Preparation by Suspension Polymerization of Porous Beads for Enzyme Immobilization

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Synopsis

Porous beads have been prepared by suspension polymerization of acrylic monomers and divinyl benzene in the presence of a porogen. The porosity varies greatly with the polymer composition, with the amount and type of porogen and with the conditions for the preparation. The enzyme binding capacity and activity has been determined for a lipase. The best results are found for crosslinked poly(methyl methacrylate) beads while other polyacrylates are less effective. Compositions based on poly(methacrylic acid) are not suitable for enzyme binding.

INTRODUCTION

The advantages of immobilization of enzymes on solid supports are known to be due mainly to the possibility of running enzymatic reactions continuously and saving time a purification step for removing enzyme from the product stream. In addition, sometimes the immobilized enzymes will have prolonged activity in comparison with the free enzyme.[1]

Various methods exist for immobilization of enzymes[1] and these may be divided into physical methods based on molecular interactions between enzyme and carrier and chemical methods based on formation of covalent bonds. In the present investigation the synthesis of porous beads has been studied which are suitable for physical binding of an enzyme. The binding capacity has been determined as a function of composition and method of preparation of the beads.

The enzyme of interest is a lipase* which may be used in connection with the synthesis of esters and transesterification of fats.

Suspension polymerization was the method of choice for preparation of the solid particles since a relatively uniform particle size distribution may be obtained if proper stabilization of the polymerizing beads is found. Such uniformity will secure the flow and minimize the pressure drop over a packed column of such beads containing the enzyme.

Acrylic polymer beads crosslinked with divinyl benzene (DVB) are prepared from various combinations of monomers, particularly methyl methacrylate (MMA), methyl acrylate (MA), and methacrylic acid (MAA), but also a few containing ethyl acrylate (EA) and *N,N*-dimethyl acrylamide (*N,N*-DMA). Procedures for suspension polymerization of acrylic monomers are described

*The lipase belongs to a class of triacyl glycorol hydrolases that is patented in connection with solid supports.[2]

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mainly in the patent literature, however, often with lack of details, although the preparation of crosslinked poly(methacrylic acid) is reasonably well described.[3]

Porosity is obtained in the present study by polymerization in the presence of an inert diluent (porogen), particularly octane, petroleum ether, or cyclohexane. Originally porous beads of crosslinked polystyrene for gel permation chromatography were prepared by Moore[4] by such a technique, and the use of several different porogens for poly(styrene/DVB) have later been investigated.[5-8] Only few reports are found concerning the preparation of porous acrylic polymers.[3]

EXPERIMENTAL

Suspension Polymerizations: General Procedures

In all preparations 50 mL of monomer with the appropriate amount of porogen was suspended in 300 mL suspension medium. Generally, the best suspension stabilization was obtained by adding a combination of 32 g hydroxyapatite [$3Ca_3(PO_4)_2 \cdot Ca(OH)_2$], freshly prepared according to a literature procedure[9] and 0.02 g poly(vinyl alcohol) (PVA), Wacker 25/140. Benzoylperoxide (0.75 g) (active ingredient) was added to the monomer prior to suspension. The DVB additions listed in the tables are based on the active DVB content in the commercial material (approx. 52%), and the other constituents are included in the total monomer charge. The polymerization was carried out in a 1-L round-bottomed flask equipped with a reflux condenser, a thermometer, and an agitator with a Teflon blade. The rate of agitation was controlled and with some minor deviations the stirrer was set generally at 220 rpm. The beads were washed thoroughly with water and in the case of the MAA and *N,N*-DMA containing beads washings were performed initially with 2*N* HCl solution. After final washings with cold methanol the beads were dried in a vacuum oven at 40°C for 4 days to remove the porogen. Generally four days was enough to remove the porogen quantitatively. Size distribution of the beads was determined with BBS standard Sieves. Resins containing MA, MMA, and EA were unable to absorb water after removal of the porogen. Therefore, they had to be treated with an icecold 50/50 methanol/water mixture in order to restore this property. After washing three times with water, the resins were stored wet. Specific conditions regarding the preparation of the individual polymers are given in the tables.

Enzyme Loading and Enzymatic Activity

The amount of enzyme bound (loaded) to the support was determined by means of lipase units (LU),[10] where 1 LU is the amount of enzyme, which under the given conditions[10] liberates 1 μmol titratable butyric acid per minute by hydrolysis of glyceryl tributyrate (tributyrin). No remaining enzyme activity in the immobilization media was set to correspond to 100% enzyme binding equal to 30000 LU/g dry polymer.

The activity of the immobilized enzyme is expressed in terms of batch interesterification units (BIU), where 1 BIU is defined as 1 μmol palmitic acid incorporated into glycerol trioleate under the standard conditions: 12 mL equimolar mixture of palmitic acid and glycerol trioleate (56.5 mmol of each)

TABLE I
Preparation and Properties of Porous Beads of MAA + DVB[a]

No.	MAA (mol %)	DVB (mol %)	Porogen[b] (wt %) on monomer	Yield (wt %)	Enzyme binding (%)	Enzyme activity (BIU/g)
1	82.9	8.9	5 T	90	17	0
2	82.9	8.9	10 T	90	23	0.3
3	86.8	6.8	34 O	94	0	0
4	86.8	6.9	50 O	94	0	0
5[c]	68.5	7.1	50 O	94	0	0
6	86.7	6.9	50 B	90	0	0
7	86.7	7.3	50 B	80	0	0
8	86.7	6.9	82 B	90	0	0

[a]Suspension polymerization carried out for 12 h, at 70°C with $CaCl_2$ solution (1.3 g/mL) as the suspension medium. General conditions are listed under Experimental.
[b]T = toluene, O = octane, B = benzin (pet. ether), bp 120–140°C.
[c]16 mol % *N,N*-DMA added.

in petroleum ether is reacted at 40°C in the presence of 275 mg immobilized enzyme.

Electron Microscopy

Beads were examined as such for surface structure and for cross-sectional features after mechanical fracture at room temperature. Standard procedures were used for preparation of the samples and they were examined on a Philips SEM 505. The magnifications are given by the scales on the micrographs.

RESULTS AND DISCUSSION

The surface properties of the porous resins were varied by combining in various ways the acrylic monomers. Initially, series of crosslinked poly(methacrylic acid)s were prepared (Table I), with the intention of investigating if carboxylic acid groups would be suitable for binding the enzyme to the porous beads. Since little or no binding was found, other series of acrylic beads were prepared where first crosslinked copolymer resins of methacrylic acid and methyl methacrylate were investigated (Table II). Although some

TABLE II
Preparation and Properties of Porous Beads of MAA + MMA + DVB[a]

No.	MAA (mol %)	MMA (mol %)	DVB (mol %)	Porogen[b] (wt %) on monomer	Yield (wt %)	Enzyme binding (%)	Enzyme activity (BIU/g)
9	77.5	6.5	8.3	4 O	90	20	
10	77.5	6.5	8.3	8 O	90	20	0.3
11	76.7	13.3	7.8	5.5 O	90	0	0
12	53.3	26.7	7.8	5.5 O	90	0.7	0
13	40.0	40.0	7.8	5.5 T	90	0.7	0

[a]Suspension polymerization carried out for 12 h at 70°C with $CaCl_2$ solution (1.3 g/mL) as the suspension medium. General conditions listed under Experimental.
[b]O = octane, T = toluene.

TABLE III
Preparation and Properties of Porous Beads of MA + DVB[a]

No.	MA (mol %)	DVB (mol %)	Porogen[b] (wt %) on monomer	Yield (wt %)	Enzyme binding (%)	Enzyme activity (BIU/g)
14[c]	73.1	6.4	35 O	90	59	1.4
15	86.7	6.9	66 B	90	62	0.7
16	86.1	7.2	65 B	80	58	1.3
17[d]	70.6	7.4	62 B	80	57	2.2
18	84.6	8.0	65 B	70	43	1.2
19[e]	70.6	7.4	62 B	90	80	7.8

[a]Suspension polymerization carried out for 20 h at 65°C with 1.0*M* NaCl as suspension medium. General conditions listed under Experimental.
[b]O = octane, B = benzin (pet. ether), bp 120–140°C.
[c]14.6 mol % MAA added.
[d]15.2 mol % EA added.
[e]15.2 mol % MMA added.

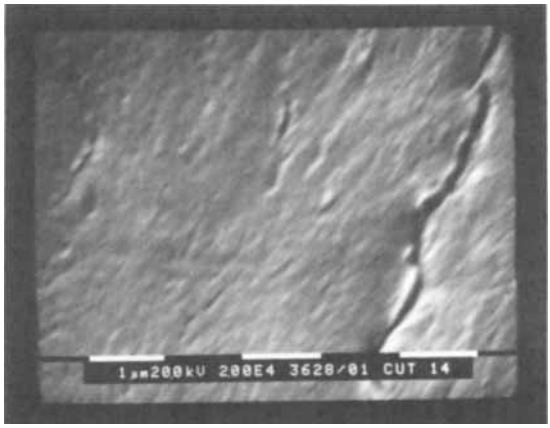
improvement in the amount of enzyme binding was found, the measured enzyme activity was essentially nil. Much better results were obtained with crosslinked polyacrylic esters without any methacrylic acid content and the first series were based on methyl acrylate (Table III), including a few copolymers with ethyl acrylate and methyl methacrylate. However, the highest activity was obtained with crosslinked poly(methyl methacrylate) as presented in Table IV.

In case of the crosslinked poly(methacrylic acid) beads in the two initial experiments only 5 and 10% porogen was added as seen in Table I, since previous work[11] had indicated that pore radii of 200–700 nm should be

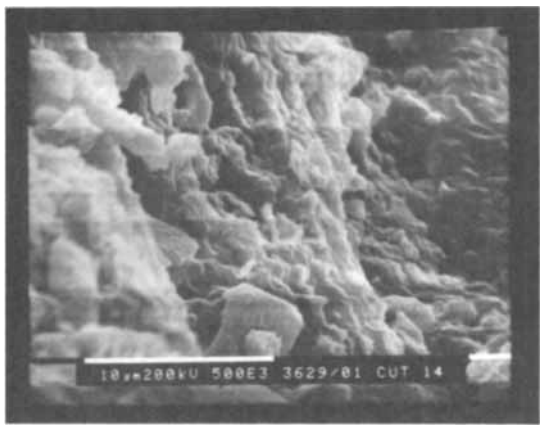
TABLE IV
Preparation and Properties of Porous Beads of MMA + DVB[a]

No.	MMA (mol %)	DVB (mol %)	Porogen[b] (wt %) on monomer	Yield (wt %)	Enzyme binding (%)	Enzyme activity (BIU/g)
20	84.2	8.2	65 B	95	100	17.1
21	85.9	7.3	65 B	80	99	15.3
22	85.9	7.3	24 B	90	1	0
23	84.8	7.9	33 B	90	19	0.7
24	84.8	7.9	50 B	90	100	1.1
25	84.8	7.9	66 B	90	100	1.5
26	79.2	10.7	51 B	90	100	2.7
27	84.8	7.9	66 C	90	0	0
28	84.8	10.7	66 C	90	0	0
29	84.8	7.9	66 C	90	100	4.4
30	83.6	8.6	66 B	90	100	4.6
31	83.6	8.6	66 C	90	81	1.4
32	83.6	8.6	50 C	90	42	7.4

[a]Suspension polymerization carried out for 20 h at 65°C (no. 29–32 at 70°C) with pure water as suspension medium except no. 20 and 21 where 1.0*M* NaCl was used. In no. 25–32 the hydroxyapatite was omitted from the stabilizer system; otherwise general conditions as listed under Experimental.
[b]B = benzin (pet. ether), bp 120–140°C, C = cyclohexane.



(a)



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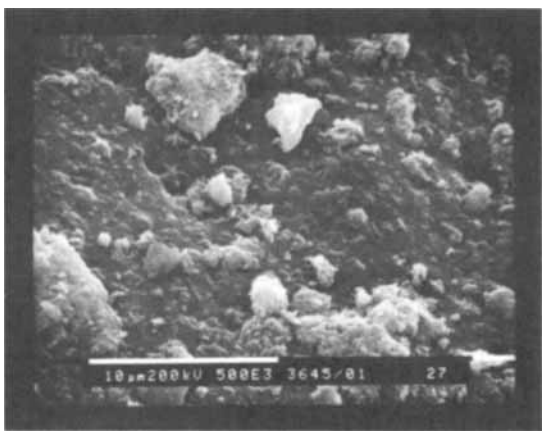
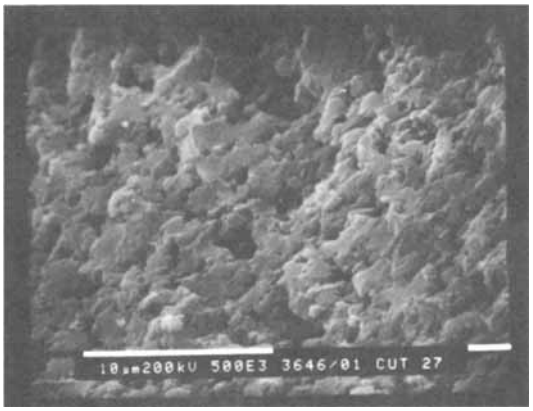


(c)

Fig. 1. Scanning electron micrographs of crosslinked poly(methacrylic acid)s: (a) polymer no. 2, surface; (b) polymer no. 2, cross section; (c) polymer no. 4, surface; (d) polymer no. 4, cross section.



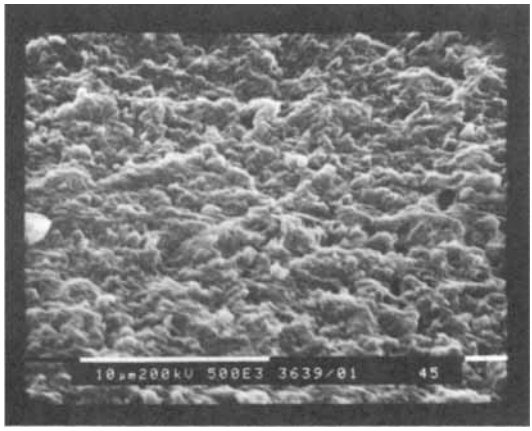
(d)

Fig. 1. (*Continued from the previous page.*)

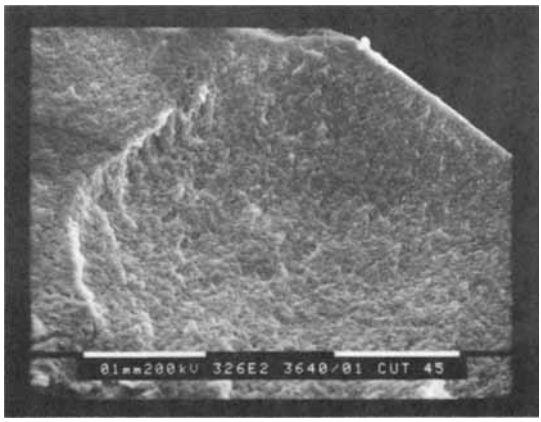
obtainable with such amounts added. Higher amounts of porogen was added in the other experiments of this series. However, it was found that the stability of the suspension of the monomers decreases with increasing amounts of porogen added and in one case with 50 parts of octane an emulsion of the aqueous phase in the organic phase resulted. The size distributions of beads were generally broad over the 0–70 BBS mesh range and it was not possible to optimize the conditions to yield a narrow product distribution. Initially, the use of $BaSO_4$ was investigated in combination with PVA; however, agglomeration and instability was predominant. Much better results were obtained with hydroxyapatite, and this in combination with PVA was found useful as a general stabilizing system also in the other series of bead preparations (see Experimental). Since the DVB will react faster than MAA, it was attempted in run no. 4 to add DVB to the polymerizing batch continuously (~ 1 mL/h). By comparing the scanning electron micrographs in Figure 1, it is seen that, although the effect of increased porogen addition combined with continuous DVB addition clearly resulted in increased porosity of the interior of the beads, no increase in the binding of the enzyme was found for polymer no. 4 as seen in Table I. On the contrary, the binding is less compared to no. 2 and may be related to the more dense surface of no. 4 arising from more crosslinking in the surface with the delayed addition of DVB.

The addition of MMA as a comonomer in the crosslinked poly(methacrylic acid)s did not improve the enzyme binding and activity (Table II). These polymerizations were fairly easy to stabilize with the hydroxyapatite/PVA system. As porogen was used only octane, a nonsolvent for PMMA. We concluded that MAA based resins were not suitable for enzyme binding.

In Table III it is seen that porous beads prepared with methyl acrylate as the major constituent in all cases shows pronounced enzyme binding properties and also enzyme activity. The suspension polymerization of MA is not adequately described in the literature; however, a procedure was adopted based on the methacrylic acid polymerizations. The same stabilizer combination was found to work very well in a suspension medium consisting of a 1M NaCl solution. Based on kinetic data for the polymerization of MA,[12] the batch time for the suspension polymerization was extended to 20 h. Much



(a)



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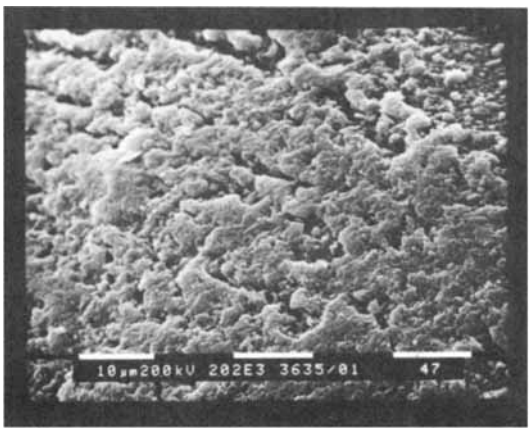
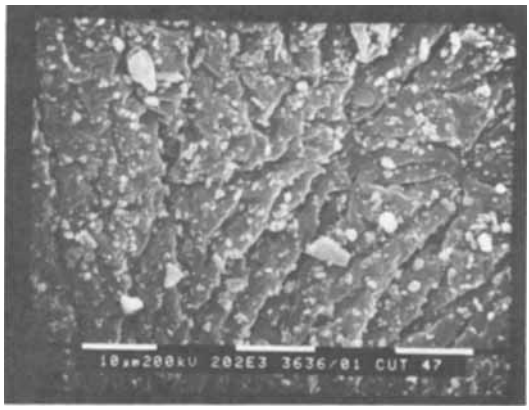


(c)

Fig. 2. Scanning electron micrograph of crosslinked copolymers of MA: (a) polymer no. 17 (comonomer EA), surface; (b) polymer no. 17 (comonomer EA) cross section; (c) polymer no. 19 (comonomer MMA) surface; (d) polymer no. 19 (comonomer MMA), cross section.



(d)

Fig. 2. (*Continued from the previous page.*)

(a)

(b)

Fig. 3. Scanning electron micrograph of crosslinked polyMMA: (a) polymer no. 21, surface; (b) polymer 21, cross section.

more uniform size distributions, around 40–45 mesh, were found for the MA systems and sieving of the products was not necessary. In three cases of this series of MA polymers, a comonomer amounting to around 15% was incorporated. In runs 14, 17, and 19, MAA, EA, and MMA were added, respectively, and only the latter appeared to have a seizeable effect on the enzyme binding and activity. For that reason the last series of preparations of porous beads are based on MMA. The scanning electron micrographs of the beads showing the highest activity, runs 17 and 19 are shown in Figure 2.

In the final series of preparations (Table IV) the highest enzyme binding and activity was found, which in runs 20 and 21 correspond to 100% binding of the enzyme within experimental error. An enzyme activity of 17.1 BIU/g is fairly high. It is seen, however, from Table IV that wide variations in the values are found depending on variations in the conditions for the preparations including the amount and type of porogen. It is necessary for a specific enzyme to optimize the conditions closely to obtain the best results. In Figure 3 is seen that polymer no. 21 has a very porous structure. The beads of preparations no. 20 and 21 had a nonspherical shape in contrast to nos. 25–32, which were very uniform in size and shape. By BET N_2-adsorption measurement on polymer no. 20 a specific area of 24.7 $m^2\ g^{-1}$ and an average pore diameter of 41,8 nm was determined. However, these numbers are associated with great uncertainty, because the measurement is carried out in a dry state quite contrary to the actual conditions under which the enzymes are loaded.

CONCLUSIONS

By suspension polymerization of acrylic monomers and DVB in the presence of a porogen may be obtained crosslinked porous beads which are suitable for enzyme binding of a lipase. It was found that the highest degree of enzyme binding and measured activity was obtained with porous crosslinked PMMA. PMA was less suitable, and compositions based on methacrylic acid or in combination with this monomer, show little or no binding capability.

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