Surface Properties and Flocculations of Globulin-Immobilized Fine Polymer Particles

HISASHI TAMAI,* HIROYUKI SAKURAI, TOSHIRO SUZAWA, and HAJIME YASUDA

Department of Applied Chemistry, Faculty of Engineering, Hiroshima University, 1-4-1 Kagamiyama, Higashihiroshima 724, Japan

SYNOPSIS

Characteristics of globulin-immobilized styrene/acrylamide/acrylic acid copolymer [poly(St/AAm/AA)] fine particles and their flocculation by an antigen–antibody reaction were investigated as a function of acrylamide (AAm) content. Immobilization of globulin was carried out through octamethylenediamine with a carbodiimide reaction. The increase in AAm content in particles decreases the amounts of bovine γ-globulin (BγG) immobilized, shifts the isoelectric points of BγG-immobilized poly(St/AAm/AA) particles to acidic pH, and increases the dispersion stability of BγG-immobilized poly(St/AAm/AA) particles. The flocculation rates of antihuman epidermal growth factor (hEGF) immunoglobulin (IgG)-immobilized poly(St/AAm/AA) fine particles of low AAm content by the antigen–antibody reaction are higher than that of anti-hEGF IgG-adsorbed polystyrene particles, whereas those of high AAm content are lower and decreased with increasing AAm content.

INTRODUCTION

Monodispersed polystyrene (PS) latex particles have been widely used as carriers for medical diagnostic tests such as detection of rheumatoid arthritis factor by an antigen–antibody reaction.[1,2] In these applications, there are two problems: (1) nonspecific flocculation independent of the antigen–antibody reaction in medium of high electrolyte concentration such as biofluid, and (2) detouchment of antigen or antibody during the antigen–antibody reaction.

In a previous article,[3] we reported that styrene/acrylamide/acrylic acid copolymer [poly(St/AAm/AA)] fine particles were colloidally stable and no flocculation occurs even at high electrolyte concentration. On the other hand, Rembaum et al.[4-6] reported that antibodies can be fixed to these particles by chemical bonding through carboxyl groups. These points suggest that poly(St/AAm/AA) particles are useful as the carriers of antigen and antibody.

In this work, from the viewpoint of practical applications, we investigated the characteristics of globulin-immobilized poly(St/AAm/AA) fine polymer particles and the flocculation behavior of the anti-hEGF IgG-immobilized poly(St/AAm/AA) particles by an antigen–antibody reaction. Especially, the effect of the amount of copolymerized acrylamide on colloidal stability of globulin-immobilized particles and the antigen–antibody reaction was considered.

EXPERIMENTAL

Materials

Styrene and acrylic acid (Wako Pure Chemical Industries) were distilled under reduced pressure in a N_2 atmosphere. Potassium persulfate and acrylamide was recrystallized from water and benzene, respectively. Octamethylenediamine (OMD) from Tokyo Kasei and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) from Wako were used without further purification. Bovine γ-globulin (BγG; Sigma Chemical Co., COHN FRACTION II) was used without further purification. Human epidermal growth factor (EGF) and anti-hEGF IgG were obtained from Wakunaga

* To whom correspondence should be addressed.

Journal of Applied Polymer Science, Vol. 51, 1277–1282 (1994)
 CCC 0021-8995/94/071277-06

Table I Preparation of Poly(St/AAm/AA) Fine Particles

	Poly-($St/AAm_0/AA$)	Poly-($St/AAm_2/AA$)	Poly-($St/AAm_{10}/AA$)	Poly-($St/AAm_{20}/AA$)	Poly-($St/AAm_{40}/AA$)	PS
Styrene (mol dm^{-3})	1	1	1	1	1	0.8
Acrylamide (mol dm^{-3})	—	0.02	0.1	0.2	0.4	—
Acrylic acid (mol dm^{-3})	0.02	0.02	0.02	0.02	0.02	—
Particle size (av, nm)	466	440	457	437	257	426

Initiator: $K_2S_2O_8$ 3×10^{-3} mol dm^{-3}. Polymerization temp: 70°C. Polymerization time: 8 h.

Pharmaceutical Co. Sodium chloride (NaCl), sodium hydroxide (NaOH), and hydrochloric acid (HCl) were all analytical grade. Distilled and deionized water was used throughout the experiments.

Preparation of Poly(St/AAm/AA) Fine Particles

Poly(St/AAm/AA) fine particles were prepared by conventional emulsifier-free emulsion polymerization using potassium persulfate as the initiator. The recipes for the preparation of the fine polymer particles are listed in Table I. Particle size was determined by electronmicroscopy. The particles obtained were centrifuged and the sedimented particles were redispersed in water by the use of ultrasonics. After this procedure was repeated three times, the fine polymer particles were dialyzed using a well-boiled Visking dialysis tube for 1 week. Further purification of the resulting particles was conducted by the ion-exchange method using Diaion PK-212-sulfonic acid and PA312-trimethylammonim (Mitsubishi Chemical Industries) as cationic and anionic ion-exchange resins, respectively.

Preparation of OMD-fixed Poly(St/AAm/AA) Particles

The introduction of OMD onto the surface of poly(St/AAm/AA) particles was carried out by a carbodiimide reaction. EDC was used as carbodiimide. EDC (2.5×10^{-3} mol) was added to 10 g of poly(St/AAm/AA) fine particles dispersed in 250 mL of water under stirring at 25°C. After 10 min, 2.5×10^{-3} mol OMD was added and the reaction was allowed to continue for 2 h. The dispersions of OMD-fixed particles [poly(St/AAm/AA)-OMD] were centrifuged to remove unreacted OMD and EDC. Then, poly(St/AAm/AA)-OMD fine particles were dialyzed for over 1 week using a Visking tube. Furthermore, the dispersions were purified by ion exchange.

Immobilization of Globulin onto OMD-fixed Poly(St/AAm/AA) Particles

The immobilization of globulin was carried out through OMD by a carbodiimide reaction as schematically shown in Figure 1. EDC (0.02 g) was added to 1 g of poly(St/AAm/AA)-OMD fine particles dispersed in 250 mL of water while stirring at 25°C. After 1 h, 170 mL of 0.5 g dm^{-3} BγG of NaCl (0.5 *M*) solution was added. The globulin-immobilized poly(St/AAm/AA)-OMD fine particles were purified by ion exchange.

Methods

Electrophoretic Mobility

Electrophoretic mobility of fine polymer particles was measured with a Rank Brothers Mark II microelectrophoresis apparatus as a function of pH. pH was varied with HCl and NaOH solutions and ionic strength was adjusted with NaCl solution.

Flocculation

The flocculation rates of poly(St/AAm/AA), poly(St/AAm/AA)-OMD, and globulin-immobilized particles were measured by the rapid mixing and stopped flow method, using a JASCO UVIDEC 610 spectrophotometer with an SFC-333 flow cell device (light path length 10 mm) connected to a Unican MX-7 sample mixing device. Equal volumes of dispersions of fine particles and electrolyte solutions or hEGF solution for the antigen–antibody reaction were taken into the two syringes and then

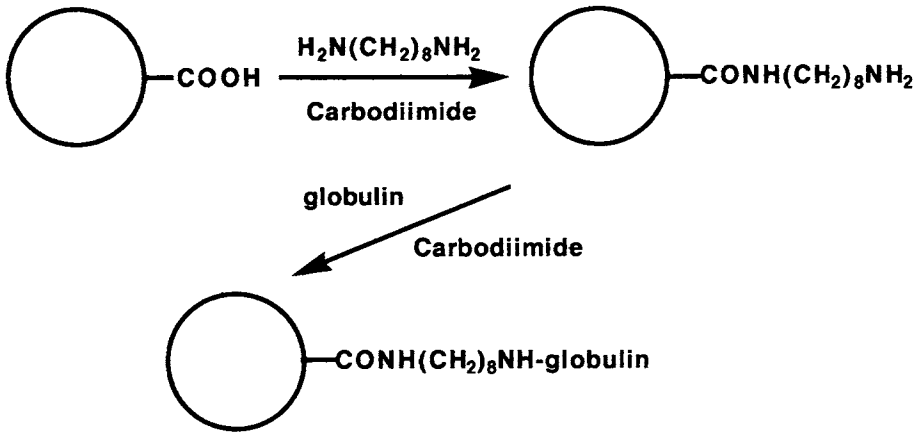

Figure 1 Preparation of globulin-immobilized poly(St/AAm/AA) fine particles.

rapidly mixed by depressing the plunger. The change of turbidity ($\Delta\tau$) of the mixture with time was recorded on an attached recorder. The rate constant (k_{11}) of flocculation between singlet particles was calculated from the initial slope of $\Delta\tau$ vs. time according to Lichtenbelt et al.[7,8]

Amount of Globulin Immobilized

The amounts of BγG immobilized on poly(St/AAm/AA)–OMD fine particles were determined by measuring the difference in soluble BγG concentration before and after immobilization. BγG concentration was determined by the Lowry method.[9]

RESULTS AND DISCUSSION

Poly(St/AAm/AA) and OMD-fixed Poly(St/AAm/AA) Fine Particles

In regard to properties of poly(St/AAm/AA) fine particles prepared without an emulsifier and using potassium persulfate as the initiator, we reported in the earlier article that the colloidal stabilities for NaCl concentration were remarkably enhanced by increasing the amount of acrylamide copolymerized[3] and suggested that this stabilization was due to a water-soluble polymer layer surrounding the particles.[3,10] In this work, similarly, the dispersion stability of poly(St/AAm/AA) particles increased with increasing AAm charged to the polymerization system, while styrene/acrylic acid copolymer, viz, poly($St/AAm_0/AA$), particles started to flocculate at 10^{-1} mol dm^{-3} NaCl solution as shown in Figure 2. In the case of poly($St/AAm_{20}/AA$) and poly($St/AAm_{40}/AA$) particles, no flocculation was observed even at 2 mol dm^{-3} NaCl solution.

Figure 3 shows the flocculation rate constants (k_{11}) and the electrophoretic mobilities of OMD-fixed poly(St/AAm/AA) fine particles as a function of pH. The isoelectric point of the particles shifts to acidic pH with increase in acrylamide concentration in the polymerization system. Maximum values for the flocculation rate constant k_{11} also shift to acidic pH. These results indicate that OMD-fixed poly(St/AAm/AA) fine particles are colloidally unstable near their isoelectric points. The small difference between the isoelectric points and the pH for the maximum flocculation rates were explained by expansion and shrinkage of OMD-fixed polymer chains.[11]

Immobilization of BγG

Figure 4 shows the immobilization isotherms of BγG onto poly(St/AAm/AA)–OMD fine particles and

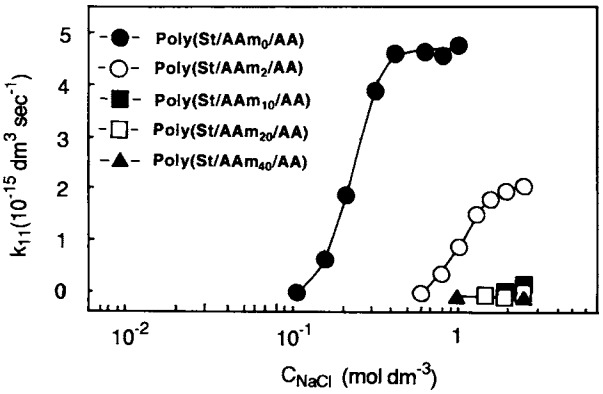

Figure 2 Rate constant for the flocculations of poly(St/AAm/AA) fine particles as a function of NaCl concentration at pH 5.9.

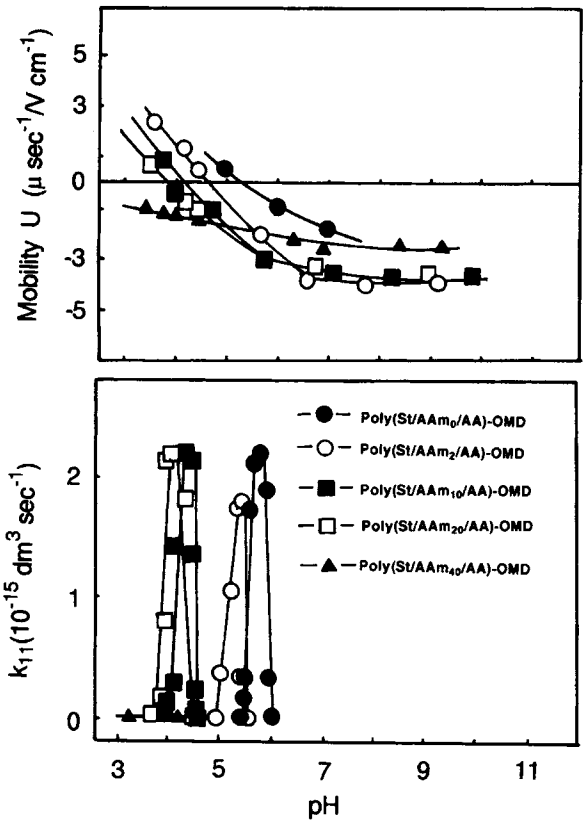

Figure 3 Electrophoretic mobility and flocculation rate constant of poly(St/AAm/AA)–OMD fine particles as a function of pH at ionic strength 10^{-3}.

polystyrene particles. BγG was immobilized onto polystyrene by adsorption. The amounts of BγG immobilized decreased with increasing the amount of AAm copolymerized. It has been reported that the amount of bovine serum albumin (BSA) adsorbed on styrene/acrylamide copolymer fine particles decreases with increasing content of AAm copolymerized, and this lowering of adsorption affinity of BSA was attributed to the effect of steric repulsion due to a water-soluble polymer layer surrounding the particles.[12] On the other hand, it was found that the copolymerization of acrylic acid increased the amounts of BSA adsorbed, and this suggested that hydrogen bonding between carboxyl groups and BSA molecules played an important role in the adsorption.[13] In the case of poly(St/AAm/AA)–OMD fine particles, the effect of carboxyl groups on the immobilization of BγG due to hydrogen bonding is low. The fixation of OMD may decrease the number of effective carboxyl groups for formation of hydrogen bonding with globulin.

Electrophoretic Mobility of BγG-immobilized Fine Particles

Figure 5 shows the electrophoretic mobilities of BγG-immobilized poly(St/AAm/AA)–OMD fine particles as a function of pH. The electrophoretic mobilities of the particles of a low content of AAm indicated the isoelectric points to be at acidic pH, while those of the particles of high content of AAm are negative at all pH. This behavior is similar to that of poly(St/AAm/AA)–OMD fine particles. The electrostatic properties of BγG-immobilized fine particles are dependent on those of original poly(St/AAm/AA) particles.

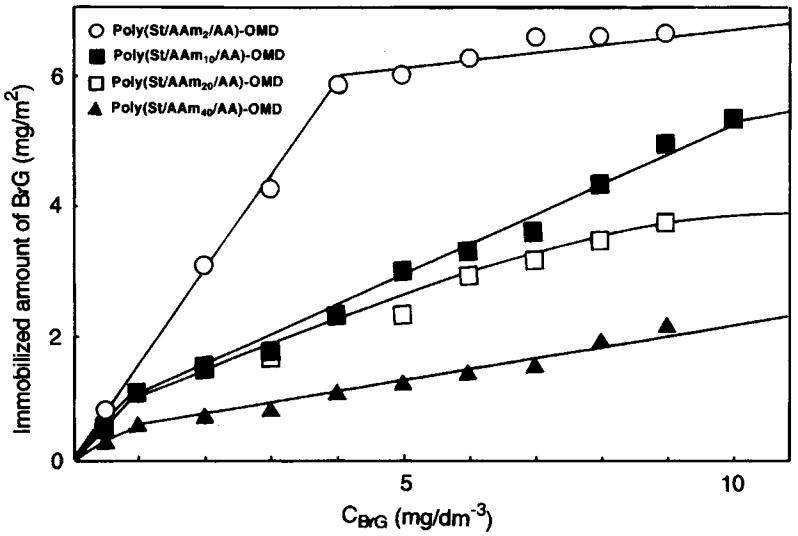

Figure 4 Amount of immobilized BγG onto poly(St/AAm/AA)–OMD fine particles at pH 7.3.

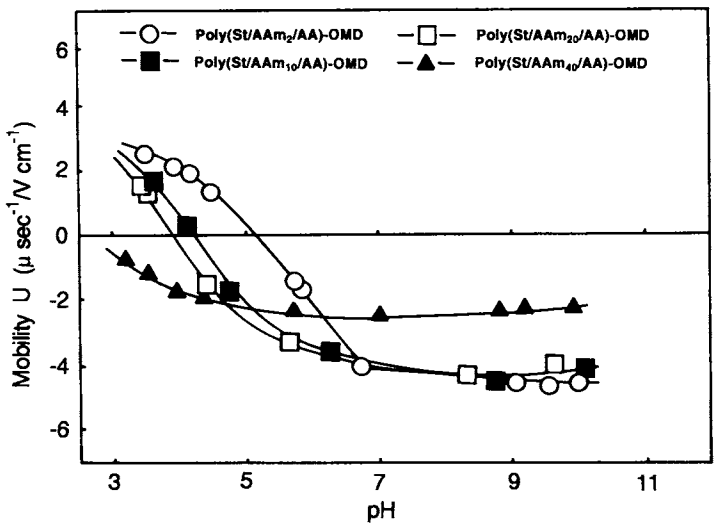

Figure 5 Electrophoretic mobility of BγG-immobilized poly(St/AAm/AA) fine particles as a function of pH at ionic strength 10^{-3}.

Colloidal Stability of BγG-immobilized Fine Particles

The flocculation rate constants of BγG-immobilized poly(St/AAm/AA) fine particles as a function of NaCl concentration are shown in Figure 6. As with the styrene/acrylamide copolymer fine particles reported in the earlier article,[10] the minimum NaCl concentration, at which the flocculation of BγG-immobilized particles take place, increases with increasing AAm content. The flocculations of BγG-immobilized poly(St/AAm_{10}/AA) and poly(St/AAm_{20}/AA) occur at a NaCl concentration of more than 1 *M*. The high dispersion stability of BγG-immobilized poly(St/AAm/AA)-OMD fine particles seems to be due to strong steric repulsion of the water-soluble polymer layer derived from acrylamide

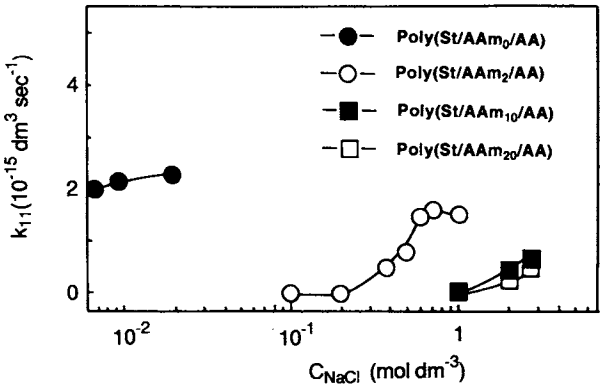

Figure 6 Rate constant for the flocculation of BγG-immobilized poly(St/AAm/AA) fine particles as a function of NaCl concentration at pH 5.9.

in spite of the fact that OMD and BγG are immobilized on the surface of the particles. These results suggest that BγG-immobilized poly(St/AAm/AA)-OMD fine particles are colloidally stable in biofluid.

Flocculation of Anti-hEGF IgG-immobilized Poly(St/AAm/AA)-OMD Particles by the Antigen-Antibody Reaction

Like BγG, anti-hEGF IgG-immobilized poly(St/AAm/AA)-OMD fine particles were prepared with the carbodiimide reaction. The flocculations of various anti-hEGF IgG-immobilized particles after rapid mixing with hEGF solution were measured. The ratio of hEGF to anti-hEGF IgG was 10 : 1. Figure 7 shows the changes of turbidity of the mixtures with time. The changes of turbidity are due to the flocculation caused by a specific antigen–antibody reaction, because the anti-hEGF IgG-immobilized poly(St/AAm/AA)-OMD particles are colloidally very stable, as expected from the stability of BγG-immobilized particles shown in Figure 6. As shown in Figure 7, the flocculation rate of anti-hEGF-immobilized poly(St/AAm_2/AA)-OMD particles is higher than that of anti-hEGF IgG-adsorbed polystyrene particles. This indicates that anti-hEGF IgG-immobilized poly(St/AAm2/AA)-OMD particles effectively react with EGF. Bonding groups of globulin molecules to fixed OMD by the carbodiimide reaction, namely, carboxyl groups, are present in the crystalline part (Fc) of globulin. Accordingly, most of the antigen binding part of IgG protrude to the bulk side, and the antigen–antibody reaction seems to occur effectively. On the other

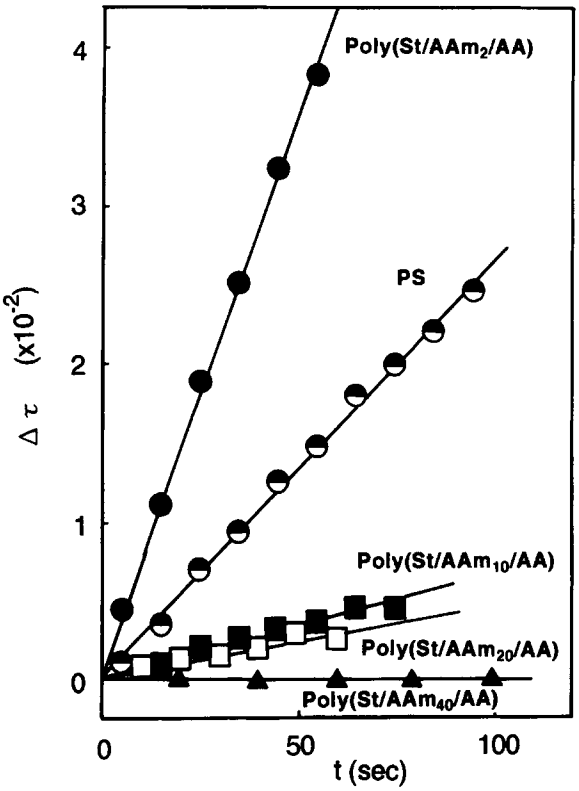

Figure 7 Changes of the turbidity of anti-hEGF IgG-immobilized poly(St/AAm/AA) fine particles by the antigen–antibody reaction.

hand, anti-hEGF IgG-immobilized poly(St/AAm_{10}/AA)-OMD and poly(St/AAm_{20}/AA)-OMD slowly flocculate after the mixing of the EGF solution. The high steric stabilization effect due to the bulky water-soluble polymer layer surrounding poly(St/$AAm_{10,20,or40}$/AA) fine particles is supposed to interfere with the antigen–antibody reaction. It is suggested that the incorporation of a small amount of hydrophilic monomer such as acrylamide into particles is a significant aid for preparation of fine polymer particles as antibody carriers.

REFERENCES

1. J. M. Singer, *Am. J. Med.*, **31,** 766 (1961).
2. C. L. Christian, R. Mendez-Bryan, and D. L. Larson, *Proc. Soc. Exp. Biol. Med.*, **98,** 820 (1958).
3. H. Tamai, M. Hasegawa, and T. Suzawa, *J. Appl. Polym. Sci.*, **38,** 403 (1989).
4. A. Rembaum and S. Margel, *Br. Polym. J.*, **10,** 275 (1978).
5. A. Rembaum, S. P. S. Yen, E. Cheong, S. Wallace, R. S. Molday, I. L. Gordon, and W. J. Dreiyer, *Macromolecules*, **9,** 328 (1976).
6. R. S. Molday, W. J. Dreyer, A. Rembaum, and S. P. S. Yen, *J. Cell. Biol.*, **64,** 75 (1975).
7. J. W. Th. Lichtenbelt, H. J. M. C. Ras, and P. H. Wiersema, *J. Colloid Interface Sci.*, **46,** 522 (1974).
8. H. Tamai, A. Fujii, and T. Suzawa, *J. Colloid Interface Sci.*, **118,** 176 (1987).
9. O. H. Lowry, N. J. Rosebrough, A. C. Farr, and R. J. Randall, *J. Biol. Chem.*, **193,** 265 (1951).
10. H. Tamai, T. Murakami, and T. Suzawa, *J. Appl. Polym.*, **30,** 3857 (1985).
11. H. Tamai, H. Kiyota, and T. Suzawa, *J. Appl. Polym. Sci.*, **45,** 85 (1992).
12. H. Shirahama and T. Suzawa, *J. Colloid Interface Sci.*, **104,** 416 (1985).
13. H. Shirahama and T. Suzawa, *Colloid Polym. Sci.*, **263,** 141 (1985).

Received June 9, 1993
Accepted June 10, 1993