heating took in all ~2 h. The remaining monomers were then removed by steam distillation for ~30 min. After cooling the mixture to room temperature, the emulsion was filtered through a filter paper (Toyo Filter Paper No. 51A). The filtered latex was centrifuged for 30 min at 10 000 rpm. The latex particles were then washed with distilled water three times by centrifugation and were finally suspended in distilled water at a suitable concentration.

Method II. Into a 100 ml glass pressure bottle were placed 9.0 g of MMA, 1.5 g of MAA, 3.0 g of HEMA, 0.9 g of EGD, 100 mg of SDBS, 10 mg of AP and 100 g of water together with a teflon-coated magnetic bar. The bottle was filled with pure nitrogen gas and was quickly closed with a screw cap. The mixture was heated gradually with stirring by the bar up to 96°C, taking ~2 h. It was further reacted at this temperature for 1 h. Then the latex particles were isolated and purified as in Method I. The yield of the particles was 47.3% and their average diameter was estimated by scanning electron microscopy to be 1.0 μm.

Preparation of antigen–latex conjugates

Antigen–latex conjugates were prepared by the carbodiimide and the cyanogen bromide methods, using latex particles of an average diameter of 1600 Å.

Carbodiimide method. To 4 ml of the latex suspension in 0.1 M NaCl (50 mg/ml) were added 5.24 mg of ε-ACA and 10 mg of EDC. The reaction was allowed to proceed for 2 h at 4°. The latex was dialysed overnight at 4°C against 0.1 M NaCl using a Visking tube and then centrifuged for 20 min at 10 000 rpm.

To the precipitate were added 5 ml of 0.01 M phosphate buffer (pH 7.0), human IgG (30 mg/100 mg latex) and 10 mg of EDC. The reaction was carried out for 2 h at 10°C with stirring. The reaction mixture was then centrifuged for 20 min at 12 000 rpm and the precipitate was washed with the buffer three times by centrifugation. The antigen–latex conjugate was stored at 4°C in the phosphate buffer at a concentration of 10–20 mg/ml. The supernatant fluid and the washings were saved for the quantitative analysis.

A similar conjugate was prepared by the same method, using DAH instead of ε-ACA. The amount of conjugated human IgG was estimated by subtracting that of uncombined protein, which was quantified by means of absorption at 280 nm, in the supernatant fluid as well as the washings, from the initial amount (30 mg). Thus it was found that 15.6 and 15.2 mg of IgG were conjugated to the latex particles by means of ε-ACA and DAH, respectively.

Cyanogen bromide method. The latex particles, 500 mg in 10 ml of water, were reacted with 10 ml of a 5% aqueous solution of cyanogen bromide at room temperature with stirring. During the reaction the pH value of the mixture was maintained in the range of 11.0–11.3 by the addition of 1 N NaOH. After 10 min the particles were separated and then washed with 0.01 M $NaHCO_3$ three times by centrifugation for 10 min at 12 000 rpm.

The CNBr treated latex particles, 50 mg in 2 ml of 0.01 M $NaHCO_3$, were allowed to react with 25 mg of human IgG for 16 h at 4°C. The reaction mixture was then treated in the same manner as in the carbodiimide method, and 22.8 mg of IgG was bound to the latex particles.

Quantitative analysis of carboxyl groups

The number of carboxyl groups per particle was estimated following the modified method of Palit and Mandal[11]. The latex particles were suspended at room temperature in a standard solution of Rhodamine 6G with stirring for 30 min. The supernatant fluid was separated by centrifugation for 15 min at 12 000 rpm and the particles were washed three times with water by centrifugation. The content of carboxyl groups per particle was calculated by dividing the amount of conjugated IgG, which was measured from the difference between the optical densities at 528 nm of the solution before and after conjugation, by the number of particles.

Scanning electron microscopy

Gold was evaporated under high vacuum onto the latex particles mounted on a 5 x 5 mm glass plate. Scanning electron microscopy was carried out on this sample using a Jeol scanning electron microscope, Model JSM 35, at a voltage of 15 kV.

RESULTS

Electron microscopic observation

Scanning electron microscopy (*SEM*) revealed that the latex particles obtained by the present method were quite globular with uniform size. *Figure 1* shows a typical *SEM* photograph of the latex particles with an average diameter

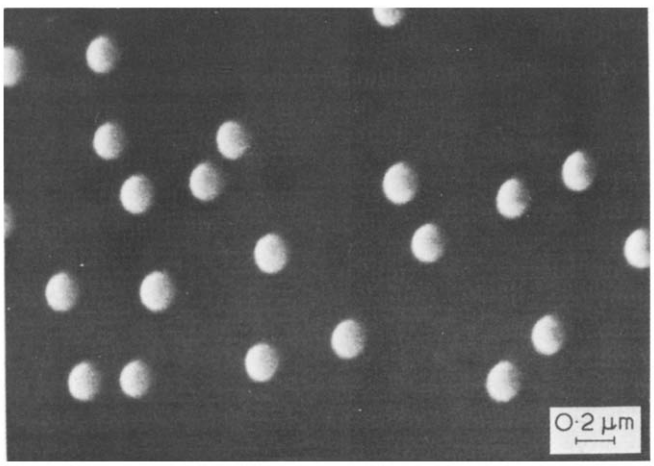


Figure 1 Scanning electron micrograph of copolymer latex spheres (1600 Å in diameter) synthesized under the conditions of Run No. 4 in *Table 2*

Table 2 Effect of quantity of emulsifier on the nature of latex particles[a]

No.	SDBS (mg)	Yield (%)		Diameter[c] (Å)	Density (g/cm^3)	–COOH (number/particle x 10^{-3})	–COOH (number/ 10^3 Å^2)
		Latex	Solid[b]				
1	194	99	0	750	1.07	5	2.8
2	97	99	0	1000	1.02	11	3.5
3	65	97	0.5	1180	1.22	26	6.0
4	49	94	0.7	1600	1.16	32	4.0
5	38	98	0.3	3500	1.21	180	4.7

[a] The reaction conditions are the same as those in *Table 1*; [b]solid polymer unfilterable through a Toyo Filter Paper No. 51A; [c]determined by scanning electron microscopy

Table 3 Binding of human IgG to latex particles

Human IgG added (mg)	Human IgG bonded (mg/100 mg latex particles)		
	Spacer		Direct binding with CNBr
	ϵ-ACA	DAH	
10	6.8	8.2	–
20	11.0	16.0	–
30	15.6	15.2	–
40	19.4	30.0	–
50	–	–	45.6

of 1600 Å. The average diameter of the particles obtained under the conditions specified in *Table 1* was estimated to be 750 Å.

Properties of latex particles

Table 2 shows that (a) the latex could be produced nearly quantitatively, although accompanied by a small amount of solid copolymer, particularly at low concentrations of the emulsifier, and (b) the particle size could be varied at will from 750 to 3500 Å by decreasing the amount of the emulsifier, with constant monomer composition. The particles with a larger average diameter have in general a higher density, and the tendency of the particles to remain as an emulsion decreases as the particle size increases. Thus particles larger than ~2000 Å in diameter precipitate easily.

The amount of carboxyl groups was found to be 5000 to 180 000 groups per particle. The larger the particle, the more groups it possessed. However, the number of the carboxyl groups in a surface area of 1000 Å^2 was 3 to 6, depending only to a small extent on the size of particles.

Conjugation of human IgG to the latex particles

The conjugation of human IgG to the latex particles was carried out by three methods, namely bonding with carboxyl groups via DAH or ϵ-ACA and direct bonding with hydroxyl groups by means of cyanogen bromide. The results are listed in *Table 3*.

From this, it is clear that (1) a parallel exists between the amount of the IgG added and that of the IgG bonded; (2) almost quantitative bonding is achieved by means of CNBr; and (3) more IgG is bonded with DAH spacer than with ϵ-ACA. However, particles conjugated with DAH spacer showed non-specific agglutination by themselves without reacting any antiserum.

Reactivity of the human IgG–latex conjugate

Figure 2 shows that the human IgG–latex conjugate

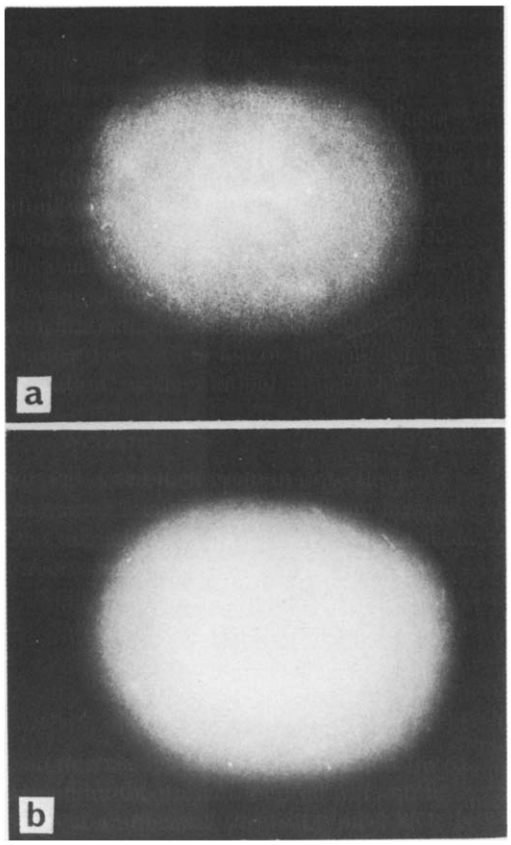


Figure 2 Photographs of (a) a human IgG–latex conjugate reacted with rabbit antihuman IgG serum, and (b) the same conjugate mixed with normal rabbit serum

thus produced via ϵ-ACA as a spacer, exhibited, just like the human IgG–nylon conjugate[4,5], a specific agglutination with rabbit antihuman IgG serum (*Figure 2a*), whereas no agglutination occurred when the conjugate was mixed with normal rabbit serum (*Figure 2b*).

Similarly, *Figure 3* illustrates that the conjugate is also specifically agglutinated by the serum of a *RA* patient (*Figure 3a*), while no reaction was observed between the conjugate and normal human serum (*Figure 3b*). No dissociation of IgG was detected in the supernatant, whereas ~420 mg/dl of IgG was found in the supernatant of the commercial latex reagent for *RA*.

DISCUSSION

The fluorescent antibody technique is a practical method which turns an invisible reaction into a visible one. If suitable

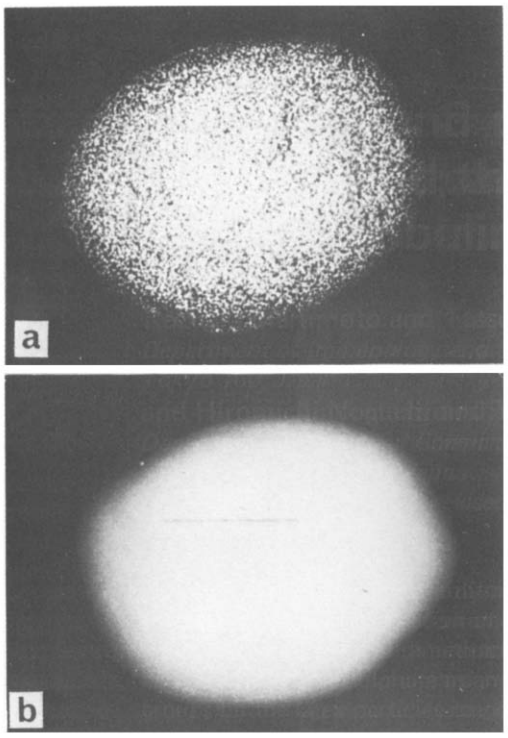


Figure 3 Photographs of (a) a human IgG–latex conjugate reacted with the serum of a *RA* patient, and (b) the same conjugate mixed with normal human serum

microspheres are used instead of fluorochromes, the reaction could be seen directly with the naked eye. Hitherto, passive agglutination tests for diagnosis of various diseases as well as pregnancy have been known which use sensitized red blood cells and a polystyrene latex. However, the bonding in these cases is achieved only by adsorption and hence once bonded the cells dissociate easily. Use of such a latex for sensitive analyses is therefore not desirable. On the contrary, the latices developed by Rembaum *et al.*[7–9] have carboxyl, hydroxyl and other groups which can be used to bond proteins firmly and thereby to form stable protein–latex conjugates.

Based on the same idea as that of Rembaum *et al.*[7–9] we had previously attempted to use a methacrylic latex and nylon particles[4,5]. However, practical use of these was hampered because, in the former case, particle aggregation occurred even at the time when the latex was synthesized by the emulsion copolymerization of MMA, MAA and divinylbenzene with potassium persulphate as an initiator and Tween 60 as an emulsifier; in the latter case, the nylon particles prepared by dispersing a 1% solution of nylon-6 in formic acid into a large amount of water with stirring were non-uniform in size even after attempted uniformalization of the particle sizes by centrifugation and besides their surface was coarse. In the present work, the latex particles were synthesized by modifying the method of Rembaum *et al.*[7–9].

In experiment no. 1 in *Table 2* we obtained particles with a diameter of 750 Å. Under almost the same conditions, Rembaum *et al.*[8] have obtained particles with a diameter of 1550 Å, using sodium dodecyl sulphate as an emulsifier and a tumbling container for the emulsion copolymerization under argon at 98°C. The diameter of the particles may depend on the method of polymerization to some extent. However, the difference in the diameters would be due primarily to the fact[12] that the critical micelle concentration of SDBS is smaller than that of sodium dodecyl sulphate and hence the particle number was larger with SDBS.

Latex particles with diameters larger than ~1 μm are especially useful to visualize immunological reactions in the optical microscope. With this particular emulsifier (SDBS) in our experiments it was difficult, however, to produce particles larger than 3500 Å unless the monomer composition or concentration was varied. In a preliminary experiment with a different monomer composition and use of a different apparatus (see Experimental, Method II) we were able to produce latex particles with an average diameter of 1.0 μm, but the yield of the latex was as low as 47%.

To characterize these synthetic latex particles, we measured the number of carboxyl groups, firstly by conductometric titration but in contrast to the results of Rembaum *et al.*[8,9], no reliable values were obtained. Therefore, quantitative measurement of these groups was performed by means of absorption spectroscopy, using Rhodamine 6G which reacts with carboxyl groups.

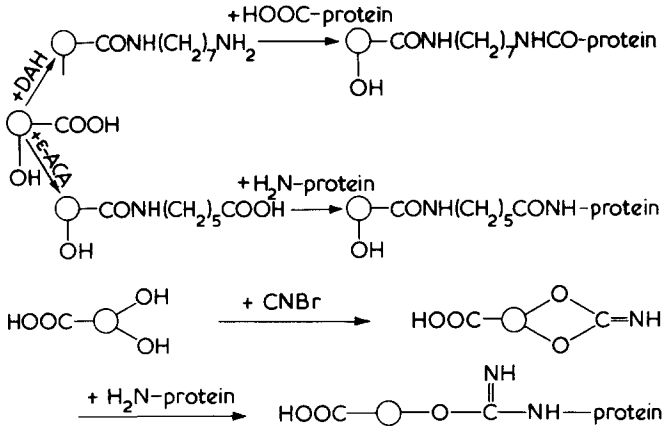
The results revealed that the larger the particle, the more carboxyl groups it had. However, the average number per unit surface area remained within a limited variation. This is a reasonable result, since the monomer composition in the feeds was the same and the yields of the latices were nearly quantitative, and besides, the Rhodamine 6G method is likely to measure the carboxyl groups on the surface of particles, since the particles are rigid due to the crosslinking, the dye is of considerably large size and the measurement was carried out under mild conditions.

No simple method is available yet to measure directly the number of hydroxyl groups. Therefore, the hydroxyl groups of the particles were reacted with phthalic anhydride in refluxing pyridine for 6 h[11] and the resulting carboxyl groups were measured by the same method as described above. However, no reliable constant values were obtained. This could be due partly to incomplete reaction of hydroxyl groups with phthalic anhydride.

Previously we have shown[4,5] that human IgG can be covalently bound to nylon particles by the carbodiimide method, making use of the terminal amino groups of nylon-6 and the carboxyl groups of human IgG, and that the resulting conjugates exhibited a specific agglutination with antihuman IgG or *RA* serum. However, as mentioned above, the present latex particles are superior to the methacrylic latex and nylon particles used in our earlier work[4,5]. The human IgG–latex conjugates produced here are particularly useful in the diagnosis of rheumatoid arthritis (*Figure 3*). Rembaum *et al.*[7–9] have also observed similar specific agglutinations of antibody–latex conjugates with antigens.

Conjugation of a protein to the present latex particles can be achieved by taking advantage of either carboxyl or hydroxyl groups. In the former case, the microspheres are first conjugated with a 'spacer' by the carbodiimide method. On the other hand, in the latter case, the latex spheres can be directly bound to a protein using the cyanogen bromide method. The reactions involved are shown in the reaction scheme below.

We have successfully prepared conjugates of the present methacrylic latex with human IgG, which has both terminal carboxyl and amino groups, by the carbodiimide method, using ε-ACA and DAH as spacers. However, when DAH



was used as a spacer, non-specific agglutination of the IgG–latex conjugates occurred to some extent even when they were prepared, which made the antigen–antibody reactions less discernible.

The most probable explanation for this is assumed to be as follows: the electric bilayer around the particles, which is primarily responsible for the prevention of aggregation of the particles, is dependent upon the net negative charges of the particles. The carboxyl groups on the particles are responsible for this. When the particles are treated with DAH, most of the carboxyl groups are converted into amino groups. Although IgG contains both carboxyl and amino groups, the former is smaller in quantity than the latter as indicated by its slower electrophoretic migration in the serum proteins. This means that when the protein is conjugated with the latex particles, the carboxyl groups on the protein are consumed, leaving the net negative charges reduced. In contrast, ϵ-ACA which has a carboxyl group at one end does not alter the net negative charge by bonding to the particles, and when conjugated with IgG, reduces amino groups on the protein and leaves some carboxyl groups unbound, thus preventing self-aggregation of the particles. This may explain why ϵ-ACA is a more suitable spacer to conjugate the latex with human IgG. It is likely that the suitable spacer varies with individual proteins. We were also able to bind human IgG to the latex spheres by the cyanogen bromide method.

It is concluded from these results that the methacrylic latex particles can be covalently bound to proteins such as antigens and antibodies by the carbodiimide and cyanogen bromide methods without depriving the proteins of their functions. Immunolatices derived from the methacrylic latex particles may therefore be expected to work as 'smart spheres'[13,14] in immunoadsorbent methods, antigen or antibody labelling methods, suicide techniques, passive agglutination tests and so forth. Recently, we have succeeded in applying this method to the analysis of the E-rosette phenomenon, which is a peculiar and characteristic reaction of human T-lymphocytes with sheep erythrocytes, details of which will be reported elsewhere.

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