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ORIGINAL CONTRIBUTION

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Capabilities of polymer-modified monodisperse colloidal silica particles as biomaterial carrier

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Abstract Polymer modification of monodispersed colloidal silica (0.5 μm) with poly(maleic anhydride-co-styrene) (P(MA-ST)) and poly (maleic anhydride-co-methyl methacrylate) (P(MA-MMA)) and application of the composite particles to biomaterial carriers were investigated. The reaction of bovine serum albumin(BSA)-immobilized P(MA-MMA)/SiO_2 with the anti-BSA antibody showed higher sensitivity in immunological agglutination test than BSA–P(MA-ST)/SiO_2, though immobilization efficiency of BSA on P(MA-MMA)/SiO_2 was lower than that on P(MA-ST)/SiO_2. Alkaline phosphatase and glucose oxidase immobilized on the composite particles exhibited extremely low activities, but α-chymotrypsin immobilized on P(MA-MMA)/SiO_2 and its derivative particles showed the relative activity of 12.5% and 16.1% to the native enzyme, respectively. Grafting of a hydrophilic polymer of poly(acrylic acid) to P(MA-ST)/SiO_2 let to an increase of the immobilized α-chymotrypsin activity to give the maximum relative activity of 55.5%.

Key words Biomaterial carrier – immunolatex – protein immobilization – monodisperse colloidal silica – polymer modification

Introduction

Recent developments in colloid chemistry have enabled to prepare many kinds of monodisperse inorganic colloids. We have been developing a new material by functionalization of monodisperse colloidal particles with polymer modification [1–3]. One application is as a biomaterial carrier in biotechnology for cell labeling, immunoassey or affinity separation. In many cases, polymer latexes, such as polystyrene microspheres of micron size, have been employed as carrier particles [4]. In latex immunoassey, polymer particles should have adequate stability and high density to attain high sensitivity and short test time. In this respect, the applications of polymer particles as carriers are limited, because it is difficult to prepare polymer spheres with high density, in spite of controlling stability and surface hydrophobicity [5]. Moreover, if composite particles, prepared by the polymer modification of inorganic colloidal particles, could be applicable for biomaterial carriers, it would be possible to develop a new separation technique by taking advantage of physical properties, such as magnetism or density, in the immunological assay or affinity chromatography [6, 7]. A previous paper reported that monodisperse colloidal silica particles, of 0.5 μm size, modified with poly(ethylene glycol), bound to bovine serum albumin (BSA) as a protein, and the hybrid particles can be used in immunological agglutination tests [8]. However, binding efficiency was low; amount of bound BSA was less than 20% of dosed protein.

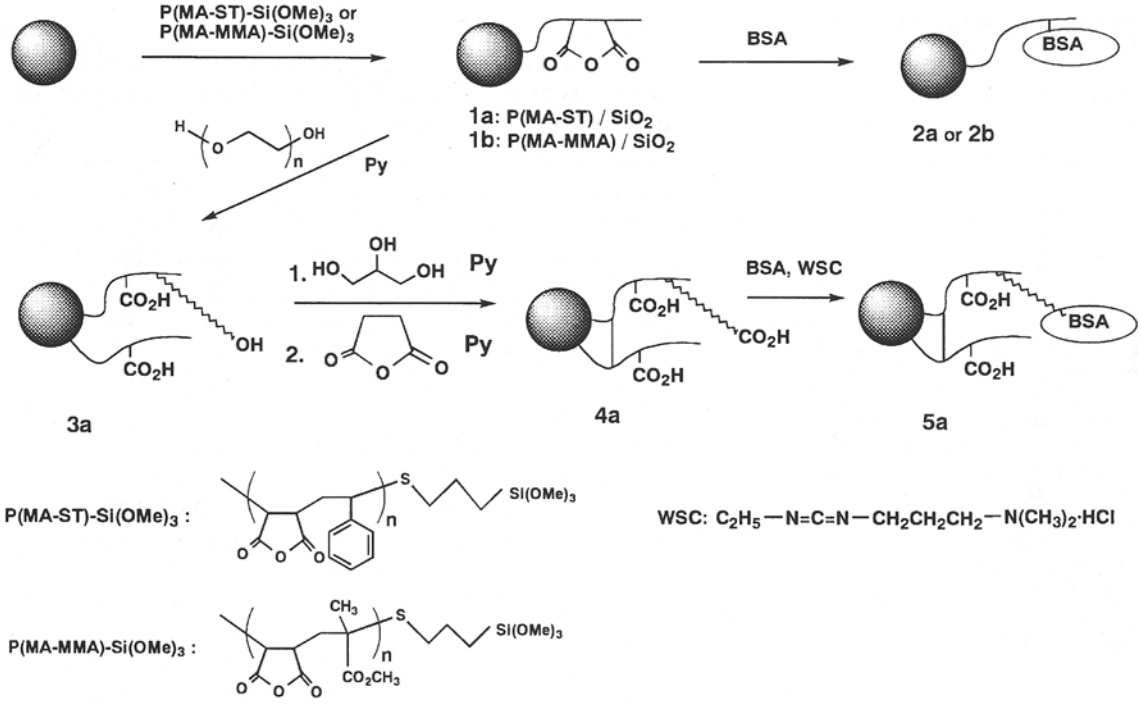
In this study, we have investigated improvement in the immobilization of protein on monodisperse colloidal silica modified with the copolymer of maleic anhydride and styrene or methyl methacrylate, and the applicability of hybrid particles to the immunological agglutination tests and supports for enzyme bindings. Polymer modification of the silica and protein bindings on the resulting particles were conducted according to the procedure shown in Schemes 1–3. The immunological tests were examined by the reaction of immobilized BSA with anti-BSA antibody. The immobilization of enzyme was also studied by using three types of enzyme; α-chymotrypsin of a simple protein [9], alkaline phosphatase of a zinc ion-contained protein [10], and glucose oxidase composed of fravine coenzyme [11].

Experimental

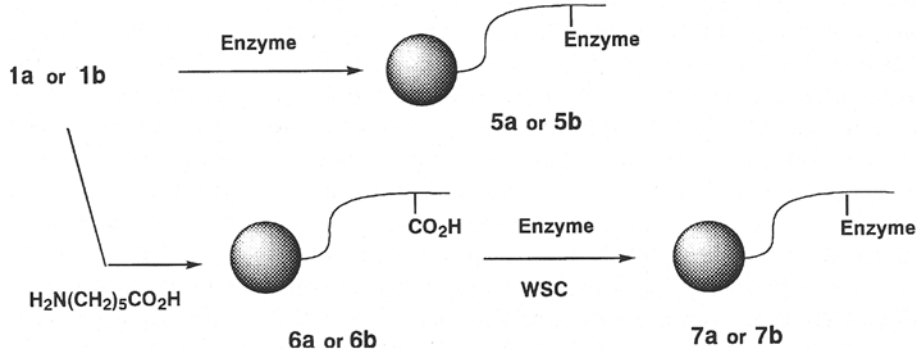
Materials

Monodisperse colloidal silica, 0.5 μm in mean diameter, suspended in ethanol was kindly offered by Catalysts & Chemicals Co. Ltd., Japan. The composites of P(MA-ST)/SiO_2 (**1a**) and P(MA-MMA)/SiO_2 (**1b**), composed of 26 mg/g poly(maleic anhydride-co-styrene) and 17 mg/g poly(maleic anhydride-co-methyl methacrylate), respectively, were prepared by the method reported previously [12]. BSA (Cohn Fraction V) and γ-globulin (Cohn Fraction II, III) were purchased from Nakarai Tesque and Sigma Chemical Co., respectively. Anti-BSA antibody was prepared according to the procedure described previously

Scheme 1



Scheme 2



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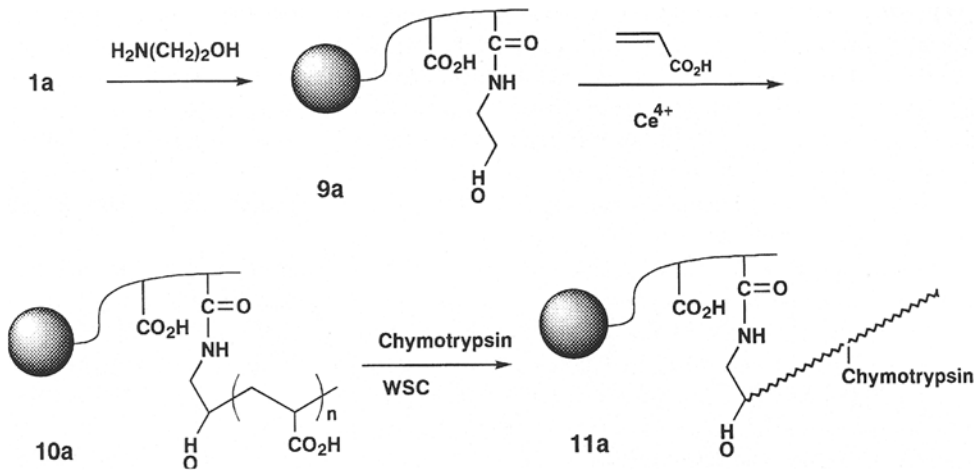
Scheme 3



[13]. Alkaline phosphatase from calf intestine was available from Sigma Chemical Co. Glucose oxidase from aspergillus and α-chymotrypsin from bovine pancreas were obtained from Toyobo Co. and Wako Chemical Co., respectively.

Measurements

Spectrophotometric measurements were carried out using a 10 mm quartz cell with a JASCO V-520. Thermogravimetric analyses were performed with a Shimadzu TGA-50. IR spectra were recorded by the diffuse reflection method on JEOL JIR-5500. Scanning electron micrographs (SEM) were taken by a JEOL JCX-733. Measurements of particle size and its distribution were made by a dynamic light scattering method on a Ohtsuka DLS-700.

Preparation of composite **3a**

A mixture of 1.5 g composite **1a**, 5.0 g poly(ethylene glycol) of molecular weight of 2000 and 3 ml pyridine in dry acetone was stirred at 70 °C for 12 h. Washing with 10 ml acetone three times and with 10 ml methanol by centrifugation, and then drying under vacuum gave 1.4 g composite **3a** grafted on to the polymer of 18 mg/g. Anal. IR (KBr, cm^{-1}) : 1861 and 1780 ($\nu_{C=O}$, maleic anhydride); 1733 ($\nu_{C=O}$, ester and carboxyl); 1454 (δ_{CH_2}, poly(ethylene glycol)); 1128 (ν_{Si-O}, silica).

Preparation of composite **4a**

The suspension of composite **3a** (0.5 g) in 20 ml dry acetone was stirred with 1 ml glycerol and 2 ml pyridine at 70 °C for 12 h. The paste, separated from the acetone solution by centrifugation, was again suspended in 20 ml dry acetone containing 0.5 g succinic anhydride and 1 ml pyridine and the mixture was stirred at 70 °C for 12 h. Washing and drying by the same procedure described above gave 0.47 g composite **4a**. Anal. IR (KBr, cm^{-1}): 1864 and 1780 ($\nu_{C=O}$, maleic anhydride); 1733 ($\nu_{C=O}$, ester and carboxyl); 1454 (δ_{CH_2}, poly(ethylene glycol)); 1140 (ν_{Si-O}, silica).

Preparation of composite **10a**

To a suspension of composite **1a** (1.0 g) in 50 ml dry acetone 3 g 2-aminoethanol was added and the mixture was stirred at 70 °C for 12 h. Washing with 20 ml acetone and with 10 ml methanol by a centrifugation, and drying under a vacuum gave 0.97 g composite **9a**, a bound 31.7 mg/g polymer. To an aqueous suspension of composite **9a** in 9.5 ml distilled water 0.5 ml of acrylic acid and 0.5 ml of 0.2 M cerium(IV) nitrate diammonium in 0.1 M HNO_3 was added. The suspension was stirred under shading at room temperature in a nitrogen atmosphere for 12 h. Washing with 10 ml distilled water three times and 10 ml methanol by centrifugation, and then drying under vacuum gave 0.2 g composite **10a**, a bound polymer of 47.1 mg/g. Anal. IR (KBr, cm^{-1}): 1708–1730 ($\nu_{C=O}$, ester and carboxyl; 1637–1662 ($\nu_{C=O}$, amide); 1427 (ν_{C-O}, carboxyl); 1132 (ν_{Si-O}, silica).

Immobilization of BSA on composite **1a** or **1b**

The reaction of BSA with the respective composite particles (0.1 g) was conducted in pH 5.6 phosphate buffer (0.03 M) solution (10 ml) at 4 °C. The hybrid particles were

stirred with 0.6 g 2-amonoethanol in pH 7.0 buffer (10 ml) at 4 °C for 5 h. The particles (**2a** or **2b**) were separated from the buffer solution by centrifugation and stored in the pH 7.0 solution containing 0.5 wt% NaN_3. The amount of bound BSA was determined by spectrophotometric absorbance of the supernatant at 280 nm.

Immobilization of BSA on composite **4a**

A suspension of 0.05 g composite **4a**, BSA and 1 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (WSC) in 5 ml of pH 7.0 phosphate buffer (0.03 M) was gently stirred at 4 °C for 12 h. Washing with the buffer three times by centrifugation, the resulting paste was stored in the buffer containing 0.5 wt% NaN_3. The amount of immobilized BSA was determined by the same procedure described above.

Immobilization of enzyme on composite **1a** or **1b**

The immobilization was carried out by the same procedure as that of BSA on the same composites.

Immobilization of enzyme on composite **6a**, **6b** or **10a**

The immobilization was carried out by the same method as that for BSA on composite **4a**.

Immunological agglutination test

Each 50 μl sample of anti-BSA antibody or antiserum diluted with 0.05 M phosphate buffer (pH 7.0) containing Triton X-100 (0.1 wt%) was placed in each well of a microplate in a series of successive two-fold dilution, starting from 20 μg/ml, and then 50 ml of BSA-immobilized silica suspension (0.1 wt%) in the buffer was added to each well. The mixture was allowed to stand for 4 h. In control experiments, γ-globulin or normal rabbit serum was used instead of the antibody or antiserum.

Assay of immobilized alkaline phosphatase [14]

Three milliliters of the suspension containing alkaline phosphatase-immobilized particles (0.1 g/15 ml) in pH 7.0 phosphate buffer (0.05 M) was added to 4 ml of the buffer immersed in the bath thermostated at 30 °C. To the mixture 1.0 ml of 50 mM *p*-nitrophenylphosphate disodium was added to start the hydrolysis reaction. An aliquot (0.5 ml) of the reaction mixture was periodically taken out and poured to 2.0 ml 0.5 N NaOH. The hydrolysis was monitored by the measurement of the spectrophotometric absorbance of *p*-nitrophenolate at 400 nm (ε = 18 000).

Assay of immobilized glucose oxidase [15]

Preliminarily, the MBTH-DEA solution was prepared by dissolving 6.8 mg 3-methyl-2-benzothiazoline (MBTH), 0.16 g N,N-dimethylaniline (DEA) and 40 mg ethylenediamine tetraacetic acid disodium salt in 400 ml of 0.1 M acetic acid. A peroxidase solution (POD) was also made from 8 mg of the peroxidase and 100 ml of 0.1 M sodium acetate. The glucose oxidase-immobilized silica suspension was adjusted to the enzyme concentration in the range of 3.4×10^{-5}–2.1×10^{-4} mg/ml by dilution with the pH 7.0 buffer. To a mixture of 2 ml MBTH-DEA solution, 0.5 ml POD solution, and 0.3 ml glucose oxidase-immobilized particles, kept at 37 °C, 0.5 ml glucose solution which was made from 15 g glucose, and 100 ml acetic acid (0.1 M) was added. After 15 min, the enzyme reaction was terminated by an addition of 1 ml 0.5 N HCl, and then a supernatant was separated by centrifugation and supplied to the measurement of the spectrophotometric absorbance at 590 nm. The net conversion in the reaction was estimated by subtraction of the absorbance in a blank test without the enzyme-immobilized particles from that with the particles.

Assay of immobilized α-chymotrypsin [16]

The suspension of α-chymotrypsin-immobilized particles was first prepared so as to contain the enzyme of less than 0.2 mg/ml in 0.05 M phosphate buffer. To 1.25 ml casein solution (10 mg/ml) in the phosphate buffer in a test tube, immersed in a bath thermostated at 37 °C, 0.25 ml of the suspension was added. After 10 min, 2.5 ml of 5% trichloroacetic acid aqueous solution was poured into the test tube to stop the reaction. The extent of the reaction was monitored by the absorbance of the supernatant at 280 nm. The activity of the immobilized enzyme relative to the native one was estimated by the absorbance ratio.

Results and discussion

Immobilization of BSA
and immunological agglutination test

Particles of composite **1a** and **1b** were stable in neutral aqueous solution, but immersing them into pH 4.0 buffer

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solution brought about the partial elimination of bound P(MA-ST) and P(MA-MMA) on the silica. Hence, the reactions of composite particles (**1a** or **1b**) with BSA were conducted in pH 5.6 phosphate buffer, though BSA has an isoelectric point at pH 4.7–4.9. The composite particles of **1a** efficiently enabled the immobilization of most of the dosed BSA at BSA concentration lower than 20 mg per unit gram of silica in the reaction solution. Increase in dose of BSA led to less immobilization efficiency: about 80% of the BSA was immobilized by BSA of 30 mg/g (Fig. 1). The amount of immobilized BSA attained a constant value of 40 mg/g, corresponding to the maximum amount for end-on packing. This was estimated by 14 nm major and 3.8 nm minor axis in an oval shape of BSA [17]. It was also observed that composite **1b** made the binding of BSA in 30 mg/g dosed protein 20–30% less efficient as compared with that on composite **1a** (Fig. 1). The high binding efficiency of composite **1a** is probably due to binding through hydrophobic interaction between P(MA-ST) and BSA. In fact, it was observed that the binding of BSA on the composite **4a** of hydrophilic polyoxyethylene chain-grafted particles using a water soluble coupling reagent, WSC, was extremely low efficient; the amount immobilized was no more than about 25% of dosed BSA. Hence, the immobilization of BSA on composite **1a** or **1b** is not only very effective, but also useful, since there is no need for coupling reagents, such as the carbodiimide.

The hybrid particles (**2a**) gave specific agglutination with the anti-BSA antibody and did not give non-specific agglutination with γ-globulin. The particles of composite **1a** and **1b** without treatment with 2-aminoethanol gave non-specific agglutination with γ-globulin, because of the reaction of the serum with maleic anhydride moiety. Figure 2 shows plots of immobilized BSA vs. the lowest concentration of anti-BSA antibody to give the agglutination. The lowest concentration of the antibody, or sensitivity, was dependent on the amount of immobilized BSA on composite **1a**. The hybrid particles containing 13 mg/g BSA exhibited the highest sensitivity. Probably BSA, less immobilized BSA than 13 mg/g on composite **1a**, undergoes degradation due to strong hydrophobic interaction between BSA and P(MA-ST), thus depresses in sensitivity. At higher BSA immobilization, crowded BSA seems to lead to steric hindrance of the recognition site in the reaction with the anti-BSA antibody. The hybrid particles of **2b** containing 14 mg/g BSA agglutinated with the antibody at concentration higher than 0.07 mg/ml, showing the highest sensitivity among composites **2a** and **2b**. The composite **2b** gave higher sensitivity in the agglutination test than the composite **2a**, in spite of lower immobilization efficiency. In the present cases, the copolymers of P(MA-ST) and (MA-MMA) function not only as surface modifier of silica, but also as linker between BSA and silica. In general, deformation of the protein adsorbed or immobilized on a polymer surface increases with surface hydrophobicity [18]. Therefore, the lower sensitivity of composite **2a** is possibly due to the extensive deformation of immobilized BSA, as compared with that of the protein on composite **2b**.

In this respect, grafting of a hydrophilic polymer, polyoxyethylene, to composite **1a** is expected to lead to increase of the sensitivity. Indeed, hybrid particles (**5a**) having polyoxyethylene linker showed almost the same sensitivity as **2b**. These results showed that the deformation of immobilized protein due to hydrophobic interaction affected the specific and intrinsic properties of the immobilized protein significantly.

Fig. 1 Immobilization of BSA on composite **1a** (○), **1b** (●), and **4a** (△)

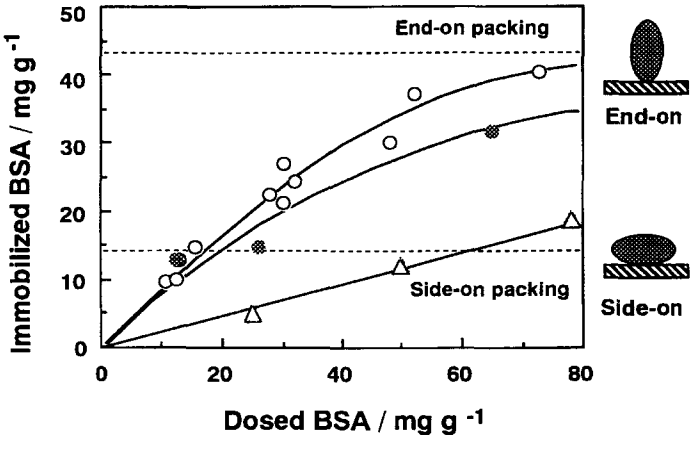

Fig. 2 Plots of immobilized BSA on composite **2a** (○), **2b** (●) and **5a** (△) vs. the lowest concentration of anti-BSA antibody to give the agglutination in immunological tests

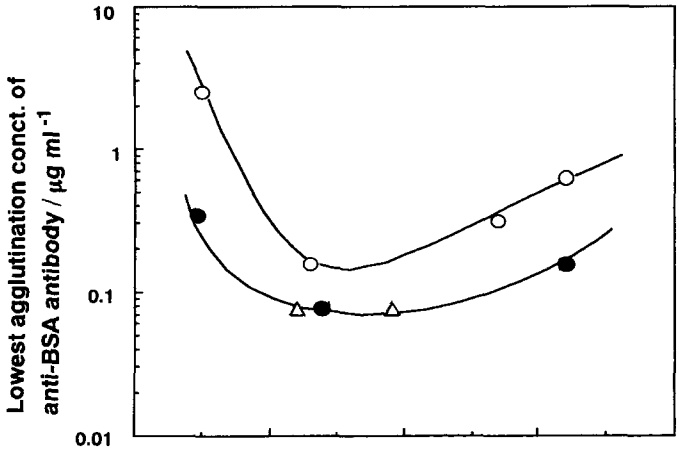

Table 1 Immobilization of enzymes onto composite **1a** or **1b** and composite **6a** or **6b**, and relative activities of immobilized enzymes

Enzyme	Composite	Buffer (pH) [ml]	Enzyme		Relative activity[a] [%]
			Dosed [mg g^{-1}]	Immobilized [mg g^{-1}]	
alkaline phosphatase	**1a**	10(5.6)	54.0	39.6	0
	1b	10(5.6)	53.0	30.5	0
	6a	10(5.6)	57.0	42.2	2.0
	6b	10(5.6)	55.0	23.3	2.0
glucose oxidase	**1a**	5(7.0)	32.2	10.0	0
	1b	5(7.0)	32.2	7.0	0
	6a	5(7.0)	31.8	5.2	2.2
	6b	5(5.0)	23.0	6.8	1.0
		5(7.0)	31.8	8.8	1.0
α-chymotrypsin	**1a**	5(7.0)	34.8	33.9	3.3
	1b	5(7.0)	34.8	9.6	12.5
	6a	5(7.0)	38.0	36.7	3.5
	6b	5(7.0)	42.0	24.2	16.1

[a] The value is the relative activity of immobilized enzyme to respective native one.

Immobilization of enzyme

For the purpose of application of composite **1a** and **1b** in fabricating biomaterials carrier, we examined immobilizations of enzymes and their properties. The immobilizations of enzymes were conducted by the procedure shown in Scheme 2. Activities of immobilized alkaline phosphatase, glucose oxidase and α-chymotrypsin on **2a**, **2b**, **7a** or **7b** were listed in Table 1. Immobilized phosphatase and glucose oxidase exhibited extremely low activities of less than 10% of respective native enzyme activity, while α-chymotrypsin immobilized on **2b** or **7b** showed 12.5% and 16.1% relative activity, respectively. Since molecular weights of α-chymotrypsin from bovine pancreas is 25000, which is relatively lower than those (89000 and 186000, respectively) of alkaline phosphatase and glucose oxidase, the activity of the immobilized enzyme probably relates with the size of the enzyme. In these cases, the immobilization is involved with multiple bindings between the composite particles and the enzyme, so that large-size enzyme presumably undergoes much deformation in the immobilization to lead to low activity. In this regard, we have reported that composite **1a** of 10 nm size was able to adsorb faster and more amount of α-amylase than composite **6a** of 10 nm size, and that the adsorbed α-amylase on **2a** showed lower activity, as compared with **7a**. The lowering of the activity was related to conformational distortion, which was estimated by a decrease of α-helix content with a circular dichroism analysis, due to the hydrophobic interaction between the enzyme and the particle surface [19].

On the above results, we investigated the immobilization of α-chymotrypsin on the composite particles, **6b**,

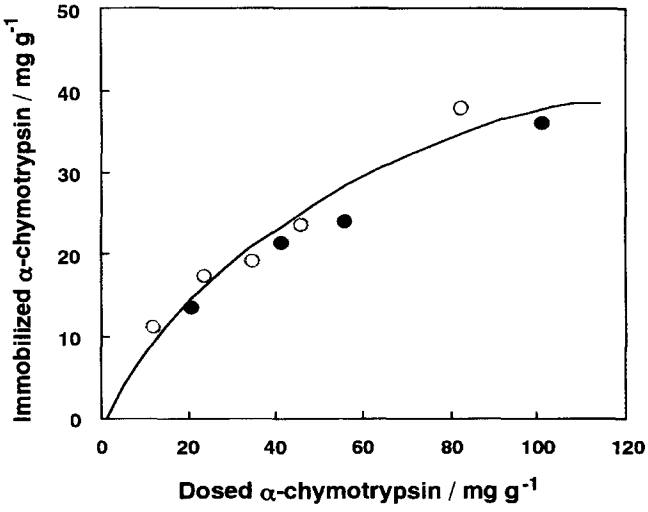

Fig. 3 Immobilization of α-chymotrypsin on composite **6b** (○) and **10a** (●)

and the properties of the bound enzyme. In the immobilization of the enzyme using WSC, about 50% of dosed enzyme was bound to the particles (Fig. 3). The immobilized α-chymotrypsin showed a maximum relative activity (16%) at about 25 mg/g of the immobilized enzyme, as shown in Fig. 4. This result is similar to the dependency of sensitivity on the immobilized BSA in the immunological agglutination tests. The low activity at less than about 20 mg/g of the immobilization is probably because of deformation due to strong hydrophobic interaction between the enzyme and the surface polymer. At higher immobilization, the crowded circumstances of the enzymes binding each other presumably lead to lowering of activity.

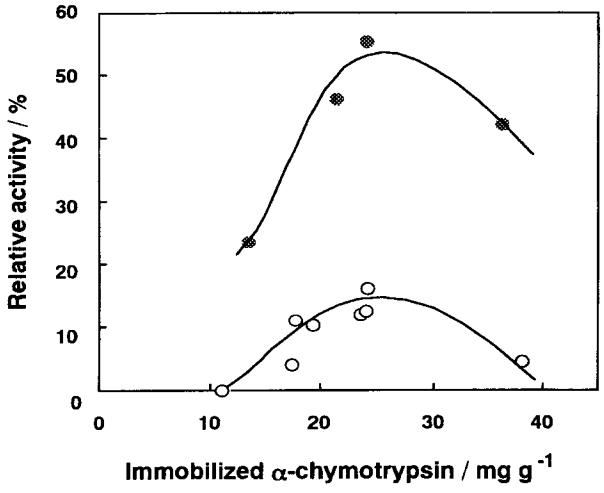

Fig. 4 Activity of immobilized α-chymotrypsin on composite **7b** (○) and **11a** (●)

In order to weaken the interaction between the enzyme and the surface polymer and to improve the enzyme activity, we prepared the composite **10a** [20], which was grafted by a redox radical polymerization of acrylic acid employing cerium(IV) nitrate as shown in Scheme 3. The amount of grafted poly(acrylic acid) was 18 mg/g. The binding efficiency of the dosed α-chymotrypsin in the immobilization on composite **10a** was almost the same as that on composite **6b** (Fig. 3). The immobilized α-chymotrypsin on composite **11a** exhibited distinctly higher activity than those of the immobilized enzyme on **7b** and gave the maximum activity of 55.5% at about 25 mg/g of immobilized amount, as shown in Fig. 4. Therefore, the introduction of hydrophilic polymer layer on **11a** effectively improved the activity of the immobilized enzyme. An increase of the activity is probably due to improvement of circumstance around the active center and/or reduction of the hydrophobic interaction between the primary polymer of P(MA-ST) and the enzyme through binding to flexible and hydrated poly(acrylic acid) chains. However, the activity of the immobilized α-chymotrypsin was still relatively low, as compared with the native one. One of the reasons for the low activity is considered as follows. The composite **10a** has many carboxyl groups on the surface, coming from grafted poly(acrylic acid), so that the enzyme is possibly bound to composite **10a** via multiple bindings. The bindings for the immobilization are probably one of the significant factors for changing the optimum circumstance, especially mobility around the active center.

Development of the composite particles from polymer-modified and monodisperse colloidal silica, which gives efficient immobilization of enzyme and enables to retain the enzyme activity without an undesirable interaction between the surface polymer, is now in progress.

Conclusions

1. The polymer-modified silica particles of P(MA-ST)/SiO_2 or P(MA-MMA)/SiO_2 were able to immobilize a protein with high efficiency. It is suggested that the hybrid particles are applicable to immunological agglutination tests.

2. Immobilized enzymes on P(MA-ST)/SiO_2 or P(MA-MMA)/SiO_2 showed extremely low activities due to changes in the circumstance around the active center via hydrophobic interaction between the enzyme and the particles surface. Introduction of a hydrophilic polymer layer between the enzyme and silica particles improved the enzyme activity.

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