

Active functional copolymeric microspheres based on 1-methacryloxybenzotriazole and *N*-acryloxysuccinimide

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Two types of copolymeric microspheres containing active functional groups were synthesized by radiation-induced dispersion copolymerization of 1-methacryloxybenzotriazole (MABt) and *N*-acryloxysuccinimide (ASu), in ethyl propionate. The formation of spherical particles is characterized by limited concentration and composition ranges, e.g. in the 60–40 wt% MABt composition region at 5 w/v% concentration, in the 60–10 wt% MABt composition region at 10 w/v% concentration, and in the 100–90 wt% MABt composition region at 20 w/v% concentration, respectively. The average diameter of particles was found to be approximately 1.6, 0.7 and 2.5 μm , in the above order of concentration. The reactivity of these active functional groups in the copolymer was evaluated from the amounts of 1-hydroxybenzotriazole (HOBt) and *N*-hydroxysuccinimide (HOSu) liberated by reaction with ethanolamine at 0°C for 40 min (pH 7.5). The maximal amount of liberated HOBt (290 mg g^{-1}) was observed in the copolymer system with a monomer composition of 50/50 wt% MABt/ASu, in contrast to homopoly(ASu) for the maximal amount of liberated HOSu (120 mg g^{-1}). This finding means that the optimal reactivity of active benzotriazole groups can be enhanced by selecting a suitable composition of MABt and ASu.

(Keywords: random copolymeric microspheres; active functional groups; 1-methacryloxybenzotriazole; *N*-acryloxysuccinimide; radiation-induced dispersion polymerization; coupling reaction)

INTRODUCTION

The immobilization of biologically active components onto polymeric microspheres containing various functional groups has become an important tool in the field of affinity chromatography for separation of biomolecules. For this purpose, poly(acrolein) microspheres with active aldehyde groups have been synthesized by means of radiation-induced suspension polymerization^{1–4} or catalyst-induced suspension polymerization⁵, and have led to the development of interesting applications with particles, such as cell labelling, cell separation and cell marking. Hydroxyl-group-containing polymeric microspheres in the size range 0.3–3 μm have been successfully prepared by emulsion polymerization of 2-hydroxyethyl methacrylate^{6–8}, for use as a visual marker for immunodiagnosis. Marumoto *et al.*⁹ synthesized polymeric microspheres with both hydroxyl and carboxyl groups in the range 0.075–1 μm , using initiator (ammonium persulphate), emulsifier (sodium dodecylbenzenesulphonate) and polymerizable monomers (methyl methacrylate, methacrylic acid and 2-hydroxyethyl methacrylate). They reported the capability in the application of particles reacted with human immunoglobulin G by carbodiimide or cyanogen bromide⁹. On the other hand, Hosaka *et al.*^{10,11} reported the preparation of polymeric microspheres in the range 0.2–4 μm by means of dispersion polymerization of glycidyl methacrylate (epoxy group), 2-hydroxyethyl methacrylate (hydroxyl group) and triethylene glycol dimethacrylate (crosslinking monomer) in the presence of organic solvent by employing 2,2-

azobis(2,4-dimethyl-4-methoxyvaleronitrile) as initiator without emulsifier. The resulting polymerization gave particles with narrow size distributions which can be used to label or mark biomolecules.

However, it is well known that, in a catalyst-induced polymerization, catalytic residues (impurities) as the terminal groups of polymers are undesirable for biomedical applications, and also that functional groups such as OH and COOH cannot be directly reacted with biomolecules, because this needs a suitable coupling agent, which inactivates biomolecules. To improve these problems, studies on the synthesis, polymerization and reactivity of active functional-group-containing vinyl monomers such as 1-methacryloxybenzotriazole (MABt), *N*-acryloxysuccinimide (ASu) and 1-methacryloylimidazole, which react directly with biomolecules under mild conditions, have been performed by many workers^{12–16}.

Recently, we have prepared polymeric microspheres in the range 0.1–20 μm by radiation-induced dispersion polymerization in the presence of a suitable organic solvent without initiator and emulsifier, in which the polymeric particles precipitate from monomer solution during irradiation^{17–19}. In this case, ASu does not form spheres in dispersion homopolymerization because of the formation of a polymeric mass, but particles can be obtained by copolymerizing with diethylene glycol dimethacrylate in very limited monomer composition ranges. In the dispersion polymerization of MABt, homopolymeric microspheres were formed. This paper describes the formation mechanism of spherical copoly-(MABt/ASu) particles by means of radiation-induced copolymerization in ethyl propionate. In addition, the difference in reactivity between active benzotriazole

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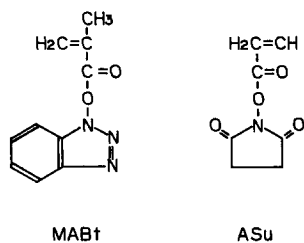


Figure 1 Structural formulae of MABt and ASu

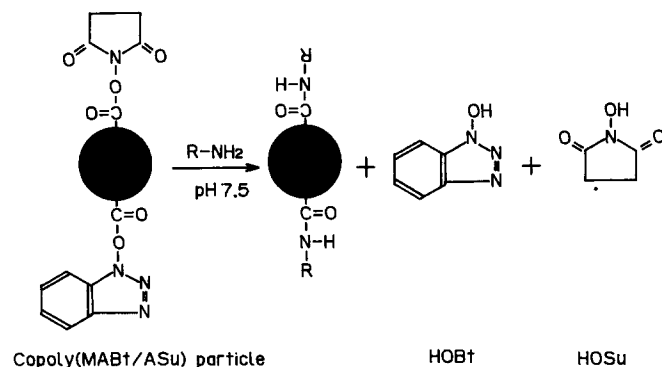


Figure 2 Model scheme for coupling reaction of ethanolamine (R-NH₂; R=CH₂CH₂OH) onto copoly(MABt/ASu) particles

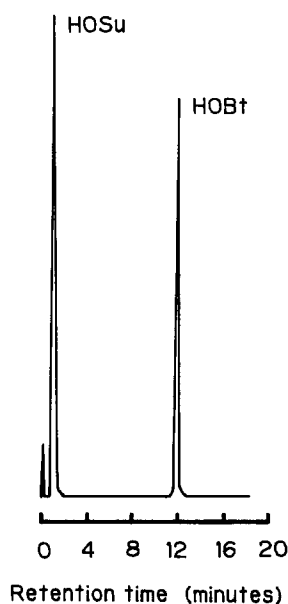


Figure 3 H.p.l.c. separation profile of HOBt and HOSu

group (Bt group) and active succinimide group (Su group) will be discussed.

EXPERIMENTAL

Materials

1-Methacryloxybenzotriazole (MABt) [$M_w=203.2$, m.p.=36–37°C, $R_f=0.80$ (chloroform/methanol/acetic acid, 95/5/3) or $R_f=0.55$ (chloroform)] and *N*-acryloxysuccinimide (ASu) [$M_w=169.2$, m.p.=62–63°C, $R_f=0.48$ (diethyl ether) or $R_f=0.65$ (n-butyl acetate)], with structural formulae as shown in Figure 1, were purchased from Kokusan Chemical Works Ltd, and recrystallized when necessary. A commercially available ethyl propionate, obtained from Tokyo Kasei Kogyo Co. Ltd,

was purified by distillation. An analytical-grade ethanolamine was obtained from Wako Pure Chemical Industries Ltd, and used without further purification. 1-Hydroxybenzotriazole (HOBt) and *N*-hydroxysuccinimide (HOSu) were reagent-grade.

Preparation of copoly(MABt/ASu) particles

The MABt/ASu monomers of desired composition were dissolved in ethyl propionate. This monomer solution was transferred into a glass vessel. After nitrogen gas was sufficiently bubbled through, the vessel was sealed. The irradiation for polymerization was performed without stirring at 25°C at a dose rate of 10 kGy h⁻¹ using γ -rays from a ⁶⁰Co source. The homogeneous monomer solution changes into an unstable emulsion during irradiation, forming polymeric particles. The deposited polymers were washed with fresh ethyl propionate and then centrifuged at 10 000 rpm. This operation was repeated several times. The polymer yield (conversion) was estimated from the ratios of weight

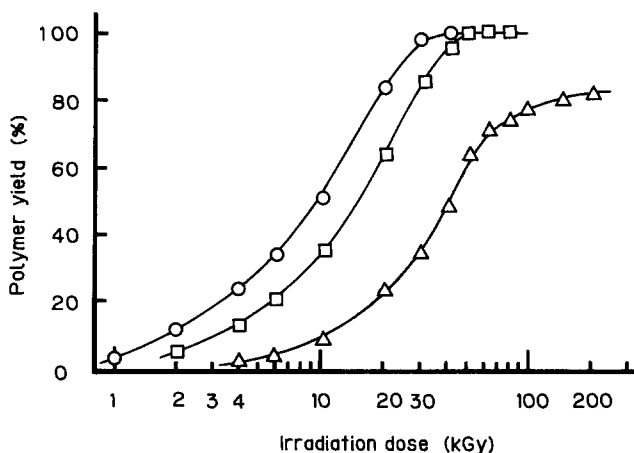


Figure 4 Effect of irradiation dose on the radiation-induced dispersion copolymerization of MABt and ASu, in ethyl propionate, as a function of monomer composition. A 10 w/v% monomer solution was irradiated at a dose rate of 10 kGy h⁻¹. Monomer composition of MABt/ASu: (Δ) 100/0 wt%; (\square) 50/50 wt%; (\circ) 0/100 wt%

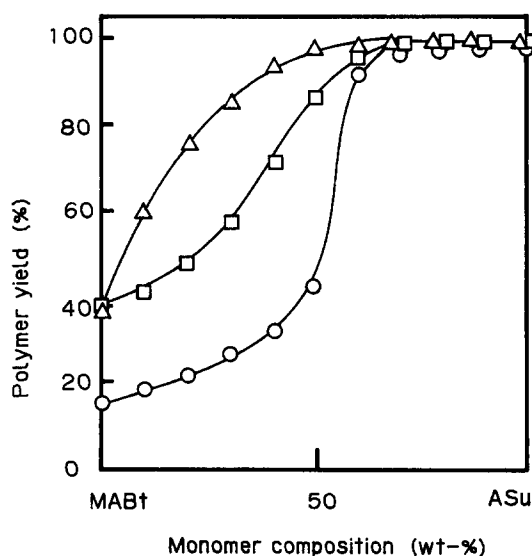


Figure 5 Effect of monomer composition on the radiation-induced dispersion copolymerization of MABt and ASu, in ethyl propionate, as a function of monomer concentration. The monomer solution was irradiated up to 30 kGy. Monomer concentration: (\circ) 5 w/v%; (\square) 10 w/v%; (Δ) 20 w/v%

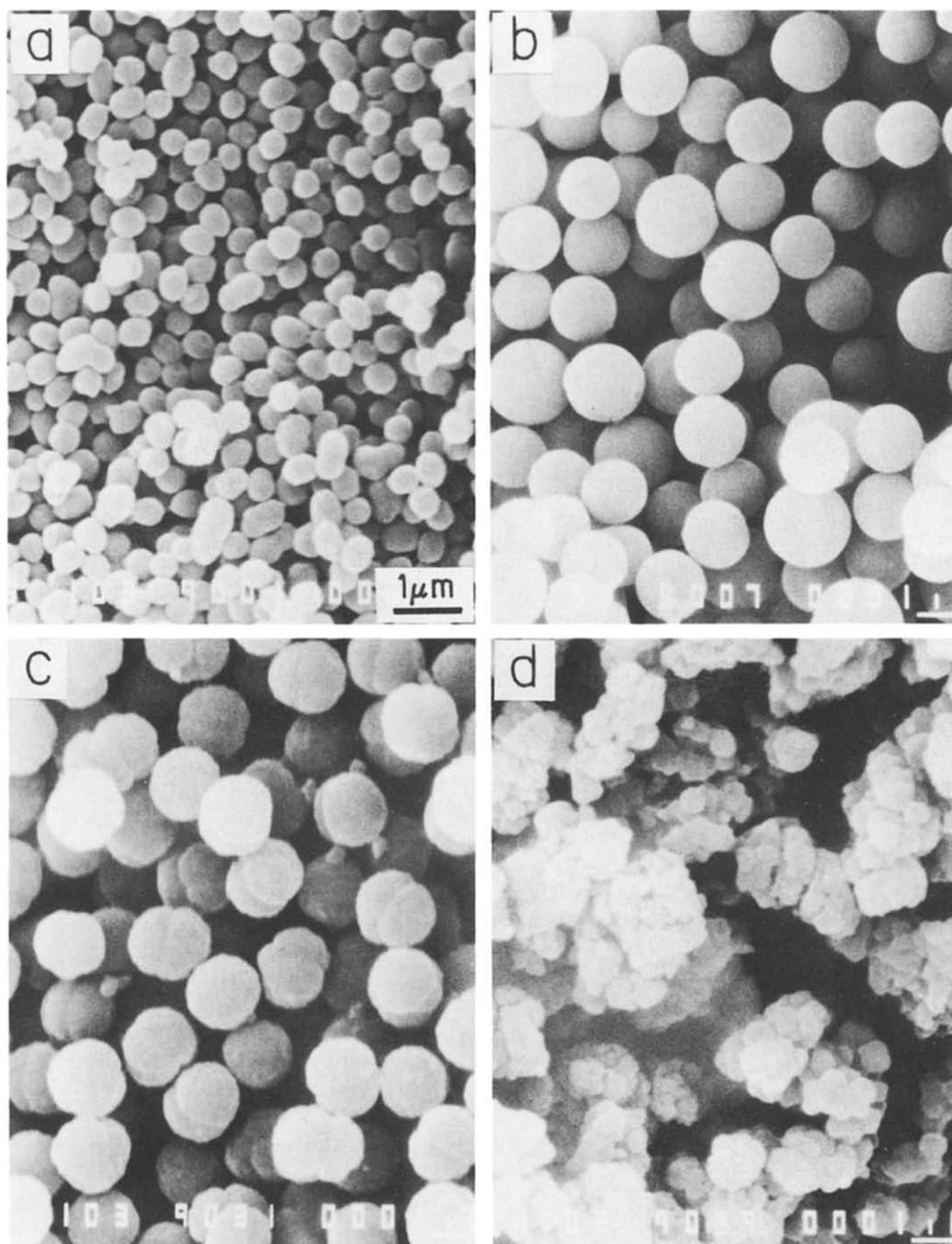


Figure 6 SEM photographs of copoly(MABt/ASu) particles obtained at 5 w/v% monomer concentration by 30 kGy irradiation as a function of monomer composition. Monomer composition of MABt/ASu: (a) 100/0 wt%; (b) 50/50 wt%; (c) 30/70 wt%; (d) 0/100 wt%

of ethyl propionate-insoluble polymer fraction against weight of initial monomer. This is because an ethyl propionate-soluble polymer fraction corresponding to the oligomers is produced during irradiation at the same time. Such oligomers readily precipitate on addition of a poor solvent, methanol, in which the precipitates do not form spheres, giving a polymeric mass.

Reactivity of copoly(MABt/ASu) particles

The model scheme for the coupling reaction of ethanolamine onto copoly(MABt/ASu) particles is shown in *Figure 2*. One hundred milligrams of the particles were added to 20 ml of 0.1 M phosphate buffer solution (pH 7.5) containing 400 mg of ethanolamine. The coupling reaction was carried out at 0°C with stirring. The particles

and solution were separated by centrifugation after reacting for the required period. The amounts of HOBt and HOSu liberated in the solution from the particles during the reaction were measured with a Shimadzu LC-6A high-performance liquid chromatograph under the following conditions: TSK-GEL OPS-80TM (4.6 mm inner diameter and 15 cm long) column, a mixture of 100 mM sodium chloride and 5 mM phosphoric acid as mobile phase, 0.8 ml min⁻¹ flow rate, 40°C column temperature and 280 nm wavelength for the detector. Under these conditions, the HOBt and HOSu peaks can be separated as seen clearly in *Figure 3*.

Microscopic observation

The particle diameter and size distribution of the

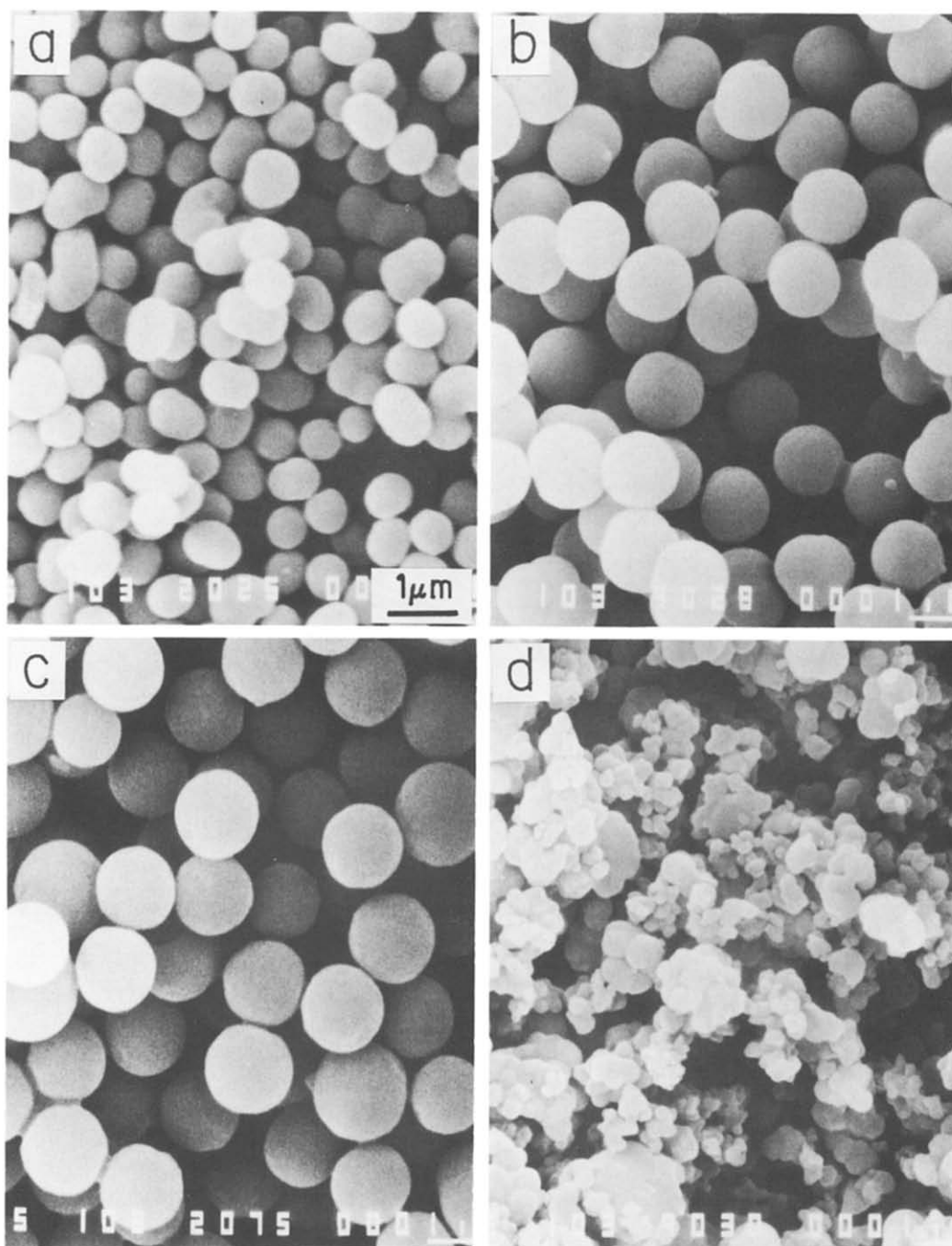


Figure 7 SEM photographs of copoly(MABt/ASu) particles obtained at 10 wt% monomer concentration by 30 kGy irradiation as a function of monomer composition. Symbols in figure refer to the MABt/ASu composition given in Figure 6

particles were measured with a Coulter TA-11 counter. The shape and surface structure of the particles were observed with a Jeol JXA-733 scanning electron microscope.

RESULTS AND DISCUSSION

Radiation-induced dispersion copolymerizability of MABt and ASu in ethyl propionate

It is well known that ASu and MABt easily polymerize not only in the solid state but also in the liquid state, using radiation or radical initiators, resulting in a radical reaction related to the carbon-carbon double bonds^{13,20,21}. The effect of irradiation dose on the radiation-induced dispersion copolymerization of MABt

and ASu at 10 w/v% monomer concentration is shown in Figure 4 as a function of monomer composition. The polymer yield in the homopolymerization of ASu reaches 100% by 30 kGy irradiation, in contrast to only 33% in MABt. It was confirmed that the dispersion homopolymerization of MABt is remarkably dependent on its monomer concentration, e.g. the polymer yield of the ethyl propionate-insoluble polymer fraction obtained by 30 kGy irradiation at 25°C was 18% at 5 w/v% concentration, 38% at 10 w/v% concentration, 36% at 20 w/v% concentration, 18% at 30 w/v% concentration and 0% at 40 w/v% concentration, respectively, in which the yield has a maximal value at an MABt concentration near 10 w/v%¹⁹. The decrease in the yield of poly(MABt) at concentrations of 10 w/v% or above is closely related to

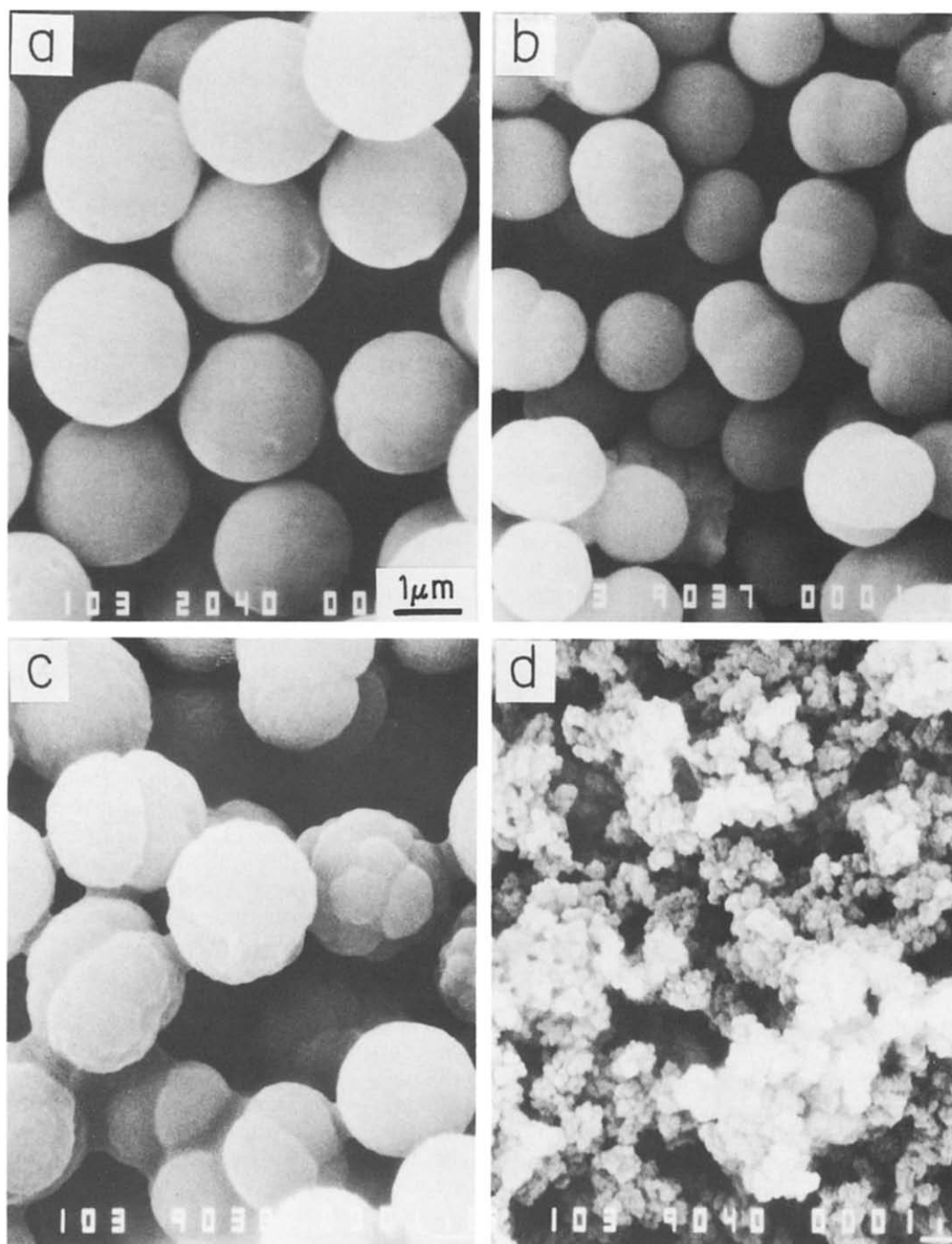


Figure 8 SEM photographs of copoly(MABt/ASu) particles obtained at 20 w/v% monomer concentration by 30 kGy irradiation as a function of monomer composition. Symbols in figure refer to the MABt/ASu composition given in Figure 6

the formation of lower-molecular-weight polymers that are soluble in the solution. On the contrary, in the dispersion polymerization of ASu, the reaction is not affected by changing the monomer concentration, and there is a constant yield of more than 90%²². These results mean that MABt and ASu have different concentration dependences for their polymerizations. As seen clearly in Figure 4, in the copolymerization system, the rate of polymerization of MABt is markedly accelerated by the introduction of ASu. The effect of monomer concentration on the copolymerizability of MABt and ASu is shown in Figure 5 as a function of monomer composition, in which the copolymerizability is changed in the region of 40 wt% MABt composition. That is, in the 0–40 wt% MABt composition region, the rate of

polymerization is apparently not influenced by either concentration or composition, giving 100% polymer yield. In contrast, the rate is strongly dependent on these two factors in the 50–100 wt% MABt composition region, in which it showed a marked increase with increasing monomer concentration and ASu composition in the monomer. However, it should be noted here that, in the 50–100 wt% MABt composition region, the oligomers that are soluble in the solution are formed as products. Such oligomers were precipitated by the addition of a poor solvent, methanol. In this case, the amount of precipitated product increases with decrease of monomer concentration, e.g. at 20 wt% MABt composition, their amounts correspond to approximately 21% at 5 w/v% concentration, 11% at 10 w/v% concentration

Table 1 Formation and particle diameter of copoly(MABt/ASu) microspheres obtained by irradiating up to 30 kGy at monomer concentrations of 5, 10 and 20 w/v%, in ethyl propionate

No.	Monomer composition (wt%)		Property of copoly(MABt/ASu) particle ^a		
	MABt	ASu	5 w/v%	10 w/v%	20 w/v%
2	100	0	DS	DS	IS (2.67 ± 0.58 μm)
2	90	10	DS	DS	IS (2.24 ± 0.72 μm)
3	80	20	DS	DS	AS
4	70	30	DS	DS	AS
5	60	40	IS (1.57 ± 0.87 μm)	IS (0.70 ± 0.37 μm)	AS
6	50	50	IS (1.62 ± 1.07 μm)	IS (0.67 ± 0.34 μm)	AS
7	40	60	IS (1.59 ± 0.98 μm)	IS (0.68 ± 0.38 μm)	AS
8	30	70	AS	IS (0.68 ± 0.38 μm)	AS
9	20	80	AS	IS (0.67 ± 0.33 μm)	AS
10	10	90	AS	IS (0.71 ± 0.34 μm)	AS
11	0	100	PM	PM	PM

^a DS, deformed spheres; IS, isolated spheres; AS, aggregated spheres; PM, polymeric mass

and 4% at 20 w/v% concentration, respectively. This means that low-molecular-weight polymers are liable to be produced abundantly at relatively low monomer concentrations.

Formation of copoly(MABt/ASu) particles

In the radiation-induced dispersion homopolymerization of MABt, in ethyl propionate, the growth of poly(MABt) particles formed by precipitation from monomer solution during irradiation can be divided into three groups: formation of deformed particles at MABt concentrations of 15 w/v% or below; formation of spherical particles in solutions ranging in MABt concentration from 18 to 35 w/v% and formation of ethyl propionate-soluble polymers at MABt concentrations of 40 w/v% or above, at which the products precipitate by the addition of a poor solvent, methanol, giving a polymeric mass¹⁹. In this case, at MABt concentrations below 15 w/v%, only deformed particles of small size are produced in the initial stage of the polymerization reaction, and the size of the particles remains unchanged during the reaction because there is no particle growth (Figures 6a and 7a). On the contrary, in the 18–35 w/v% MABt concentration region, the spherical particles of small size, produced in the initial stage, grow with reaction time, giving large spherical particles due to a multi-coating effect, which is closely related to newly produced polymer. On the other hand, in an ASu homopolymerization system, the formation of spherical particles did not occur, and the products gave a polymeric mass (Figures 6a, 7a and 8a).

The aim of this report is to prepare copolymeric microspheres by selecting a suitable composition of MABt and ASu, by means of radiation-induced dispersion copolymerization in ethyl propionate. For this purpose, the polymerization reactions were performed at various monomer concentrations of 5, 10 and 20 w/v%, as a function of monomer composition. The copolymers of MABt and ASu were precipitated from all monomer solutions during irradiation. The shape of the particles was observed by SEM, and the SEM photographs are shown in Figures 6, 7 and 8. The shapes of the particles are classified into four groups: deformed spheres (DS), isolated spheres (IS), aggregated spheres (AS) and polymeric mass (PM), as listed clearly in Table 1. Completely sphered particles are formed over a

very wide range of compositions by copolymerizing rather than homopolymerizing, but they are strongly dependent on the concentration, e.g. in the 60–40 wt% MABt composition region at 5 w/v% concentration, in the 60–10 wt% MABt composition region at 10 w/v% concentration, and in the 100–90 wt% MABt composition region at 20 w/v% concentration, respectively. This finding means that the morphology of homopoly(MABt) particles and polymerizability play an important role in order to obtain spherical copoly(MABt/ASu) particles over a wide range of compositions. However, at present, it cannot be sufficiently explained why these polymerization reactions gave the products with such spherical particles by copolymerization.

The size distributions of copolymer particles with a composition of 50/50 wt% MABt/ASu, which obtain at monomer concentrations of 5, 10 and 20 w/v%, are shown in Figure 9. At 5 and 10 w/v% concentrations, the shape of the particle consists only of isolated spheres, in contrast to aggregated spheres for the copolymer obtained at 20 w/v% concentration. These polymerizations gave particles with relatively narrow size distributions, in which the diameter of particles was found to be 1.24 ± 0.92, 0.67 ± 0.34 and 2.46 ± 0.56 μm, in the above order of concentration (see Table 1). The result in Table 1 shows that the size of spherical particles obtained at each concentration is not widely changed by monomer composition, e.g. approximately 1.6 μm in the 60–40 wt% MABt composition region at 5 w/v% concentration, 0.7 μm in the 60–10 wt% MABt composition region at 10 w/v% concentration, and 2.5 μm in the 100–90 wt% MABt composition region at 20 w/v% concentration, respectively. Therefore, it was concluded here that spherical copoly(MABt/ASu) particles with two active functional groups can synthesize in the widest range of compositions by copolymerizing at 10 w/v% concentration.

Reactivity of copoly(MABt/ASu) particles

The reactivity of two active functional-group-containing copolymeric microspheres was evaluated by reacting with ethanolamine. The effect of reaction time on the amounts of HOBt and HOSu liberated from copolymer particles with a composition of 50/50 wt% MABt/ASu, which are reacted at 0°C in 0.1 M phosphate buffer solution (pH 7.5) containing ethanolamine, is shown in Figure 10. It

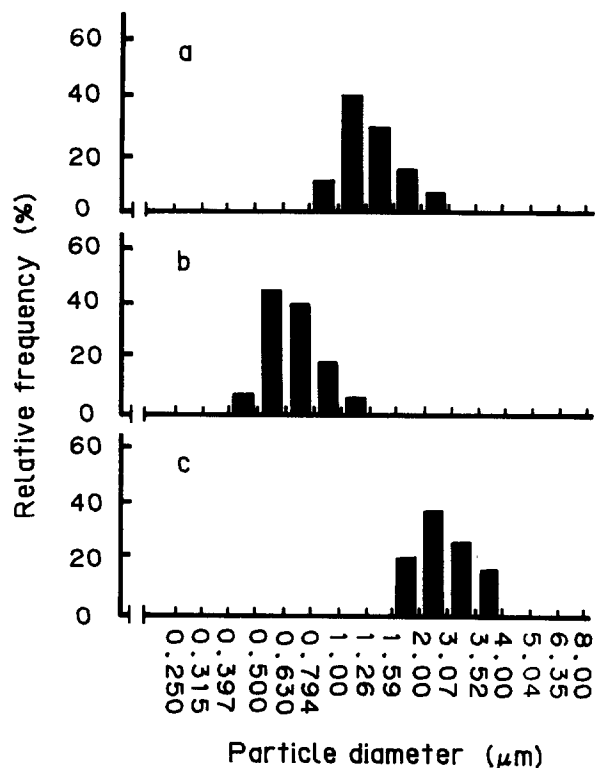


Figure 9 Size distribution of copoly(MABt/ASu) (50/50 wt%) particles obtained in ethyl propionate by 30 kGy irradiation as a function of monomer concentration. Monomer concentration: (a) 5 w/v%; (b) 10 w/v%; (c) 20 w/v%

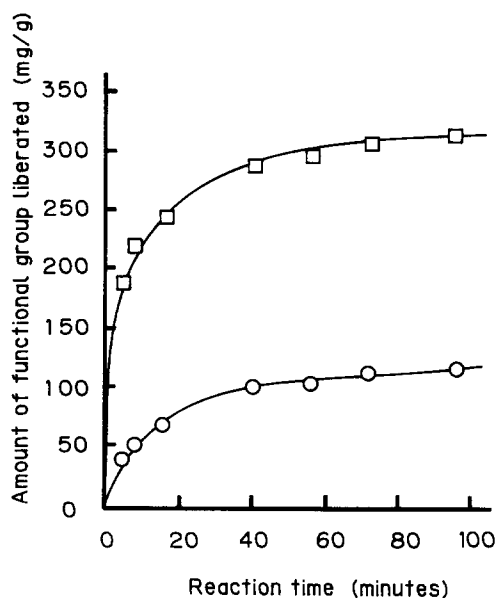


Figure 10 Effect of reaction time on the amounts of HOBT and HOSu liberated from copoly(MABt/ASu) (50/50 wt%) particles, which are reacted with ethanolamine at 0°C in 0.1 M phosphate buffer solution (pH 7.5). The copolymer was obtained by irradiating up to 30 kGy at 10 w/v% monomer concentration. Kind of liberated functional group: (□) HOBT; (○) HOSu

was confirmed that the polymers used in this study are insoluble in ethanolamine solution throughout the experimental period, but such polymers are soluble if they are treated in dimethylformamide at 60°C. The amount of liberated functional groups showed a gradual increase with the passage of time after increasing rapidly during the first 20 min. It was found that the rate of the coupling

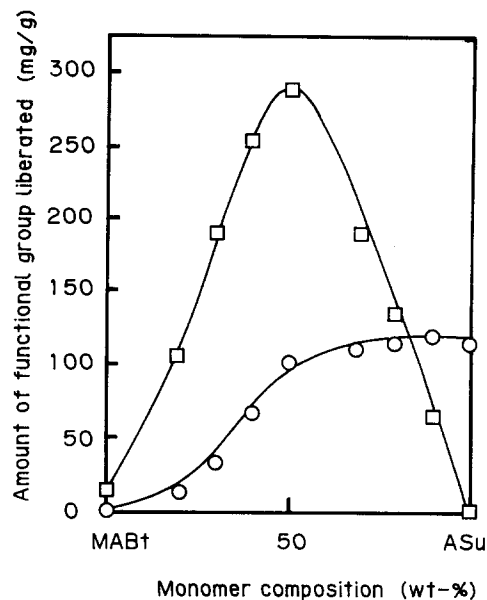


Figure 11 Effect of monomer composition on the amounts of HOBT and HOSu liberated from copoly(MABt/ASu) particles, which are reacted with ethanolamine for 40 min at 0°C in 0.1 M phosphate buffer solution (pH 7.5). The copolymer was obtained by irradiating up to 30 kGy at 10 w/v% monomer concentration. Symbols in figure refer to the kind of liberated functional group given in Figure 10

reaction of active Bt groups in the copolymer is 2.5 times faster than that of active Su groups. The effect of monomer composition on the amounts of HOBT and HOSu liberated from copoly(MABt/ASu), which are reacted at 0°C for 40 min with ethanolamine, is shown in Figure 11. According to this result, especially in the case of each homopolymer system, the amount of HOBT liberated from poly(MABt) was found to be 20 mg g⁻¹ (3% of initial amount of HOBT), in contrast to 120 mg g⁻¹ for the homopoly(ASu) (18% of initial amount of HOSu). The reactivity of homopoly(MABt) was much lower than that of homopoly(ASu). However, the reactivity of active Bt groups is markedly accelerated by the addition of ASu, and the resulting amount of HOBT liberated by reacting with ethanolamine reaches a maximum in the copolymer system with a composition of 50/50 wt% MABt/ASu, while the maximal amount of liberated HOSu was observed in a homopolymer of ASu. From these results, it was concluded that the reactivity of active Bt groups can be greatly enhanced by the copolymerization of MABt and ASu.

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Copolymeric microspheres: M. Yoshida et al.

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