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Monodisperse Microspheres of Copolymers of Glycidyl Methacrylate and its Derivatives as Materials for Biomedical Application*

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Abstract: Monodisperse microspheres of copolymers of glycidyl methacrylate were prepared by dispersion polymerization in organic media. The microsphere diameter could be adjusted in the range from 0·5 μm to 5 μm by changing the monomer concentration, the type of dispersion medium and the content of the comonomers. Terpolymers of glycidyl methacrylate, 2-hydroxyethyl methacrylate and tri(ethylene glycol) dimethacrylate were analysed by thermal decomposition gas chromatography and the compositions of the polymers agreed well with those of the monomer mixtures. The epoxide of the polymer microspheres was hydrolysed to α,β-diol with dilute sulphuric acid without side reactions except the slight formation of sulphate. It was confirmed by the ^{13}C FT-NMR spectrum that the main structure of the hydrolysate was that of poly(glyceryl methacrylate). In the reaction of the epoxide with ammonia, the predominant production of tertiary amine was presumed by the relationship between the conversion of the epoxide and the nitrogen content of the reaction product. The amination of the epoxide with secondary amines resulted in the quantitative formation of the corresponding tertiary amines.

Key words: monodisperse microspheres, polymer microspheres, glycidyl methacrylate, hydroxylation, amination.

INTRODUCTION

The authors have found that monodisperse microspheres of μm size could be prepared by copolymerization of glycidyl methacrylate (abbreviated to GMA hereafter) with other methacrylates added as minor components in an appropriate organic medium without a stabilizer.[1,2] Conventionally, μm-sized monodisperse polymer microspheres have been prepared by methods of seeding polymerization,[3] polymerization under non-gravitational conditions,[4] polymerization in the state of activated swelling[5] and dispersion polymerization.[6] The above mentioned conventional methods except dispersion polymerization require a multistep polymerization process. One of the advantages of the polymerization method of the present report is the capability of producing microspheres larger than 1 μm in one step. Conventional dispersion polymerization can also give μm-sized monodisperse microspheres, but the stabilizer would remain in the product as a contaminant. The chemical reactivity of the epoxy group in the polymer microspheres is another advantage compared with conventional polymer microspheres, most of which are polystyrene. The authors have studied the binding of proteins and other substances to the microspheres of

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GMA copolymer and its derivatives, as well as their biomedical application. It has been already reported that these microspheres can be usefully applied to cell labelling,[1] immunological agglutination tests,[2] the measurement of phagosomal reactive oxygen,[7-16] and the measurement of phagolysosomal enzyme activity.[17-20] The present report describes the methods of preparing these polymer microspheres and a discussion of their properties.

EXPERIMENTAL

Materials

GMA (Tokyo Kasei Kogyo, Tokyo), 2-hydroxyethyl methacrylate and methacrylic acid (Kishida Chemical, Tokyo), tri(ethylene glycol) dimethacrylate (Shin-Nakamura Chemical, Wakayama), *N,N'*-azobis(2,4-dimethylvaleronitrile) (Wako Pure Chemical Industries, Tokyo), ethyl propionate and *n*-butyl acetate (Kishida Chemical, Tokyo) were used without further purification.

Polymerization and chemical derivation of the polymers

GMA, 2-hydroxyethyl methacrylate (HEMA), tri(ethylene glycol) dimethacrylate (3G) and a polymerization initiator were dissolved in a solvent and the solution was allowed to stand under nitrogen atmosphere at a constant temperature. Precipitated polymer microspheres were separated from residual monomers and solvent by centrifugation, washed sequentially with acetone and petroleum ether by repeated resuspension and centrifugation, dried and stored in a desiccator.

For the purpose of introducing hydroxyl groups into the polymer microspheres by hydrolysing the epoxy group, the microspheres were suspended and stirred in a dilute acidic or alkaline solution.

For the purpose of introducing amino groups into the polymer microspheres by ammonilysing the epoxy group, the microspheres were suspended and stirred in an ammoniacal water solution.

Amination of the epoxy group with secondary amine was carried out as follows:

(1) A sample (1 g) of the microspheres of a copolymer (GMA/HEMA/3G = 95·7/9·5/4·8 in molar ratio, 2 μm) was dispersed in 15 ml of dried dioxane, then the dispersion was mixed with 20 g of diethylamine and stirred at 50°C for 16 h. Thereafter, the microspheres were washed three times with dioxane and five times with water.

(2) A sample (2 g) of the microspheres as above was dispersed in 30 ml of dioxane, then the dispersion was mixed with 30 g of diethanolamine diluted with 12 ml of dried dioxane and stirred at 50°C for 16 h. Thereafter, the microspheres were each washed twice with dioxane and water.

Analysis and measurement

The shape and diameter of the polymer microspheres were observed and measured with a field emission scanning electron micrograph, HITACHI S-800.

Thermal decomposition gas chromatographic analyses of the polymer microspheres were performed as follows: the sample was decomposed at 500°C, and the decomposition products were analysed with a gas chromatograph (Shimadzu GC-6M) using a column of PEG-HT(5%)/Gaschrom Q(60/80 mesh) in a glass tube (2 m × 3 mm), the temperature being raised from 100°C to 220°C at a rate of 10°C/min.

^{13}C NMR spectra of GMA copolymer microspheres and the hydroxylated derivative were measured in the state swollen by DMSO-d_6 with a JEOL FX-100 (25·05 MHz) spectrometer in the pulsed Fourier transform mode.

RESULTS

Polymerization

Table 1 shows the results of polymerization experiments performed with varied monomer compositions and reaction media. The selection of the polymerization medium is the most important factor from the view point of obtaining monodisperse microspheres. Ethyl propionate, *n*-butyl acetate, and methyl *n*-butyl ketone are favourable media for the polymer to precipitate as monodisperse microspheres. In other media such as benzene, toluene, ethyl benzene, acetone, methyl ethyl ketone and *n*-nonyl acetate, the polymer gelated, or precipitated as aggregates. The state of the polymer produced also depends on the monomer composition. In ethyl acetate, for example, the copolymer containing 48 mol% HEMA precipitated as monodisperse microspheres with a diameter of 0·7–0·8 μm, whereas the copolymers containing 9·7 mol% HEMA and no HEMA formed aggregates. The latter copolymers gelated in γ-butyrolactone and acetone.

Table 2 shows the dependence of the diameter of the polymer microspheres on polymerization conditions. The most effective factor was the type of medium, and fine adjustment of the diameter could be made by altering the monomer concentration. In addition, the medium affects not only the diameter but also the shape of the polymer microspheres. For example, polymer particles were irregular in shape when produced in DOP and DOS, though they were spherical in all other cases.

A typical scanning electron micrograph of polymer microspheres is shown in Fig. 1.

The composition of the copolymers was determined by gas chromatographic analysis of thermal decomposition products. Analytical results of thermal decomposition products were in good agreement with the composition of

TABLE 1. Relationship of the appearance of polymer with monomer composition and the type of medium

Monomer composition (mol.%)			Medium	Polymer
GMA	HEMA	3G		
95·2	—	4·8	EtPr[a]	Slightly aggregated
			Toluene	Aggregated
			Acetone	Gelated
			Ethyl acetate	Aggregated
85·7	9·7	4·6	EtPr	Dispersed
			Benzene	Aggregated
			Toluene	Aggregated
			Ethyl benzene	Aggregated
			Ethyl acetate	Aggregated
			EtPr/CCl_4	Dispersed
			n-Butyl acetate	Dispersed
			Methyl ethyl ketone	Gelated
			Methyl *n*-propyl ketone	Gelated
71·5	23·9	4·6	EtPr	Dispersed
48·0	48·0	4·0	Benzene	Aggregated
			Toluene	Aggregated
			Ethyl benzene	Aggregated
			Ethyl acetate	Dispersed
			EtPr	Dispersed
			n-Butyl acetate	Dispersed
			n-Nonyl acetate	Aggregated
			γ-Butyrolactone	Gelated
			Acetone	Gelated
			Methyl ethyl ketone	Aggregated
			Methyl *n*-butyl ketone	Dispersed

[a] EtPr: ethyl propionate.

TABLE 2. Dependence of the diameter of the microspheres on polymerization conditions

Monomer conc. (%)	Temperature (°C)	Time (h)	Initiator (mg/g)	Medium	Yield (%)	Diameter (μm)
10	50	16	V-65[a] 1	EtPr/CCl_4 (1:1)	67	2·2–2·3
15	50	16	V-65 1	EtPr/CCl_4 (1:1)	78	2·0–2·7
20	50	16	V-65 1	EtPr/CCl_4 (1:1)	85	2·0–3·0
26	50	16	V-65 1	EtPr/CCl_4 (1:1)	90	2·7–3·0
35	50	16	V-65 1	EtPr/CCl_4 (1:1)	94	4·0–4·6
26	50	16	V-65 0·5	EtPr/CCl_4 (1:1)	87	2·7–3·0
26	45	16	V-65 1	EtPr/CCl_4 (1:1)	94	2·7–3·0
28	45	16	V-65 1	EtPr/CCl_4 (1:1)	90	2·8–3·2
29	45	16	V-65 1	EtPr/CCl_4 (1:1)	90	3·0–3·7
30	45	16	V-65 1	EtPr/CCl_4 (1:1)	91	3·3–3·7
26	45	16	V-65 1	DOP[c]	90	0·5
26	45	16	V-65 1	DOS[d]	90	0·9
26	40	3	V-70[b] 1	BuAc[e]	60	1·7–2·0

[a] Azobis(2,4-dimethylvaleronitrile).
[b] Azobis(2-methyl-4-methoxyvaleronitrile).
[c] 2-Ethylhexyl phthalate.
[d] 2-Ethylhexyl suberate.
[e] *n*-Butyl acetate.

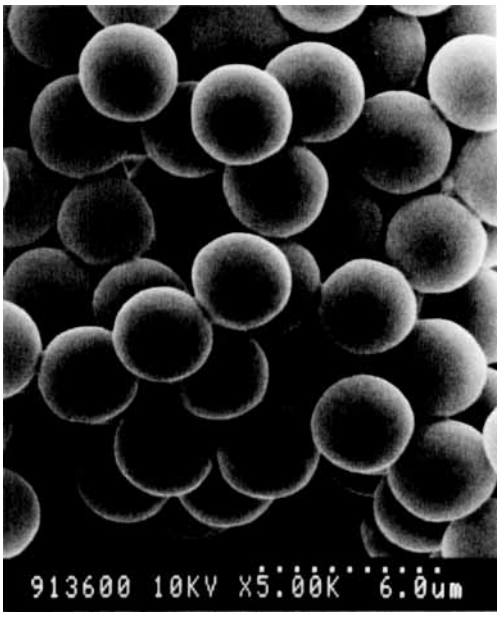

Fig. 1. Scanning electron micrograph of microspheres of a copolymer of glycidyl methacrylate.

the monomer mixtures before polymerization, as shown in Table 3.

Hydroxylation

The polymer microspheres were hydrolysed with dilute sulphuric acid or perchloric acid. Results of thermal decomposition gas chromatographic analysis are shown in Table 4.

The ^{13}C NMR spectrum of the hydrolysate of the copolymer (GMA/HEMA/3G = 85·7/9·5/4·8 in molar ratio) is shown in Fig. 2. The peak at 48·9 ppm assigned to the CH of the epoxy group in the copolymer of GMA (spectrum not shown) completely disappeared, while peaks at 62·9 ppm and 69·1 ppm appeared and were assigned respectively to methenyl and methylene carbons adjacent to hydroxyl groups derived from the epoxy group.

Elemental analysis revealed that 0·1% sulphur was contained in polymer microspheres hydrolysed with 0·06 N sulphuric acid. A very small portion of the epoxy group was converted into sulphate by the addition of

TABLE 3. Comparison of the composition of thermal decomposition products with that of the monomer mixture

Composition of monomer mixture (mol.%)			Thermal decomposition GC weight ratio			Composition of the thermal decomposition product (mol.%)		
GMA	HEMA	3G	GMA	HEMA	3G	GMA	HEMA	3G
89·1	9·9	1·0	10·5	1	0·23	89·7	9·3	1·0
88·2	9·8	2·0	11·2	1	0·46	89·4	8·8	1·8
85·7	9·5	4·8	10·2	1	1·17	85·8	9·3	4·9

TABLE 4. Thermal decomposition GC analysis of hydroxylated polymer microspheres

Reaction conditions			Thermal decomposition GC weight ratio		
Catalyst	Temperature (°C)	Time (day)	GMA	HEMA	3G
0·06 N H_2SO_4	40	3	0·39	1	1·06
		5	0·36	1	1·15
		7	0·40	1	1·10
		10	0·32	1	0·91
0·06 N $HClO_4$	30	3	0·90	1	1·20
		5	0·19	1	1·20
		7	0·07	1	1·23
		10	0·04	1	1·27
Before reaction	—	–	7·98	1	0·93
			10·94	1	1·20

Reaction temperature: 30°C.

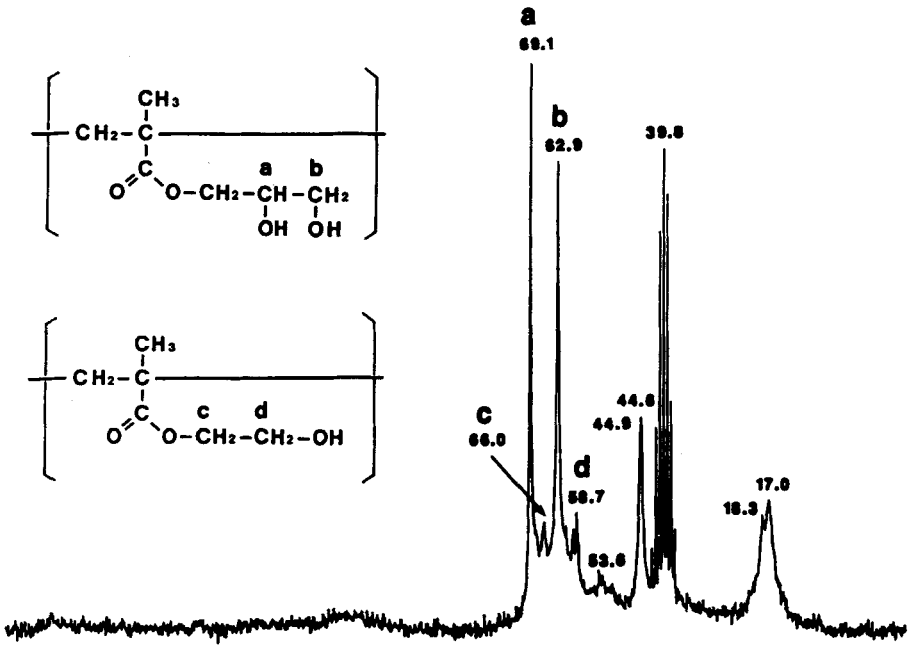

Fig. 2. ^{13}C FT-NMR spectrum of the hydrolysate of microspheres of a copolymer of glycidyl methacrylate (GMA/HEMA/3G = 85·7/9·5/4·8).

sulphuric acid. It was confirmed that the ester bonds of methacrylate were hardly cleaved by hydrolysis with 0·06 N sulphuric acid by the fact that neither ethylene glycol nor glycerine were detected by the gas chromatographic analysis of the hydrolysis reaction solution. (Detection limit: 10 ppm for ethylene glycol and 100 ppm for glycerine.)

Amination

GMA copolymer microspheres were aminated with ammoniacal water solutions. The dependence of nitrogen content of the reaction product on ammonia concentration is shown in Fig. 3. In this experiment, nitrogen contents were determined after hydrolysis. Tables 5 and 6 show the results of thermal decomposition gas chromatographic analysis of animated microspheres before and after hydrolysis, respectively.

When GMA copolymer microspheres were reacted with secondary amines in dioxane, the amination reaction proceeded quantitatively (Table 7).

DISCUSSION

It is essential in the polymerization system of the present report, so-called precipitation polymerization, that the growing polymer microspheres should not collide with each other, because the agitation of the polymerization

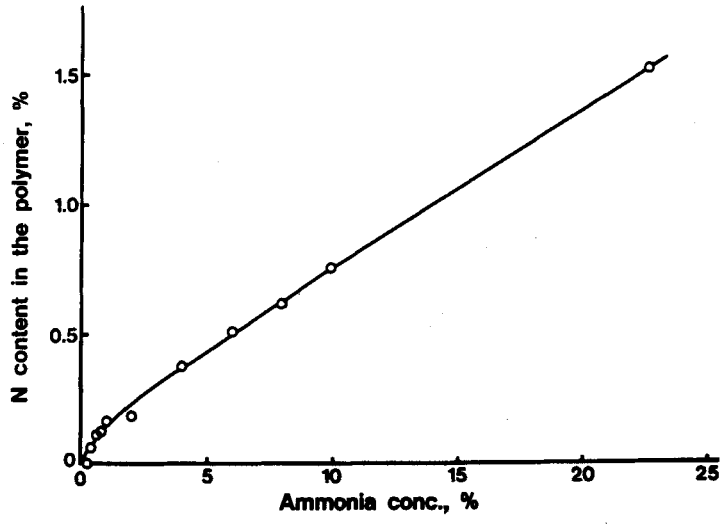

Fig. 3. Dependence of nitrogen content in the polymer on the concentration of ammonia in the amination solution.

TABLE 5. Thermal decomposition GC analysis of aminated polymer microspheres

Ammonia (%)	Thermal decomposition GC weight ratio		
	GMA	HEMA	3G
0·2	6·8	1	0·83
10·0	3·6	1	0·77
Before amination	7·4	1	1·0

Amination condition: 40°C, 1 h.

TABLE 7. Results of amination with secondary amines

Secondary amine	N content in polymer (%)	
	Observed	Calculated
Diethyl amine	5·42	5·48
Diethanolamine	4·88	5·05

Microsphere diameter: 2 μm.
Concentration: microspheres 3%, secondary amine 57%.
Reaction medium: dioxane.
Reaction temperature and time: 50°C, 16 h.

mixture caused without exception aggregation of the polymer microspheres. Precipitation polymerization differs from dispersion polymerization mainly in this point.

The mechanism of the formation of monodisperse microspheres in precipitation polymerization is assumed to be similar to that of dispersion polymerization, which some authors have discussed in detail.[6,21] Presumably, oligomer radicals and polymer molecules formed in the homogeneous phase and deposit on the surface of polymer microspheres without the formation of new polymer particles, once the number of polymer microspheres per unit volume of polymerization mixture has reached a certain level. Then, all the polymer microspheres will grow at the same rate because each polymer microsphere has equal probability of trapping oligomer radicals and polymer molecules newly formed in the homogeneous phase. Hence, the diameter of the polymer microspheres is determined by the number of seed polymer microspheres per unit volume, under the condition that the generation of new seed polymer particles can be neglected, provided the initial concentration of monomers and the polymer yield are the same.

In terms of the rate of conversion of newly formed polymer molecules from the dissolved state to the precipitated state, presumably, the ratio of the rate of new polymer particle formation to that of the deposition of newly formed polymer molecules on the existing polymer microspheres depends upon the affinity of the medium for the polymer. Therefore, the most important factor determining the diameter of the polymer microspheres is the affinity of the medium for the polymer. If the affinity is too high, the polymer is dissolved, or gelated when cross-linked. If the affinity is too low, the polymer forms aggregates. Monodisperse microspheres would be formed only when the affinity is within an appropriate range.

Copolymerization of HEMA facilitated the production of monodisperse microspheres in a well dispersed state as shown in Table 1. It was assumed that the affinity of copolymers containing HEMA with these esters had entered an appropriate range. A similar phenomenon was reported by Kawaguchi *et al.*[22] in the case of the precipitation polymerization of acrylamide in alcohols. In that polymerization system the addition of a certain amount of methacrylic acid gave fine and monodisperse microspheres having a diameter around 1 μm, whereas without the addition of methacrylic acid the products of polymerization were commonly coarse and bulky particles having a diameter about 100 μm. They argued that methacrylic acid units contribute to the stabilization of the particles formed at the initial stage of polymerization and the enhancement of swelling of the particles by monomers and alcohols. We have not yet measured the swelling of the polymer with monomers and media precisely, but presume that the contribution of swelling is

TABLE 6. Results of hydrolysis under varied conditions following amination

Catalyst	Hydrolysis condition		Thermal decomposition GC, weight ratio		
	Medium	Temperature (°C)	GMA	HEMA	3G
0·06 N H_2SO_4	Water	50	0·52	1	1·54
0·05 N HCl	Acetone/water (2:1)	30	0·66	1	1·41
0·03 N $HClO_4$	Water	50	0·54	1	1·09
0·5 N NaOH	Water	50	5·08	1	1·28
Neither amination nor hydrolysis	—	—	11·2	1	1·04

Amination condition: 8% ammonia solution, 40°C, 1 h.
Time of hydrolysis: 1 day.

not so important in our case because by qualitative observation the polymer was hardly swollen.

The hydrolysis of the polymer resulted in substantially selective hydroxylation of the epoxide into α,β-diol when catalysed by a dilute strong acid. Although the cleavage of ester bonds in the methacrylate units could be expected, the ratios of such side reactions were calculated to be less than 1%, because from the analytical results on the hydrolysis reaction solution neither glycerine nor ethylene glycol were detected.

The conversion of a very small portion of the epoxide into sulphate was the only side reaction that was detected. The sulphur content of 0·1% in the polymer means that about 0·8% of the epoxide was converted into sulphate, the content of which was calculated to be 0·03 meq/g in the polymer. This is the reason why polymer microspheres hydrolysed with sulphuric acid are more dispersible than those hydrolysed with perchloric acid.

The amination reaction is more complicated than the hydroxylation. From Table 5, the conversion of the epoxide was calculated to be 51% when the copolymer was treated with 10% ammonia solution at 40°C for 1 h.

$$\text{Conversion of epoxide} = (1 - 3{\cdot}6/7{\cdot}4) \times 100\% = 51\%$$

The nitrogen content of the copolymer was 0·76% after additional treatment with 0·06 N sulphuric acid (Fig. 3). If all of the reacted epoxy groups were converted into primary amino groups, the nitrogen content should be 3·3%, and 1·1% if tertiary amine was the only product. In the above calculation, it was assumed that the amines were in the state of sulphate salt because treatment with dilute sulphuric acid followed the amination. The observed value of 0·76% is even less than that calculated for tertiary amine. Although the hydrolysis of epoxide would reduce the yield on amination, the rate of hydrolysis of epoxide with alkali is very low as seen in Table 6. Therefore, it is tentatively concluded that the amination of epoxide with 10% ammonia solution produces tertiary amine rather than primary and secondary amines.

When 23% ammonia solution was used, the yield of primary and secondary amines might increase because the nitrogen content of the product was about double as seen in Fig. 3.

However, even polymer microspheres aminated with 8% ammonia solution could bind sufficient antigens and antibodies for application in immunological examination using glutaraldehyde as binder, as reported in previous papers.[1,2] Presumably, primary amine is predominant on the surface of the microspheres because the rate of the reaction of ammonia with epoxy groups is higher and the number of epoxy groups surrounding the primary amino group produced is less on the surface than in the inner part of the microspheres. In addition, a relatively low surface density of primary amino groups would be sufficient for binding proteins because of the bulkiness of a protein molecule.

In the amination with a secondary amine, the first product, that is a tertiary amine, does not further react with surrounding epoxy groups. This is, without doubt, the main reason for quantitative amination, though other factors must also be considered such as no possibility of hydrolysis as a side reaction, higher temperature, longer reaction time and the swelling of the polymer with dioxane.

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