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Carriers from triacrylate for penicillin acylase immobilization

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The properties of carriers made of acrylonitrile (AN) or butyl acrylate (BA) and crosslinked by trimethylolpropane trimethacrylate monomer (TMPMA) or trimethylolpropane triacrylate monomer (TMPA) are discussed. The extent of aminolysis of triacrylate copolymers depends on the comonomer composition. The carriers crosslinked with TMPMA have greater porosity and larger mean pore size than carriers that were crosslinked by TMPA. The enzyme activity on carriers that were obtained by ester group aminolysis in BA copolymers is higher than on carriers made of AN. It is found that TMPMA/BA carriers are the best for immobilization of peniccilin acylase. Copyright © 1996 Elsevier Science Ltd.

(Keywords: TMP tri(methyl)acrylates; aminolysis; penicillin acylase)

INTRODUCTION

The properties of carriers made of triacrylate copolymers, namely trimethylolpropane‡ triacrylate (TMPA) and acrylonitrile (AN) with a degree of crosslinking that amounts to 30% of TMPA, and the activities of immobilized enzymes in relation to native enzymes, i.e. acylase, glucoamylase and peroxidase, have already been described[1]. The best activities of enzyme–carrier systems were observed for carriers with the widest pore size distribution. Generally, the pore radii determined by inverse steric exclusion chromatography (i.s.e.c.) are very small (about 2.8 nm).

It follows from the research of Flodin[2,3] and Reinholdsson[4] that increasing the monomer dilution with inert diluents (above 1 : 1 v/v) in suspension polymerization causes an increase of the mean pore size of trimethylolpropane trimethacrylate (TMPMA) polymers.

It has been shown by our experiments[5,6] as well as those of other research workers[7–9] that enzyme activity on carriers that were obtained by ester group aminolysis in butyl acrylate (BA) copolymers and ethylenedimethacrylate is higher than on carriers made of acrylonitrile (AN)[1]. On the other hand, it is much more difficult to aminolyse the ester group of acrylates than the nitrile group, but the hydrolysis of esters occurs very frequently when the reaction is carried out under more severe conditions. It was found that the network obtained using pentaerythritol triacrylate (PENTA) as the crosslinking monomer undergoes degradation down to dissolvable polymer, even under mild conditions. The reason is aminolysis and hydrolysis of ester groups, and because of these reactions, *N*-amidoamine groups, carboxyl groups and equivalent amounts of hydroxyl groups are made[10].

An attempt was therefore made to 'stiffen' the polymer network using, as the crosslinking substance, TMPMA monomer instead of TMPA.

The aim of this work is to compare the properties of carriers, for immobilization of acylase, made of acrylonitrile or butyl acrylate and crosslinked by trimethacrylate or triacrylate monomer.

MATERIALS AND METHODS

The copolymers, i.e. TMPMA/AN, TMPMA/BA, TMPA/AN and TMPA/BA (60/40), and terpolymers, i.e. TMPMA/VA/AN, TMPMA/VA/BA, TMPA/VA/AN and TMPA/VA/BA (40/20/40) (VA = vinyl acetate), have been obtained by suspension polymerization as described previously. The ratio of volume of diluent mixture, i.e. cyclohexanol and hexadecane (CHD), cyclohexanol and 2-ethylhexanol (CE), toluene and hexadecane (THD), and cyclohexanol and dodecanol (CD), to monomer volume was 1 : 1 or 2 : 1, as was coded in symbols[11]. The reactants were purchased from Aldrich.

The matrices were modified by treating them with a boiling solution of ethylenediamine (EtDA) in water or toluene[1,5]. The concentration of EtDA and the aminolysis time are shown in *Table 1*. After this reaction the carriers were washed with methanol and then rinsed three times successively with 0.1 M solution of HCl and NaOH.

Amine group concentration was determined according to the method of Hecker[12] and calculated from the content of organic nitrogen. Carboxylic and amine group concentrations were measured also according to our own method[13].

The carrier characterization methods have been

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‡ The IUPAC name for TMP is 2-ethyl-2-hydroxymethyl-1,3-propanediol

Table 1 Parameters of aminolysis

Symbol	Amine concentration (%)	Diluent[a]	Time (h)
A	20	t	1.5
B	20	t	3.5
C	50	t	5.0
D	75	t	4.5
E	95	t	5.0
F	95	w	5.0
G	75	w	5.0
H	75	w	2.5
I	75	w	3.5
K	95[b]	w	5.0
L	95	w	3.5
M	95	w	10.0
N	20	t	5.0

[a] Toluene (t) or water (w)
[b] Swelling time was 72 h

described previously[1]. True density was measured pycnometrically in cyclohexane. The density of the samples in the swollen state was determined by a pycnometric method in water. Porosity was calculated from these densities. Water regain was evaluated by the centrifugation method. Inverse steric exclusion chromatography (i.s.e.c.) was used to characterize the copolymers swollen with water[14].

Enzymes were immobilized by the glutaraldehyde method. The procedures for penicillin acylase attachment and enzyme assay have been published elsewhere[7]. For the purposes of this work, the methods were not modified. The amount of enzyme that catalysed the formation of 1 μmol of 6-aminopenicillanic acid from penicillin G within 1 min under the test conditions (37°C, pH = 7.8) was defined as one activity unit (U). Because a crude penicillin acylase (0.73 U mg^{-1}) was used for immobilization, the preferential bonding ability of the active enzyme was detected. This parameter was expressed as the ratio of specific activities of bound to native enzyme.

RESULTS AND DISCUSSION

As shown from the introductory experiments, the TMPA and TMPMA networks and copolymers of acrylonitrile and butyl acrylate, crosslinked with triacrylates (just as were PENTA networks[10]), undergo degradation because of a hydrolysis reaction during aminolysis with ethylenediamine. A linear polymer is an intermediate product of this reaction (*Figure 1*). As a result of aminolysis, *N*-amidoamine groups and hydroxyl groups, in the stoichiometric amount, are made. Carboxyl and hydroxyl groups result from the hydrolysis reaction. The hydrophilicity of the network increases with the progress of aminolysis; the amine diffuses deep into agglomerates

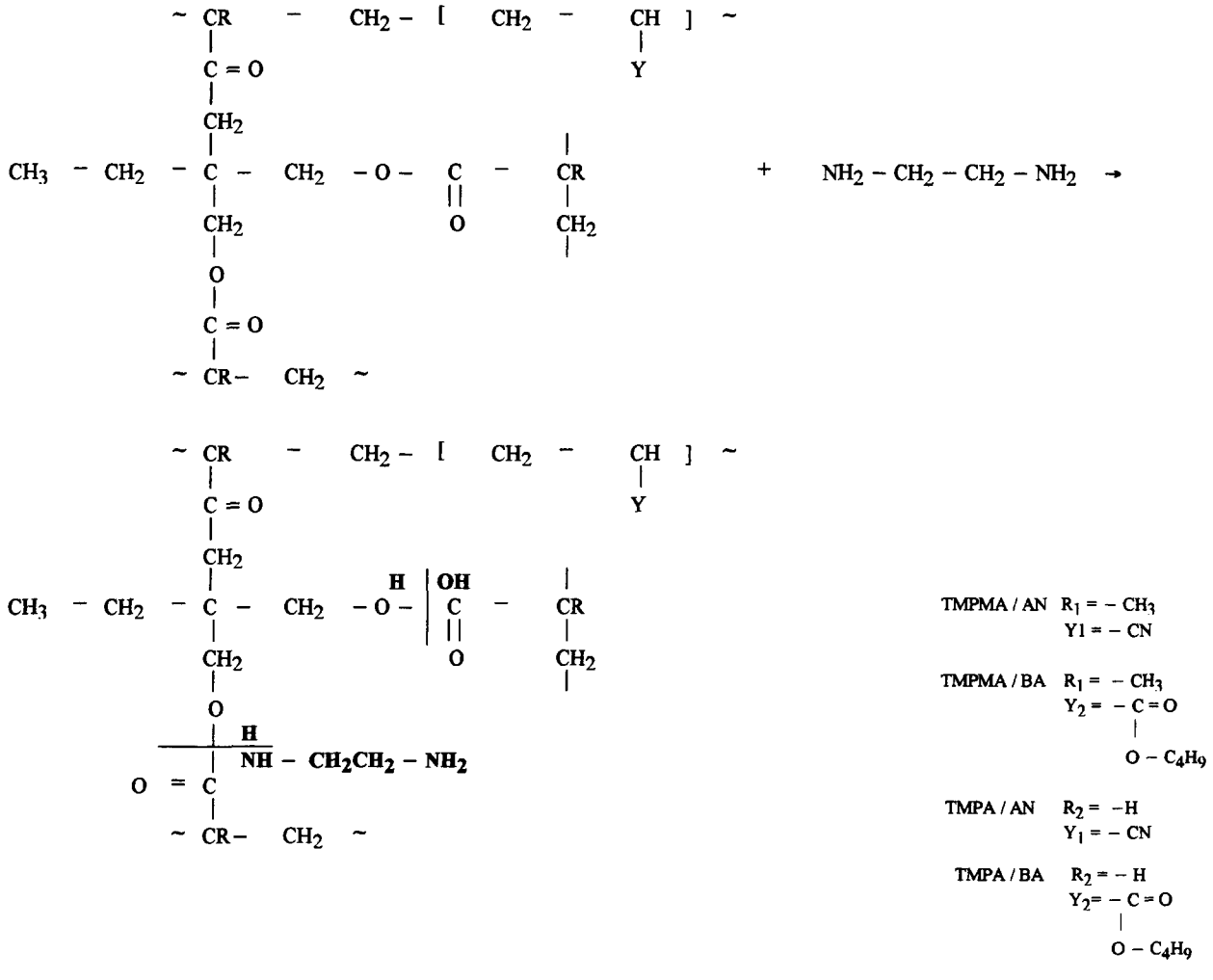

Figure 1 Reaction of hydrolysis during aminolysis with ethylenediamine

and destroys them, and even, for porous copolymers, hydrogels are made.

The extent of aminolysis of triacrylate copolymers, carried out under the same conditions, depends significantly on the comonomer composition. The nitrile group undergoes aminolysis much easier than the ester group[1,5,6].

The aminolysis of TMPA/AN copolymers was carried out in 20% toluene solution of amine for a short time from 1.5 to 3.5 h, in order to limit hydrolysis of the ester groups[1]. The carriers obtained had an appropriate concentration of amine groups on the agglomerate surface and retained a porous structure or they had the structure of an expanded gel[6].

As shown recently, a high concentration of amine groups on carriers for the immobilization of enzymes is a disadvantage, because a large amount of protein is bound on them, even by means of a few groups, and blocks enzymes. The enzyme loses its activity, because of deformation of an active centre[1].

To obtain a low comparable concentration of amine groups in the carriers investigated, the aminolysis of groups in copolymers was performed under various conditions, which are shown in *Table 1*.

In water solution, the aminolysis must be carried out for a long time and at high concentrations of amine; while in toluene solution, the conditions of the reaction are very mild.

According to the aim of this work, the group of carriers made of methacrylic and acrylic acid esters and crosslinked with TMPMA or TMPA were chosen for comparison. Acrylonitrile and butyl acrylate were the comonomers and, in this particular case, a terpolymer composed of vinyl acetate mers was also aminolysed. The ester groups in the mers of vinyl acetate were hydrolysed under the conditions of aminolysis[15].

The properties of TMPMA/AN and TMPA/AN carriers obtained from copolymers with crosslinking monomer concentration of 40% and synthesized in the presence of diluents that had various solubility parameters are collected in *Table 2*. The aminolysis was performed in 20% ethylenediamine solution in toluene.

The concentration of amine groups in the carriers obtained was less than 1 mmol g^{-1}, while the concentration of carboxyl groups varied and depended on the structure of the carriers and the conditions of aminolysis. CE/N and C2E/N copolymers were synthesized in the presence of very good diluents, cyclohexanol and 2-ethylhexanol, except that in making the latter the network was made with a higher dilution of monomers. The C2E/N carrier is characterized by high water regain; and C2EV/N, modified AN/VA/TMPMA terpolymer, has similar water regain and porosity to the C2E/N carrier, though there are more hydroxyl groups in this terpolymer after hydrolysis of the acetate groups in the mer of vinyl acetate[15]. The porosities of all carriers determined by pycnometry in the swollen state are similar, as shown in *Table 2*.

Table 2 Properties of TMPMA or TMPA and AN carriers

Symbol[a]	Water regain (g g^{-1})	Group concentration (mmol g^{-1})		Porosity
		$-NH_2$	$-COOH$	
TMPMA + AN				
CHD/N	0.99	0.92	1.45	0.51
CE/N	1.14	0.73	1.20	0.55
C2E/N	2.51	0.85	0.61	0.74
C2EV/N	2.76	0.84	0.96	0.76
TMPA + AN				
CHD/N	0.94	0.99	0.63	0.53
CE/B	1.36	0.70[b]	–	0.49
C2E/B	2.92	0.23[b]	–	0.68

[a] *Notation*: for example, C2EV/N means C ≡ cyclohexanol, 2 ≡ 2 : 1 (v/v) diluent : monomer ratio, E ≡ 2-ethylhexanol, V ≡ terpolymer containing VA, N ≡ symbol for amine concentration in *Table 1*

[b] Hecker method

It may be possible that the networks that are made under better solvation conditions are composed of smaller agglomerates but are characterized by a greater amount of cycles, so swelling is bigger. The structure of the expanded gel is made after aminolysis, just as in other types of carriers, in which the primary agglomerates of the copolymer are broken up but the fluctuation of gel density is still big, so it is possible to distinguish regions filled by diluent—'pores'[6].

Copolymers of TMPA/AN undergo aminolysis more easily than copolymers crosslinked by TMPMA. The C2E/B carrier obtained from the copolymer that was synthesized in the presence of high dilution of monomers is characterized by greater porosity and higher water regain than the CE/B carrier. In comparison with the TMPMA/AN carriers, TMPA/AN are much more elastic, so their porosity is smaller. This is the result of a bigger capacity for whirls in the network. Differences of carrier properties, which arise from employing various diluents during copolymerization of monomers, are not significant.

The properties of carriers obtained from TMPMA and TMPA copolymers and from butyl acrylate are presented in *Table 3*. From all four groups, the aminolysis of TMPMA/BA copolymers is the most difficult. The copolymers containing about 0.5 mmol g^{-1} amine groups are barely synthesized after long heating (10 h) in 95% water solution of amine. The aminolysis is easier when the amount of diluents is large. The TMPMA/BA carriers are characterized by higher swelling and greater porosity in the swollen state than the others. A carrier made of C2EV/L terpolymer, which undergoes aminolysis easier, has the greatest porosity.

The network of TMPMA homopolymer is much easier to aminolyse than its copolymers. It is enough to heat it in 20% solution of amine in toluene to obtain the carrier with functional groups (*Table 3*).

The porosity of all TMPMA/BA carriers is very great. It can be assumed that they are in the expanded gel state (*Table 3*).

The most favourable parameters for the aminolysis of TMPA/BA carriers were sought by changing the reaction time and amine concentration. The concentration of amine groups was low when the reaction was carried out in 20 and 50% toluene solution of amine for 1.5 and 5 h. The very small water regain of these carriers was surprising, because it was significantly smaller than the water regain value of corresponding carriers crosslinked with TMPMA (*Table 3*). Only aminolysis performed under severe conditions, in 95% water solution of amine for 5 h (F in *Table 1*), allowed one to obtain carriers with large swelling but with high concentration of carboxyl groups too. It can be suspected that these carriers are gels

Table 3 Properties of TMPMA or TMPA and BA carriers

Symbol	Water regain ($g\,g^{-1}$)	Group concentration ($mmol\,g^{-1}$) $-NH_2$	$-COOH$	Porosity
TMPMA + BA				
THD/L	1.35	0.30	1.10	–
/M	1.68	0.40	0.89	0.66
CE/L	0.87	0.20	1.50	–
/M	0.96	0.46	0.76	0.52
C2E/L	1.83	0.20	1.20	–
/M	2.09	0.54	1.17	0.70
C2EV/L	2.58	0.61	0.74	0.74
C2E/N[a]	1.71	0.22	0.70	0.60
T2HD/N[a]	1.78	0.53	0.45	0.67
TMPA + BA				
THD/A	0.96	0.11[b]	–	–
/C	1.03	0.23[b]	–	–
/F	1.31	0.90[b]	–	–
CE/A	0.35	0.07[b]	–	–
/C	0.32	0.34[b]	–	–
/F	2.93	1.80	1.20	–
CD/A	0.57	0.06[b]	–	–
/C	0.56	0.42	1.07	–
/I	1.07	1.18	0.85	–
/F	1.69	1.40[b]	–	–
CEV/G	12.40	ND[c]	ND	–
/I	11.20	3.73	1.78	–

[a] Homopolymer
[b] Hecker method
[c] Not possible to determine

Table 4 Characteristics of carriers in the swollen state (i.s.e.c. method)

Symbol	Porosity	Surface area[a] ($m^2\,cm^{-3}$)	Pore radius, mean (nm)
TMPMA + AN			
CHD/N	0.56	321	2.76
CE/N	0.66	213	4.16
C2E/N	0.89	164	5.40
TMPA + AN			
CHD/N	0.49	347	2.55
CE/B	0.64	242	3.65
TMPMA + BA			
THD/M	0.62	244	3.63
CE/M	0.63	267	3.32
C2E/M	0.84	245	3.62
C2EV/L	0.85	230	3.85
C2E/N	0.76	244	3.62
TMPA + BA			
CD/F	0.62	423	2.09
CE/F	0.71	361	2.45

[a] Square metres per cubic centimetre of polymer

with a relatively regular disposition of chains in unit volume.

The terpolymer CEV/G undergoes aminolysis under milder reaction conditions than the other TMPA/BA copolymers. The network of this copolymer swells very strongly and it is 'impervious'. The carrier is a hydrogel.

Copolymers obtained from a mixture of monomers that was diluted by a double volume of diluents during aminolysis underwent dissolution as a result of network hydrolysis.

Additional information about the structure of carriers in the swollen state was supplied by applying the i.s.e.c. method. Results of the experiments are set out in *Table 4*. The porosity determined by the i.s.e.c. method is in most cases bigger than the porosity determined in the swollen state by the pycnometric method (*Tables 2–4*). The carriers crosslinked with TMPMA, especially those with AN, have greater porosity and mean pore size than carriers crosslinked by TMPA. The CHD/N carrier, which was obtained under bad solvation conditions, is the exception.

It is very interesting that all TMPMA/BA carriers have very similar mean pore radii (about 3.6 nm), though the pore volumes of carriers obtained from copolymers that were synthesized in the presence of a double amount of diluents are much bigger.

The carrier made of TMPA/BA copolymer (CD/F) is characterized by especially small pores. Measurement by the i.s.e.c. method confirms its gel character (*Table 4*).

The molecular structure of carriers influences the capacity for immobilization of acylase and its activity. TMPMA/AN carriers (*Table 5*) adsorb large amounts of

Table 5 Activity of acylase on TMPMA or TMPA and AN carriers

Symbol	Bound protein ($mg\,cm^{-3}$)	Enzyme activity ($U\,cm^{-3}$)	Ratio of active to bound protein (%) From protein balance	From activity balance	Specific activity ($U\,mg^{-1}$)
TMPMA + AN					
CHD/N	6.0	3.6	76.6	52.0	0.61
CE/N	7.3	7.3	126.1	80.4	1.00
C2E/N	7.1	5.7	101.6	77.3	0.80
C2EV/N	8.9	5.0	71.8	55.3	0.57
TMPA + AN					
CHD/N	3.6	4.5	147.5	–	1.12
CE/B	5.6	3.9	88.6	–	0.70
C2E/B	3.6	0.3	9.6	–	0.03

Table 6 Activity of acylase on TMPMA or TMPA and BA carriers

Symbol	Bound protein ($mg\,cm^{-3}$)	Enzyme activity ($U\,cm^{-3}$)	Ratio of active to bound protein (%) From protein balance	From activity balance	Specific activity ($U\,mg^{-1}$)
TMPMA + BA					
THD/L	6.4	3.7	73.1	46.1	0.58
THD/M	8.7	7.8	114.6	78.1	0.91
CE/L	7.2	6.7	117.3	86.7	0.93
CE/M	8.4	7.7	116.3	84.0	0.92
C2E/L	7.2	3.2	56.9	38.0	0.45
C2E/M	9.6	8.2	108.3	71.3	0.86
C2EV/L	9.7	9.6	125.0	77.4	0.99
C2E/N[a]	8.3	5.1	77.8	57.5	0.62
THD/N[a]	6.4	3.4	67.9	37.9	0.54
TMPA + BA					
THD/A	2.2	0.5	–	–	–
/C	2.3	0.9	–	–	–
/F	2.8	0.7	31.2	–	0.24
CE/A	1.6	0.1	–	–	–
/C	3.4	2.2	81.4	–	0.64
/F	6.8	4.3	80.5	–	0.63
CD/A	4.0	2.5	79.6	–	0.62
/C	5.7	4.2	93.3	–	0.74
/I	4.6	3.8	106.1	–	0.83
/F	7.2	7.2	127.0	–	1.00
CEV/G	12.0	8.7	92.2	–	0.73
/I	8.1	4.9	76.4	–	0.60

[a] Homopolymer

protein but are not very active. The best activity and selectivity regarding acylase were observed for the CE/N carrier with the biggest mean pore size. It is surprising that the C2E/N carrier, with greater volume of pores, binds a similar amount of protein but its activity is smaller. TMPA/AN carriers bind the least amount of active protein, though the porous structure parameters of both carriers (CE/N and CE/B) are similar.

Finally, TMPMA/BA (*Table 6*) proved the best carriers for acylase. Carriers with high concentration of amine groups bind more enzyme and the protein is then very active. The highest activity of acylase is observed for the C2EV/L enzyme–carrier system, which is obtained from BA/VA/TMPMA terpolymer with the highest water regain and volume of pores and with large mean pore radius (3.85 nm). It is a similar situation with C2E/N carrier, which has large water regain and volume of pores too, though the activity of the immobilized enzyme is lower. The properties of TMPMA/BA carriers are similar to the characteristics of G-1 carrier, which binds a lot of active acylase[5–9].

TMPA/BA carriers with a degree of crosslinking that amounts to 40% of TMPA are not suitable for immobilization of acylase, because of their small pores, which is connected with the gel character of the network. This fact was surprising, because carriers obtained by aminolysis of TMPA/AN copolymers with 30% degree of crosslinking (e.g. CE-30) were very good for immobilization[1]. This can be explained by the 'stiffening' influence of acrylonitrile mers. The concentration of these mers is higher in carriers that have 30% of TMPA (CE-30). The presence of stiff AN mers allows the structure of the expanded gel to be retained after aminolysis. If mers of AN are exchanged for elastic BA mers, such copolymers are gels after aminolysis.

The hydrogel CEV/G, which is characterized by very large water regain, just like PENTA carriers[10], binds plenty of acylase but it is not very active after immobilization.

CONCLUSIONS

By comparison of four types of carriers (TMPMA/AN, TMPA/AN, TMPMA/BA and TMPA/BA), which were obtained by aminolysis of functional groups, it was shown once again that carriers having the structure of an expanded gel are the most favourable for keeping the activity of immobilized acylase[6–9]. They are made by aminolysis of a fraction of the groups (10–20%) on the surface of porous agglomerates of copolymers. If the copolymer network is built up of a crosslinking substance that has 'stiff' crosslinks (divinylbenzene, ethylene dimethacrylate, TMPMA) or consists of stiff AN mers, swelling of carriers takes place owing to separation of agglomerates modified superficially. This is possible due to the large expansion of chains in the network. This is why the water regain pore volume and mean pore radius of TMPMA/AN carriers, obtained in the presence of thermodynamically good diluents, are bigger than for TMPA/AN. Carriers made of BA/VA/TMPMA terpolymer have the greatest swelling and mean pore sizes, because of additional hydrolysis of acetate groups and formation of hydroxyl groups.

TMPA/BA carriers with large elasticity of the network (made of elastomers) are not useful for acylase immobilization, because they are characterized by small mean pore size.

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