Preparation of Porous Polymeric Structures for Enzyme Immobilization

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ABSTRACT: Porous polymethacrylic acid-*co*-triethylene glycol dimethacrylate (MAA-*co*-3G) and polyacrylic acid-*co*-triethylene glycol dimethacrylate (AA-*co*-3G) were prepared by four different polymerization techniques, namely, suspension, dispersion, seed, and microemulsion polymerization using an inert diluent (*n*-hexane and polystyrene). The morphology and porosity of the obtained polymers were examined by means of a scanning electron microscope. The surface areas of the obtained polymers were determined colorimetrically. The copolymers were modified by hydroximation and chlorination using hydroxyl amine and thionyl chloride, respectively. The effect of polymerization type, surface area, modification, and pH on the protease enzyme immobilization over poly(MAA-*co*-3G) and poly(AA-*co*-3G) was examined. The enzyme activity was measured by means of a spectrophotometer. The reactivity ratios of the two monomers MAA and 3G were determined by means of the elemental analysis method. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 76: 594–601, 2000

Key words: porous; enzyme immobilization; suspension; dispersion; seed; microemulsion; methacrylic acid; acrylic acid; triethylene glycol dimethacrylate; protease

INTRODUCTION

Because the recovery yield and the reusability of free enzymes as industrial catalysts are quite limited, attention has been paid to enzyme immobilization on solid supports,[1,2] which offers advantages over free enzymes in the possibility of running enzymatic reactions continuously, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture (thus saving time of a purification step for removing enzyme from the product stream), adaptability to various engineering designs, prolonged activity, and obtainability of a concerted or sequential reaction of several enzymes by the use of mixed or stratified beds.[3–6] Furthermore, the interest in the immobilized enzymes and their application to bioprocessing,[7,8] analytical systems,[9] and enzyme therapy[10] has steadily grown in the past decade. Thus, many approaches to the preparation of water-soluble enzymes have been explored[11–14] to study the enzyme reaction in biphasic systems similar to those existing in vivo. Various methods exists for immobilization of enzymes,[15,16] and these may be divided into physical methods based on molecular interactions between the enzyme and carrier, and chemical methods based on formation of covalent bonds. When immobilizing an enzyme to a surface, it is most important to choose a method of attachment that will prevent loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site of the enzyme. In other words, attach the enzyme, but do as little damage as

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possible. Considerable knowledge of the active site of the enzyme will prove helpful in achieving this task. It is desired to avoid reaction with the essential binding site group of the enzyme. Alternatively, an active site can be protected during attachment as long as the protective groups can be removed later on without loss of enzyme activity. In some cases, this protective function can be fulfilled by a substrate or by a competitive inhibitor of the enzyme. The surface on which the enzyme is immobilized is responsible for retaining the structure in the enzyme through hydrogen bonding or through the formation of electron transition complexes. These links will prevent vibration of the enzyme and thus increase thermal stability. The microenvironment of surface and enzyme has a charged nature that can cause a shift in the optimum pH of the enzyme of up to 2 pH units. This may be accompanied by a general broadening of the pH region in which the enzyme can work effectively, allowing enzymes that normally do not have similar pH regions to work together. The most intensely studied of the immobilization techniques is the formation of covalent bonds between the enzyme and the support matrix. When trying to select the type of reaction by which a given protein should be immobilized, the choice is limited by two characteristics: first, the binding reaction must be performed under conditions that do not cause loss of enzymatic activity; and second, the active site of the enzyme must be unaffected by the reagents used.

In this study, a protease was selected as a hydrolytic enzyme. Proteolytic enzymes have different applications in the field of industry and medicine. It is widely used in meat tendering, leather tanning, synthetic detergents, and pharmaceutical preparations (for treatments of burns, reducing dental plaque, depilation).[17,18] *Aspergillus* spp are classically used to produce proteolytic enzymes.[19] Some of these enzymes, particularly semialkaline proteases, are used in therapeutics as antiinflammatory agents[20] or to promote healing. They have been used in the preparation of immunizing agents,[21,22] collagen derivatives, and various proteolysates. Other applications are being explored such as the conversion and upgrading of plant proteins to achieve novel functional and nutritional properties.[23]

EXPERIMENTAL

Materials

Methacrylic acid, acrylic acid, styrene and triethylene glycol dimethacrylate supplied from Redel de Haen (Germany), were purified by being passed through active alumina. Poly vinylalcohol (M_w 70,000), hot water-soluble (USA) and benzoyl peroxide reprecipetated from chloroform solution by methanol were supplied from Sigma Chemical Company. *n*-Hexane and all the solvents used are supplied from El Nasr Pharmaceuticals Company. Methacrylate-terminated phthalate glycol polyester used as a steric stabilizer in dispersion polymerization, of an intrinsic viscosity of $\eta_{int} = 1.15$ at 20°C in MEK, was prepared.[24] Monodisperse polystyrene seed particles were prepared of average particle size 9 μm by the emulsifier-free emulsion polymerization.[25] Protease enzyme from fungus (*Aspergillus oryzae*) was obtained from Sigma Chemical Co. (St. Louis, MO) (P-4755).

SURFACE AREA MEASUREMENTS[26]

The copolymer particles surface area is determined colorimetrically. A polymer sample 1.0 g is left for 24 h in methylene blue solution 100 mg/L. The decrease in light absorbance at 670 nm is a measure of polymer surface area (each mg of methylene blue adsorbed is equivalent to 2.4 m^2/g.

Polymerization Methods

Suspension Polymerization[27,28] of MMA and 3G

The reaction ingredients comprises MAA (15 mL), 3G (15 mL) as the monomer phase, and benzoyl peroxide as monomer soluble initiator (0.5 g). *n*-Hexane (5 mL) was used as the inert diluent, PVA (1.0 g) as the protective colloid dissolved in distilled water (100 mL). The monomer phase was introduced to the reactor in a step-wise manner to ensure good monomer phase dispersion. The copolymerization was carried out in a 500-mL round-bottom flask fitted with a mechanical stirrer, nitrogen inlet, and a condenser at 80°C, and was shown to be completed after ~ 150 min where spherical beads were separated. Figure 1 shows the porous structure of the formed beads. The

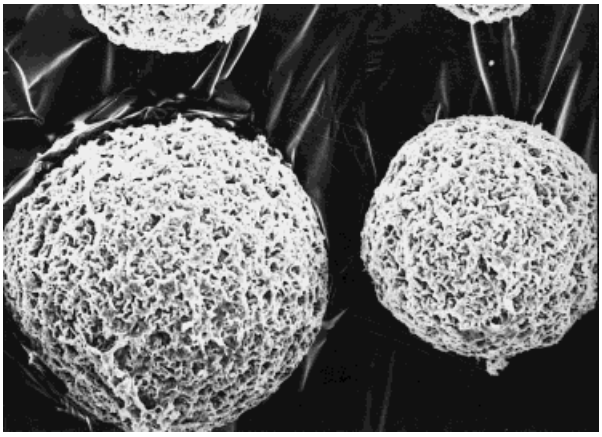

Figure 1 Porous poly(MAA-*co*-3G) obtained from suspension polymerization (magnification ×112.5).

beads were found to have an average particle diameter 324 μm, and specific surface area 18 m^2/g.

Dispersion Polymerization[29] of MAA and 3G

The copolymerization was carried out in a 250-mL round-bottom flask fitted with a mechanical stirrer, nitrogen inlet, and a condenser at 70°C.

The polyester stabilizer, which was previously prepared, was first dissolved in the chloroform mixture. Methacrylic acid was then introduced as one shot, and benzoyl peroxide was added to initiate the polymerization. After ~ 100 min the crosslinker (3G) was added, and stirring was stopped. The dispersion was heated for another 80 min, where a solid mass was obtained. The resulting copolymer was subjected to air drying followed by freeze drying to obtain a porous structure. Figure 2 shows the porous structure of the prepared poly(MAA-*co*-3G).

Seed Polymerization[30]

To a dispersion of polystyrene particles 7.5 g, an emulsion of a swelling agent (*n*-hexane 5 mL) is added, containing a free radical initiator (benzoyl peroxide) in an aqueous solution of dodecyl benzene sodium sulphonate. The mixed dispersion is stirred over a magnetic stirrer at room temperature for 24 h until the drops of the swelling agent have disappeared. A mixture of MAA 10 mL, 3G 5 mL, and a porogenic agent *n*-hexane emulsified by 0.5% aqueous solution of dodecyl benzene sodium sulphonate is added to the dispersion. The mixture is stirred at room temperature at 200 rpm for another 24 h to allow complete swelling of the monomer phase into the activated PS particles. The final dispersion is supplemented by a 1% aqueous solution of poly vinyl pyrrolidone to reach the required concentration for dispersion stabilization during polymerization. The reactor content was flushed with nitrogen to remove the dissolved oxygen, and the reactor was closed and heated with a stirring rate 100 rpm to 65°C for 24 h. As the reaction proceeds, the monomers are transformed into the crosslinked polymer chains, and a phase separation occurs between the MAA-3G and the linear PS and the nonsolvent. The resulting copolymer particles were dried and subjected to soxhlet extraction for 36 h using chloroform as the solvent, to remove the the diluent mixture (PS and *n*-hexane). Figure 3 shows the resultant porous structure.

Microemulsion Polymerization[31]

The polymerization components (i.e., water 100 mL, surfactant, dodecyl benzene sodium sulphonate 5.0 g, monomer phase, MAA 10 mL + 3G 3 mL, cosurfactant butan-1-ol 25 mL, initiator ammonium persulphate 0.5 g) were successively introduced into the glassy reactor and heated to 65°C. The polymerization mixture was stirred at 300 rpm for 3 h until a solid mass was separated. The resulting copolymer was subjected to air drying followed by freeze drying to restore the porous structure. Figure 4 shows the porous structure of poly(MAA-*co*-3G).

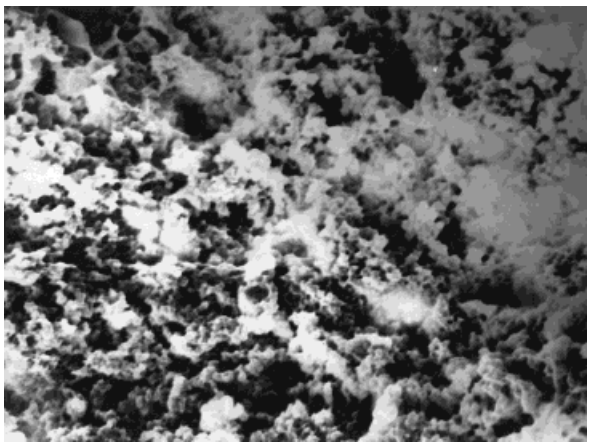

Figure 2 Porous poly(MAA-*co*-3G) obtained from dispersion polymerization (magnification ×187.50).

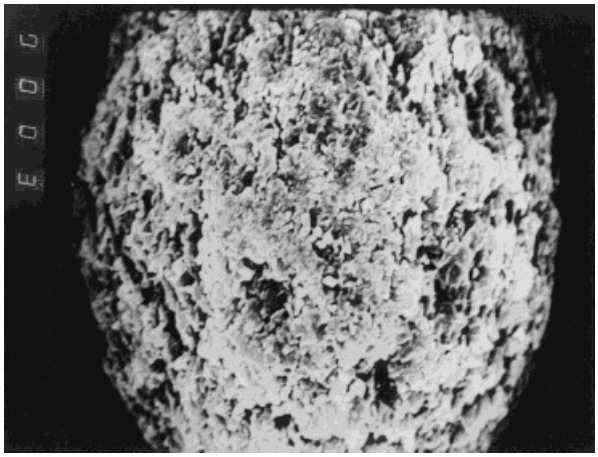

Figure 3 Porous MAA-*co*-3G obtained from seed polymerization (magnification ×1500).

Chlorination[32] of the Formed Poly(MAA-*co*-3G)

One sample of each of the prepared copolymers was subjected to chlorination using thionyl chloride as the chlorinating agent.

$$RCOOH + SOCl_2 \rightarrow RCOl + SO_2 + HCl$$

Hydroximation[33]

One sample of each of the prepared copolymers was subjected to hydroximation using hydroxyl amine, triethylamine, and freshly prepared sodium methoxide as a base.

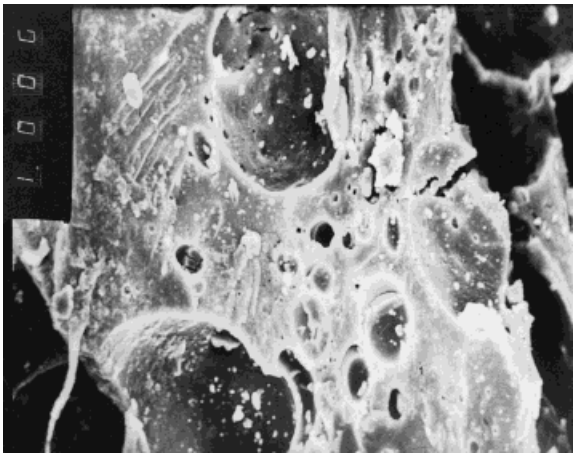

Figure 4 Porous poly(MAA-*co*-3G) obtained from microemulsion polymerization (magnification ×375).

Table I Reaction Ingredients of the Dispersion Polymerization of MAA

Ingredients	Concentration wt %
MAA	11
Chloroform	65
Ethanol	12
Polyester stabilizer	6.5
Benzoyl peroxide	0.5
3G	5.0

$$RCOOR' + RCOOH \xrightarrow[-2-3-3-]{NH_2OH/CH_3OH/CH_3ONa} R'OH + RCONHOH.$$

Enzyme Assay[34]

Protease activity was assayed by the modified method of Anson.[34] An enzyme solution 0.5 mL suitably diluted was mixed with 2.5 mL of 0.6% Hammarsten Casein (Merck & Co., Darmstadt, Germany) solution, pH 10.5, made up with 50 m*M* Glycine : NaCl : NaOH buffer. After incubation for 20 min at 30°C, 2.5 mL of trichloroacetic acid (TCA) solution (consisting of 0.11*M* TCA, 0.22*M* sodium acetate, and 0.33*M* acetic acid) was added to stop the reaction. The mixture was further incubated at 30°C for 30 min and then filtered with Toyo Roshi filter paper No. 5C (Toyo Roshi Co., Tokyo, Japan). To 0.5 mL of filtrate 2.5 mL of 0.5*M* Na_2CO_3 solution and 0.5 mL twofold-diluted Folin-Ciocalteau reagent[36] was added. After standing for 30 min at room temperature, the absorbance was measured at 660 nm. One unit of protease enzyme activity was defined as the amount of enzyme to produce 1 μg tyrosine/min.

RESULTS AND DISCUSSION

A series of polymethacrylic acid (PMAA) and polyacrylic acid (PAA) crosslinked with triethyleneglycol dimethacrylate (3G) were prepared, and at different monomer/crosslinker ratios, a series of copolymer samples were prepared by immersing 0.1 g solid in an enzyme solution 250 mg/10 mL water. The data given in Table I, shows a comparison between two different copolymers PMAA-*co*-3G and PAA-*co*-3G. The effect of the method of preparation on the the enzyme immobilization of PMAA-*co*-3G was included in Table II, and finally

the effect of polymer surface modification by a stochiometric ratio, chlorination, and hydroximation was examined. From Table II, PMAA gave a higher enzyme adsorption (92.96)% than that of PAA 45.57% from the enzyme solution 250 mg/10 mL water. The protease enzyme adsorption was found to increase with increasing the triethylene glycol dimethacrylate content in both PMAA-*co*-3G and PAA-*co*-3G. This indicates that the enzyme fixation on these polymers is mainly attributed to the physical electrostatic adsorption rather than the chemical adsorption through the carboxylic groups of PMAA and PAA. The highest enzyme adsorption value belonging to PAA-*co*-3G with a monomer ratio of AA : 3G (2 : 8) was found to be 97.26%, which is similar to its corresponding PMAA-*co*-3G (2 : 8), 97.00%, which means that the diffusion of the enzyme solution through the PMAA-*co*-3G copolymer is similar to that of PAA-*co*-3G. The modification of PMAA-*co*-3G and PAA-*co*-3G into the corresponding polyhydroxamic acid (PHOX) gave unlogical enzyme adsorption values (higher than 100%). These results are attributed to the hydrolyzing effect of the protease enzyme on the peptide linkages. Different amino acids were liberated in the adsorption medium and detected in the residual solution, so (PHOX) copolymers are not suitable for protease enzyme immobilization. The modification of PMAA-*co*-3G and PAA-*co*-3G into the corresponding polyacid chloride gave very good results. The chlorination raised the adsorption capacity for the protease enzyme to $\sim$ 200%, as shown in Table I. The increased adsorption is attributed to the transformation from the physical electrostatic bond into a chemical adsorption bond due to the interaction between the acid chloride of the prepared copolymer and the amino group of the protease enzyme.

$$R—CO—Cl + NH_2—\text{-Protease} \rightarrow$$

$$R—CO—NH—\text{-Protease} + HCl$$

From Table II PMAA-*co*-3G prepared by the seed polymerization technique gave the lowest adsorption values with respect to PMAA-*co*-3G samples prepared by suspension, dispersion, and microemulsion polymerization. These results can

Table II Enzyme Immobilization on Different Crosslinked Acrylate Copolymers (0.1 g) from Protease Solution 250 mg/10 mL Water

No.	Polymer Substrate	Method of Preparation	Surface Area m^2/g	Residual Activity U/mL	Activity Adsorbed on Polymer	
					U/mL	%[b]
1a	Control	—	—	0.1931	0.0000	00.00
2	PMAA	Dispersion	17.0	0.0900	0.1000	92.96
3	PMAA-3G(8-2)	Suspension	21.3	0.0195	0.1736	89.90
4	PMAA-3G(2-8)	Suspension	22.9	0.0058	0.1873	97.00
5	PMAA-3G(8-4)	Suspension	23.7	0.0136	0.1795	92.96
6	PAA	Dispersion	15.5	0.1051	0.0880	45.57
7	PAA-3G (2-8)	Suspension	29.6	0.0053	0.1878	97.26
8	PAA-3G (8-2)	Suspension	33.5	0.1114	0.0817	42.31
9	PMAA-3G(5%)	SEED Emulsion	48.0	0.1001	0.0930	48.16
10	PMAA-3G(10%)	SEED Emulsion	40.5	0.0782	0.1149	59.50
11	PHOX(PMAA-3G)	SEED Emulsion	48.0	(0.3406)	—	—
12	PMAA(Base) Hydrolized	Dispersion	17.0	(0.4266)	—	—
13	PHOX(PMAA-3G)	Suspension	22.9	(0.2876)	—	—
14	PHOX(PAA-3G)	Suspension	23.9	(0.3793)	—	—
15	PMAA-3G(2-8)	Microemulsion	35.6	0.0353	0.2639	88.20
16	PMAA-3G(8-2)	Microemulsion	43.4	0.0558	0.2434	76.92
1b	Control	—	—	0.2992	0.0000	00.00
17[a]	Chlorinated PMAA-3G	Suspension	21.3	0.0097	0.2895	96.76
18[a]	Nonchlorinated PMAA-3G	Suspension	21.3	0.1950	0.1042	34.83

[a] Trials 17 and 18 are subjected to higher (500 mg/10 mL water) enzyme concentration than the remainder of the trials.
[b] %: U of enzyme loaded/100 U initial.

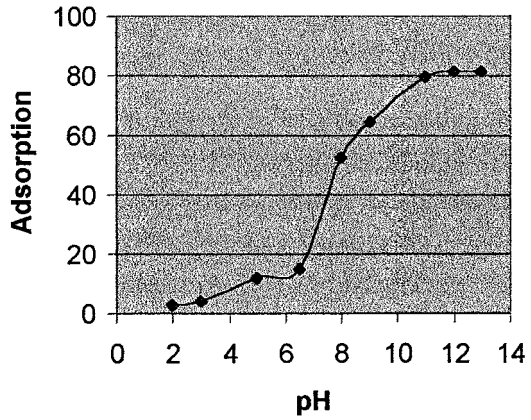

Figure 5 Effect of pH on the adsorption of protease enzyme over PMAA-*co*-3G.

be explained in terms of the increased hydrophobocity of the copolymers prepared by the seed polymerization method. This hydrophobocity is attributed to the remaining polystyrene in the core of the PMAA-*co*-3G particles. Samples 9 and 10 in Table II show the protease adsorption at 5 and 10% *n*-hexane (diluent), respectively. From Table II PMAA-*co*-3G with the higher diluent concentration gave higher adsorption than that of the lower one. This is attributed to the decrease in the amount of the remaining polystyrene.

Effect[34] of pH on the Adsorption of Protease Enzyme Over PMAA-*co*-3G

The adsorption of the protease enzyme over the prepared copolymer PMAA-*co*-3G was investigated at various pHs. The copolymer beads (0.01 g) were incubated in an protease enzyme solution (25 mg/10 mL water) at room temperature for 24 h in a range of pH values. The data are given in Figure 5. From Figure 5 the adsorption in the acidic pH range is very small, which is attributed to the instability of the protease enzyme in the pH range of 1–7. The adsorption of the protease enzyme over the copolymer beads increases markedly in the alkaline medium due to the increased stability and activity of the protease enzyme in the pH range.[8–13]

Determination[37,38] of the Reactivity Ratios r_1, r_2 of MAA and 3G

The reactivity ratios of MAA and 3G are determined by the elemental analysis method to know the amount of each monomer in the copolymer samples, which in effect, influences the various applications. The data given in Table III represents the mol fraction of the monomers MAA and 3G in the feed and the copolymer composition, prepared by the bulk polymerization technique to avoid the effect of adsorbates on the elemental analysis.

Purification

The copolymer beads were first washed with distilled water, and then subjected to a soxhlet extraction with acetone followed by benzene for 8 h each to dissolve any homopolymer formed during copolymerization, and finally dried at 40°C for 24 h.

The following equation derived by Kelen Tudos has been used to calculate the reactivity ratios r_1 (MAA) and r_2 (3G):

$$x(y-1)/(\alpha y + x2) = \{r_1 + r_2/\alpha\}x^2/\alpha y + x^2 - r_2/\alpha \quad (1)$$

where x is the ratio of mol fraction of monomer 1 (M_1) and monomer 2 (M_2), and y is the mol frac-

Table III Reactivity Ratios of MAA (M_1) and 3G (M_2)

	Feed Composition (mol)		Copolymer Composition (H%)			
No.	MAA	3G	MAA	3G	Feed ($X = M_1/M_2$)	Copolymer ($Y = dM_1/dM_2$)
1	0.20	0.80	1.41	6.20	0.25	0.23
2	0.40	0.60	2.81	4.65	0.67	0.60
3	0.50	0.50	3.51	3.87	1.00	0.91
4	0.60	0.40	4.21	3.10	1.50	1.35
5	0.80	0.20	5.62	1.55	4.00	3.63

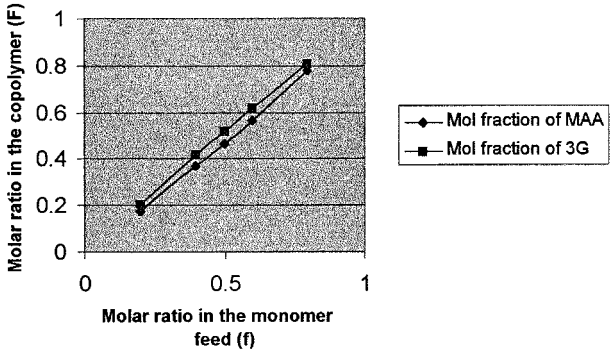

Figure 6 Plot of mol fraction of MAA and 3G in the feed (f) vs. the mol fraction in the copolymer composition (F).

tion of M_1 and M_2 in the copolymer, and the parameter α is given by:

$$\alpha = x_{\min} \cdot x_{\max}/(y_{\min} \cdot y_{\max})^{0.5}. \quad (2)$$

Figure 6 shows that the mol fractions of both MA and 3G in the feed and the copolymer composition are very close and symetrically distributed around the base line, which indicates similar reactivity ratios. The reactivity ratios r_1 and r_2 were shown to be 0.60 and 0.65 for MAA and 3G, respectively. This result indicates that the copolymerization of MAA and 3G approaches the ideal copolymerization behavior, i.e., the two propagating species M_1 and M_2 show near the same preference for adding one or the other of the two monomers.

CONCLUSIONS

We have shown that, porous polymethacrylic acid-*co*-triethylene glycol dimethacrylate (MAA-*co*-3G) was prepared by four different polymerization techniques, namely, suspension, dispersion, seed emulsion, and microemulsion polymerization. The prepared poly(MAA-*co*-3G) has shown highly crosslinked structures, moderate surface area, and high binding capacity towards the protease enzyme. The chlorinated form of poly(MAA-*co*-3G) has given the highest binding capacity towards the protease enzyme over the rest of the modified forms of poly(MAA-*co*-3G). The binding capacity of the prepared poly(MAA-*co*-3G) towards the protease enzyme was shown to be much higher in the basic medium than the acidic medium. The reactivity ratios of methacrylic acid and triethylene glycol dimethacrylate were determined and found to be 0.6 and 0.65, respectively.

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