0273-2289/98/7403—0191$11.25

Beaded Reactive Polymers 3

Effect of Triacrylates as Crosslinkers on the Physical Properties of Glycidyl Methacrylate Copolymers and Immobilization of Penicillin G Acylase

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Received December 11, 1997; Revised April 27, 1998; Accepted June 4, 1998

ABSTRACT

Various glycidyl methacrylate (GMA) copolymers were synthesized by suspension polymerization, using pentaerythritol triacrylate (PETA), trimethylolpropane triacrylate (TMPTA), and trimethylolpropane trimethacrylate (TRIM) as crosslinking comonomers. These copolymers were evaluated for the immobilization of penicillin G acylase. Broad pore-size distribution that was observed was in the range 5–300 nm. Both surface area and pore volume increased with increase in the mole fraction of crosslinking comonomer (increasing crosslink density). The pore volume of the copolymers was more than doubled by including lauryl alcohol as porogen. Binding of penicillin G acylase (PGA) was quantitative on highly crosslinked copolymers. The expression of bound PGA was better on the relatively more hydrophilic GMA-TMPTA and GMA-PETA copolymer supports compared to the GMA-TRIM copolymers. Among the different copolymers studied, GMA-TMPTA copolymer 7411 exhibited highest activity of immobilized penicillin G acylase (167.4 IU/g) with 35.1% expression.

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Index Entries: Penicillin G acylase; immobilized penicillin G acylase; glycidyl methacrylate; pentaerythritol triacrylate; trimethylolpropane trimethacrylate; trimethylolpropane triacrylate; immobilization; pore size distribution.

INTRODUCTION

Functionalized synthetic macroporous polymeric supports have found application in diverse fields *(1–3)*. One such application has been the immobilization of enzymes, which has eased the retrieval and recycling of enzymes and has minimized the cost. Immobilization of enzymes generally decreases their initial activity. This is attributed in part to the influence of the properties of support/matrix and in part to alterations in the enzyme structure *(4,5)*. Although native enzymes are inactivated soon, immobilized enzyme preserve their activity over much longer periods. The growing demand for 6-amino penicillanic acid (6-APA) justifies the search for newer carriers for immobilization of penicillin G acylase (PGA), an enzyme that hydrolyzes the linear amide bond in the penicillin G molecule.

While selecting a carrier for a given system, such factors as properties of enzyme, substrate(s), and product(s) under the conditions of catalysis, which contribute to the economics of the process, have to be taken into account. The design of optimal porous and beaded copolymers for enzyme immobilization necessitates an in-depth examination of the effect of synthesis variables, such as type and mole fraction of crosslinking comonomer, diluent type and volume, and so forth. The interrelation between synthesis variables and material properties, such as surface area, porosity, and mechanical strength of the carrier, have been thoroughly examined for beaded glycidyl methacrylate-ethylene glycol dimethacrylate (GMA-EGDM) *(6–8)* and glycidyl methacrylate-divinyl benzene (GMA-DVB) *(9,10)* copolymers. It was shown that porosity and surface area are influenced by the crosslink density, the type and volume of porogen, initiator concentration, and polymerization temperature. Macroporous reactive copolymers comprise three-dimensional structures formed by coupling vinyl monomers with crosslinking multifunctional (di- or trivinyl) comonomers. In nonswelling systems, the desired macroporous structure is formed at a very high relative concentration of the crosslinker. The hydrophilic/hydrophobic character of the crosslinker dictates this in the copolymer. Therefore, the crosslinking comonomer would influence the extent of immobilization of an enzyme.

Here we report the synthesis and properties of glycidyl methacrylate (GMA) copolymers supports crosslinked with trifunctional hydrophobic pentaerythritol triacrylate (PETA), marginally hydrophobic trimethylol-

propane triacrylate (TMPTA), and moderately hydrophobic trimethylolpropane trimethacrylate (TRIM) vinyl crosslinking agents, specifically as supports for the binding of penicillin G acylase (PGA), an enzyme of commercial importance.

MATERIALS AND METHODS

Materials

GMA was obtained from Merck (Darmstadt, Germany). PETA, TMPTA, TRIM, and azo *bis*(isobutyro nitrile) (AIBN) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Poly (*N*-vinyl pyrrolidone) (PVP) was obtained from Polysciences (Warrington, PA). Partially purified PGA preparation from *Escherichia coli* was from the production unit of Hindustan Antibiotics Ltd., Pimpri, Pune, India. All other chemicals were of analytical grade from local suppliers.

Synthesis of Polymer Matrices

The porous GMA-PETA, GMA-TMPTA, and GMA-TRIM copolymers were synthesized by suspension polymerization in a cylindrical polymerization reactor at 70°C using a constant-speed stirrer by a procedure described previously *(9)*. A series of copolymers of differing crosslink densities (CLD) were synthesized at constant volume of the porogen, cyclohexanol. The relative mole ratio of crosslinking comonomer PETA, TMPTA, or TRIM was varied from 10 to 300% of glycidyl methacrylate. The mole percent of the crosslinking comonomer relative to the moles of the comonomer (GMA) is termed CLD. Two sets of GMA-PETA copolymers of a specific CLD were synthesized. In the first set, the porogen, cyclohexanol was partially replaced with lauryl alcohol to alter the pore size distribution, and in the second set, the crosslinking comonomer PETA was partially replaced with ethylene glycol dimethacrylate (EGDM) to alter the hydrophilicity.

Immobilization of PGA

Polymer beads (5 g) were suspended in 0.05 *M* phosphate buffer (pH 7.5) containing 2500 IU of PGA. The method for immobilization was as described earlier *(11,12)*. The difference between the units of enzyme loaded and the units of enzyme in the supernatent solution indicated the amount of enzyme bound on the copolymer matrix. The expression of the enzyme is the comparison of the activity of the immobilized enzyme to that of the enzyme bound on the matrix.

Analytical Methods

The pore size distribution, pore volume, and surface area of the porous copolymers were studied by mercury intrusion porosimetry in the pressure range of 0–4000 kg/cm^2 using Autoscan 60 mercury porosimeter from Quantachrome, USA. The mercury contact angle was 140°. The activity of the soluble and the immobilized PGA was determined by measuring 6-APA formation *(12)*.

RESULTS AND DISCUSSION

Oxiranoyl polymers provide reactive epoxy groups for immobilization of enzymes through amino, carboxylic, or phenolic groups present in the enzymes. The effect of changes in the copolymer supports owing to the change in the crosslinking comonomer on the immobilization of PGA is the focus of the present study. Of the several multivinyl derivatives used as crosslinking agents, most literature pertains to TRIM. Flodin and coworkers have described TRIM homopolymers *(13–15)* as well as the copolymers with methyl methacrylate *(15–17)* and GMA *(18)*. The synthesis of copolymers of TMPTA with acrylonitrile by suspension polymerization has also been reported *(19)*. Porous GMA-PETA and GMA-TMPTA copolymers have not been examined.

The surface area, pore-size distribution, and pore volume of copolymers generated with GMA and PETA, TRIM, or TMPTA are presented in Table 1. The porosity studies of GMA-PETA copolymers reveal that the pore-size distribution broadened with increase in mole fraction of PETA, and the fraction of pores with larger radii and pore volume showed an increase. Similarly, the pore volume as well as surface area increased with increasing mole ratio (CLD) of TRIM in the GMA-TRIM copolymers, with a concurrent broadening of pore-size distribution. The fraction of pores with larger radii increased with CLD. This trend was observed in GMA-TMPTA copolymers as well. Thus, the pore-size distribution widened with increasing mol% of TMPTA, and the fraction of macropores also showed an increase.

PETA possesses a hydroxyl group. The hydrophilicity of the GMA-PETA copolymers increases with increasing PETA content (i.e., increasing CLD) against GMA-DVB, where the hydrophobicity increases with CLD. The PETA comonomer possesses three polymerizable double bonds of equal reactivity. Therefore, two variables are simultaneously present here. In addition to the enhanced hydrophilicity, the trifunctional crosslinking agent generates higher crosslinking at lower concentration, thereby enabling higher loading of reactive epoxy groups needed for anchoring the enzyme to the polymer support.

Table 1
Pore Size Distribution, Pore Volume, and Surface Area in Copolymers

Polymer no.	CLD[a] %	Pore size distribution, volume percent, radius in nm <5	5–10	10–15	15–20	20–30	30–50	50–100	100–300	>300	PV[b] mL/g	SA[c] M^2/g
GMA-PETA copolymers[d]												
PE8	50.0	48.4	24.0	7.7	3.0	3.3	3.4	3.1	1.6	5.5	0.17	72.2
PE10	67.5	41.2	33.3	11.2	5.0	4.4	3.6	1.3	0.0	0.0	0.24	96.4
PE11	75.0	41.4	29.4	11.8	4.6	4.6	3.2	2.1	1.3	1.6	0.23	86.3
PE12	87.5	34.0	34.5	18.2	6.6	6.2	0.50	0.0	0.0	0.0	0.31	63.0
PE13	100.0	28.4	34.0	18.5	7.5	6.5	3.6	1.5	0.0	0.0	0.35	114.2
PE14	133.3	26.0	29.2	18.2	9.6	9.1	4.6	2.7	0.6	0.0	0.41	124.0
GMA-TRIM copolymers[e]												
TM5	25	14.2	32.8	31.0	7.6	5.0	3.1	2.4	1.5	2.4	0.38	93.0
TM6	30	20.6	29.5	22.5	10.8	7.0	4.4	2.5	1.5	1.1	0.48	130.0
TM7	40	18.5	24.2	18.6	13.6	10.4	7.9	6.8	0.0	0.0	0.55	138.0
TM8	50	16.2	21.5	17.5	14.5	15.4	7.3	7.6	0.0	0.0	0.65	148.0
TM10	67.5	17.4	22.0	17.5	13.3	15.1	7.3	4.2	2.0	1.2	0.69	159.0
GMA-TMPTA copolymers[e]												
7410	67.5	54.3	28.5	6.6	2.6	1.8	1.0	1.1	1.0	3.0	0.23	110.5
7411	75.0	48.2	30.5	11.0	3.7	2.7	1.6	2.1	0.2	0.0	0.26	114.0
7412	87.5	41.8	29.5	12.1	4.5	3.7	2.3	1.5	1.5	3.1	0.32	126.4
7413	100	39.4	31.3	13.7	4.4	3.3	2.4	1.7	1.3	2.5	0.33	131.7
7414	133.0	37.0	31.0	14.6	6.6	5.5	3.1	2.2	0.0	0.0	0.35	134.8
7415	167.0	31.5	26.7	18.0	7.7	7.8	4.4	2.8	1.0	0.0	0.41	137.9

[a]CLD: Crosslink density.
[b]PV: pore volume.
[c]SA: surface area.
[d]GMA-PETA copolymers were synthesized by using 65.4 mL of cyclohexanol, 5 g of PVP, 0.6 g AIBN.
[e]GMA-TRIM and GMA-TMPTA copolymers were synthesized by using 65.4 mL of cyclohexanol, 5 g of PVP, 0.8 g AIBN.

Immobilization of PGA on GMA-PETA, GMA-TRIM, and GMA-TMPTA Copolymers

The immobilization of PGA on GMA-PETA, GMA-TRIM, and GMA-TMPTA copolymers was investigated. In GMA-PETA copolymer series PE2–PE7, with low relative mole ratio of crosslinker PETA (10–40% CLD), exhibited low binding of PGA. Only 20–30% of the enzyme was bound, even though these copolymers possess relatively very high concentrations of epoxy groups needed for enzyme binding. The data for copolymers with high CLD from PE7 onward are presented in Table 2. Although epoxy concentration decreased with increasing CLD, the amount of enzyme bound showed an increase, and near quantitative binding was estimated beyond 100% CLD. This can be attributed to an increase in the surface area, pore volume, and number of pores with larger radii in the copolymers with high CLD, as seen in Table 1. The expression of bound enzyme was maximum in copolymers with 67.5–100% CLD. Beyond 100% CLD, the binding remained high, whereas the expression dropped to slightly lower values.

In the GMA-TRIM series, copolymers TM2–TM4 exhibited low binding of PGA (Table 2). As the CLD increased beyond 25%, the binding increased and was almost quantitative beyond 50% CLD. In GMA-TMPTA copolymers (Table 2), the binding was lower relative to GMA-TRIM copolymers over similar values of crosslinking up to 67.5% CLD. This is owing to lower pore volume in the GMA-TMPTA copolymers (Table 1). However, the expression of bound PGA was relatively greater on GMA-TMPTA copolymers than on GMA-TRIM. This behavior is related to the differences in the hydrophobicity. GMA-TRIM copolymers are relatively more hydrophobic relative to GMA-TMPTA copolymers because of the long sequence of methyl side groups. PGA has a greater preference for hydrophobic environment *(20)* and binds to a greater extent on more hydrophobic matrices, such as GMA-TRIM copolymers. However, increased hydrophobicity may involve penicillin side-chain binding region of PGA in the process of immobilization, thereby rendering it inactive and resulting in lower activity of bound enzyme.

Since the internal surface of a macroporous copolymer carrier is approx 100- to 1000-fold relative to the external surface, which is dependent on the diameter of the particle, approximately all the enzyme is bound in the interior of the carrier, and the activity of the enzyme immobilized onto the external surface may be neglected. Enzyme immobilization being irreversible is dominated by slow diffusion of enzymes into the pores of the polymer matrices *(21)*. In addition, the diameter of the pores should be large enough to accommodate the enzymes and subsequently the substrate molecules. These GMA-PETA copolymers have pores with radii in the range 5–50 nm. Since the diameter of the PGA molecule *(22)* is

Table 2
Binding and Expression of PGA onto GMA-PETA[a] (PE Series), GMA-TRIM[c] (TM Series), and GMA-TMPTA[d] (74 Series) Copolymers

Polymer no.	CLD, %	Enzyme bound, IU/g[b]	Binding %	IME activity, IU/g	Expression %
PE7	40	158.5	31.7	ND[c]	ND
PE8	50	340.0	68.0	101.6	29.9
PE9	60	115.0	23.0	ND	ND
PE10	67.5	461.0	92.2	143.8	31.2
PE11	75	407.5	81.5	123.9	30.4
PE12	87.5	445.5	89.1	139.4	31.3
PE13	100	457.0	91.4	135.3	29.6
PE14	133	468.5	93.7	130.7	27.9
PE15	167	478.0	95.6	126.7	26.5
PE16	200	463.0	92.6	103.2	22.3
PE17	300	480.0	96.0	116.6	24.3
TM2	10	106.5	21.3	4.6	4.3
TM3	15	195.5	39.1	36.7	18.8
TM4	20	145.0	29.0	25.6	17.7
TM5	25	251.5	50.3	59.3	23.5
TM6	30	343.5	68.7	113.3	33.0
TM7	40	418.0	83.6	138.8	33.2
TM8	50	471.5	94.3	131.1	27.8
TM9	60	482.5	96.5	124.5	25.8
TM10	67.5	486.0	97.2	121.5	25.0
TM11	75	462.5	92.5	116.1	25.1
TM12	87.5	483.0	96.6	107.2	22.2
TM13	100	483.5	96.7	97.6	20.2
TM14	133	484.0	96.8	80.8	16.7
TM15	167	484.0	96.8	84.7	17.5
TM16	200	484.0	96.8	62.4	12.9
TM17	300	485.0	97.0	47.5	9.8
748	50	182.0	36.4	39.7	21.8
749	60	363.0	72.6	98.4	27.1
7410	67.5	450.0	90.0	151.6	33.7
7411	75	477.0	95.4	167.4	35.1
7412	87.5	479.0	95.8	157.6	32.9
7413	100	480.0	96.0	147.8	30.8
7414	133	485.0	97.0	136.8	28.2
7415	167	486.0	97.2	134.6	27.7
7416	200	491.0	98.2	108.0	22.0
7417	300	491.0	98.2	89.4	18.2

[a]GMA-PETA copolymers of varying CLD were prepared by taking mole ratios of GMA and PETA, 65.4 mL cyclohexanol, 5 g PVP, and 0.6 g AIBN.

[b]500 IU of PGA were loaded/g of polymers.

[c]ND; not determined.

[d,e]GMA-TRIM and GMA-TMPTA copolymers of varying CLD were prepared by taking mole ratios of GMA and PETA, 65.4 mL cyclohexanol, 5 g PVP, and 0.6 g AIBN.

Table 3
Pore Size Distribution, Pore Volume, and Surface Area in GMA-PETA Copolymers[a]

Polymer no.	CH:LA V/V	Pore size distribution, volume percent, radius in nm									PV[b] mL/g	SA[c] M^2/g
		<5	5–10	10–15	15–20	20–30	30–50	50–100	100–300	>300		
PE10	100:0	41.2	33.3	11.2	5.0	4.4	3.6	1.3	0.0	0.0	0.24	96.4
521	95:5	20.0	24.0	18.8	11.8	10.7	7.8	4.2	1.8	0.9	0.49	125.6
522	90:10	9.7	19.8	17.6	13.2	17.7	14.0	6.7	1.3	0.0	0.51	92.5
523	85:15	5.2	14.1	11.7	10.2	15.6	22.5	14.6	6.1	0.0	0.65	87.4
524	80:20	4.5	7.6	10.0	8.9	13.5	19.4	24.8	10.0	1.3	0.71	77.0

[a]GMA-PETA copolymers with 67.5% CLD were synthesized by using a mixture of cyclohexanol (CH) and lauryl alcohol (LA) as porogen at the indicated ratio, 5 g PVP, 0.6 g AIBN.

around 5 nm, large amounts of enzyme can be bound. High loading of enzyme causes internal diffusion limitations, ie barriers to free diffusion within the porous matrix *(23)*. Also, since the hydrophilicity of the matrix increases at higher CLD: (1) the matrix has lower affinity for the nonpolar substrate creating a partitioning, and (2) the product and side products being relatively hydrophilic may remain adsorbed, thereby creating product inhibition. As a result, the substrate concentration gradually decreases from bulk solution to the inside of the matrix. Thus, substrate depletion in the vicinity of the bound enzyme decreases the measured activity. This is manifested as a lower percent of expression in the highly crosslinked matrices. The expression of PGA bound on GMA-PETA copolymers is reasonably high, even at high values of CLD, which is opposite to those observed for GMA-DVB copolymers over similar compositions *(10)*. This is because of the increased hydrophilicity in these copolymers. Here the possibility of change in active conformation of the enzyme is lessened in contrast to the highly hydrophobic GMA-DVB copolymers.

Effect of Incorporation of Lauryl Alcohol as Porogen on Binding and Expression of PGA

A series of GMA-PETA copolymers were synthesized using a mixture of lauryl alcohol and cyclohexanol as porogen to study the effect of the nature of porogen on pore size, pore-size distribution, and correspondingly binding of PGA. The pore volume increased greatly (two to three times), whereas the surface area decreased when copolymers of a fixed CLD of 67.5% were synthesized with increasing relative volumes of lauryl alcohol (Table 3). The pore size distribution too broadened, with a greater increase in the density of larger pores. The amount of PGA bound on these copolymers was greater obviously owing to the great increase in pore volume (Table 4). The expression of bound enzyme, however, decreased. At a fixed CLD, the expression of bound PGA decreases with the increase in the relative volume of lauryl alcohol owing to greater steric crowding of the bound enzyme. As discussed earlier, this limits the permeability of the substrate into the product and out of the environment of bound PGA. The lower perceived apparent activity is owing to a decrease in the substrate concentration around the enzyme.

Immobilization of PGA on GMA-PETA-EGDM Terpolymers

Varied amounts of EGDM were incorporated in copolymer PE10 to subtly modify the hydrophilic character of GMA-PETA copolymers to obtain a series of GMA-PETA-EGDM terpolymers. The hydrophobicity of EGDM is marginally greater than PETA, but is less than DVB. The binding of PGA was seen to drop (relative to the corresponding GMA-PETA

Table 4
Binding and Expression of PGA onto GMA-PETA Copolymers Generated by Using a Mixture of Cyclohexanol and Lauryl Alcohol as Porogen[a]

Polymer no.	CLD, %	CH:LA V/V	Enzyme bound, IU/g[b]	Binding %	IME activity, IU/g	Exp., %[c]
521	67.5	95:5	472.5	94.5	137.0	29.0
522	67.5	90:10	463.5	92.7	107.5	23.2
523	67.5	85:15	459.5	91.9	106.1	23.1
524	67.5	80:20	408.5	81.7	74.3	18.2
525	75	95:5	475.5	95.1	102.2	21.5
526	75	90:10	480.0	96.0	104.1	21.7
527	75	85:15	471.5	94.3	92.9	19.7
528	75	80:20	424.5	84.9	70.0	16.5
529	87.5	95:5	477.5	95.5	95.9	20.1
5210	87.5	90:10	478.5	95.7	74.6	15.6
5211	87.5	85:15	468.5	93.7	79.6	17.0
5212	87.5	80:20	421.0	84.2	75.3	17.9
5213	100	95:5	478.0	95.6	102.3	21.4
5214	100	90:10	475.0	95.0	80.7	17.0
5215	100	85:15	475.0	95.0	86.5	18.2
5216	100	80:20	447.0	89.4	89.4	20.0
5217	133	95:5	477.5	95.5	87.4	18.3
5218	133	90:10	480.5	96.1	79.7	16.6
5219	133	85:15	479.0	95.8	59.9	12.5
5220	133	80:20	473.0	94.6	66.2	14.0

[a]GMA-PETA copolymers with indicated CLD were prepared by using a mixture of cyclohexanol (CH) and lauryl alcohol (LA) as porogen at indicated ratio, 5 g of PVP and 0.6 g AIBN.
[b]500 IU of PGA were loaded/g of polymer.
[c]Expression.

copolymer) as the relative mole ratio of EGDM was increased, but the expression of bound enzyme increased marginally when the EGDM amount exceeded 70% (Table 5). The terpolymer GPE8 shows better activity of bound PGA enzyme than the GMA-EGDM copolymer GPE10.

To summarize, a comparison of the results of binding and activity of bound PGA obtained with the various copolymers, namely GMA-DVB *(9,10)*, GMA-PETA, GMA-TRIM, and GMA-TMPTA, is presented in Fig. 1. TRIM is more hydrophobic than PETA and TMPTA, but less than DVB. Greater enzyme binding occurs on GMA-TRIM and GMA-DVB copoly-

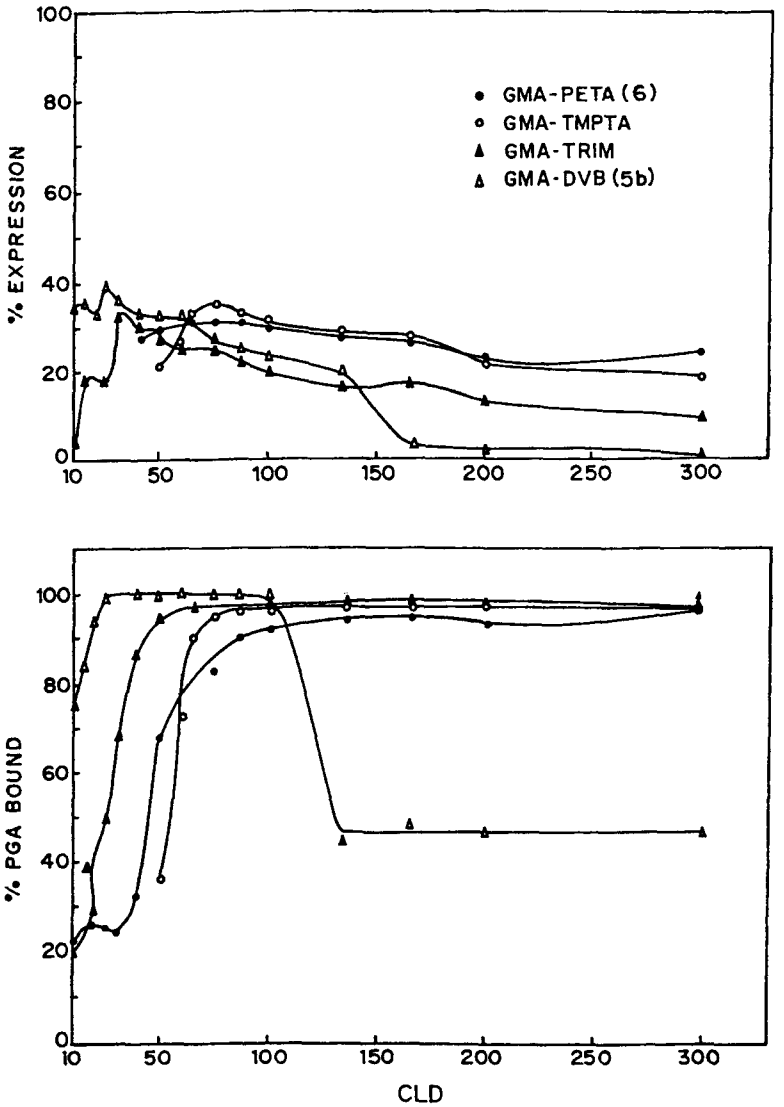

Fig. 1. Comparison of binding and expression of bound PGA on different polymer matrices.

mers compared to the relatively hydrophilic GMA-PETA and GMA-TMPTA copolymers of similar CLD. In GMA-DVB and GMA-TRIM, the enzyme expression decreased with the relative mol% of crosslinking comonomer. The excessive hydrophobicity interferes with the enzymatic catalysis, leading to loss in activity.

Table 5
Binding and Expression of Enzyme PGA onto GMA-PETA-EGDM Terpolymers[a]

Polymer no.	PETA: EGDM mol/mol	Enzyme bound[b], IU/g	Binding, %	IME activity, IU/g	Expression, %
PE10	100:0	461.0	92.2	143.8	31.2
GPE1	90:10	453.5	90.7	140.6	31.0
GPE2	80:20	452.0	90.4	136.9	30.3
GPE3	70:30	453.0	90.6	141.3	31.2
GPE4	60:40	419.0	83.8	118.6	28.3
GPE5	50:50	462.5	85.3	139.7	30.2
GPE6	40:60	453.5	90.7	132.4	29.2
GPE7	30:70	422.5	84.5	135.6	32.1
GPE8	20:80	427.5	85.5	147.5	34.5
GPE9	10:90	433.0	86.6	142.9	33.0
GPE10	0:100	373.0	74.6	123.4	33.1

[a]GMA-PETA-EGDM terpolymers with 67.5% CLD were prepared using the indicated amount of PETA and EGDM, 65.4 mL cyclohexanol, 5 g of PVP and 0.6 g AIBN.
[b]500 IU of PGA were loaded/g of polymers.

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