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A study of enzyme and protein microencapsulation—some factors affecting the low apparent enzymic activity yields

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Microencapsulation of aqueous solutions of enzymes, α-chymotrypsin (EC 3.4.21.1) and histidase (EC 4.3.1.3), with semipermeable polyamide membranes resulted in a loss of enzymic activity. The low yields (less than 40%) found with both enzymes were typical of others reported in the literature. The activity of broken histidase-containing microcapsules was greater than that of the microcapsules before breaking, and this was interpreted as being due to a simple diffusional restriction on the substrate and product. The maximum of the apparent pH-activity curve of α-chymotrypsin was found to be shifted one unit to more alkaline pH when the enzyme was encapsulated. This phenomenon was explained in terms of the hydrogen ion concentration in the microenvironment surrounding the enzyme being different from that in the bulk solution. Microencapsulation of aqueous solutions of enzymes is accomplished by in situ polymerization reactions at the interface of a water-in-oil emulsion. ^{125}I-labelled proteins (albumin and fibrinogen) were encapsulated under similar conditions to determine the efficiency of the microencapsulation process. About one third of these proteins was lost during the overall preparation procedure and a further fraction was attached to the membranes of the microcapsules.

Currently there is a considerable amount of research into the use of enzymes and other proteins in a wide range of biomedical applications. In addition to microencapsulation by interfacial polymerization, other approaches including the use of liposomes (Kurosaki et al 1981), erythrocyte 'ghosts' (Fiddler et al 1977) and microparticles within a hydrogel implant (Edman & Sjoholm 1981) are being studied. Microencapsulation of enzymes overcomes a number of the problems associated with their therapeutic use, and several in vivo studies have been reported, particularly by Chang (1972) pursuing his concept of 'artificial cells'. Thus microencapsulation of enzymes, cell contents, cells, vaccines, antigens, antisera, cofactors, hormones and proteins (Chang 1977a) provides potential therapy for hereditary enzyme deficiency conditions, substrate-dependent tumours and organ failure (Chang 1977b).

In this approach, an aqueous solution of enzyme is emulsified in an organic solvent and a 'semipermeable' membrane is produced by in situ polymerization of added monomers which react at the interface. The encapsulated droplets are then separated from the organic solvent and washed to produce an aqueous suspension of 'artificial cells'.

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The most frequently used class of polymers for semipermeable microcapsule preparation has been the polyamides, particularly nylon 6,10 (Chang 1972). An alkaline diamine solution (of relatively high pH and ionic strength) containing enzyme is emulsified in an organic solvent and a diacid chloride is subsequently added. The two co-reactants diffuse to the interfacial region and rapidly polymerize, resulting in an insoluble polymeric membrane surrounding the aqueous droplets.

At this time little is known about the ultimate degradation and excretion of polyamide microcapsules in vivo, while macroscopic implants of nylon 6 are reported to be resistant to bioabsorption (Roggendorf 1976). We have recently reported preliminary attempts to prepare microcapsules from butyl 2-cyanoacrylate monomers (Wood et al 1981) which are potentially biodegradable.

In this laboratory the microencapsulation of histidase has been studied (Wood et al 1979). Histidinaemia (LaDu 1978) first reported in 1961 (Ghadimi et al 1961), is one of many rare genetic disorders of metabolism (Raivio & Seegmiller 1972) for which enzyme replacement would offer a treatment other than dietary control. The low yields of enzymic activity (less than 40%) found in this study are typical of others quoted in the literature (e.g. Chang & Poznansky 1968; Mori et al 1972, 1973; Kondo &

Muramatsu 1976). The high cost of enzymes, such as histidase, for replacement therapy prompted the investigation into the possible reasons for the loss of proteins and enzymic activity during microencapsulation.

There have been few reports of investigations into the factors involved in the loss of enzymic activity during microencapsulation and because different enzymes have been encapsulated under different conditions, some results appear to be conflicting. Chang (1964) recognized that denaturation of enzymes and haemoglobin occurred during microencapsulation and recommended the addition of haemoglobin to protect the enzyme from, for instance, interfacial denaturation, when exposed to the conditions prevailing during microencapsulation.

Mori et al (1972) compared the protective effect of several materials (e.g. albumin, casein, gelatin and haemoglobin) and found the optimal yield of asparaginase to be 37% when casein was included in the aqueous phase. In a simailar study Kondo & Muramatsu (1976) compared the loss of arginase activity in the presence of a range of concentrations of albumin and found maximal yields of about 40 with 1% albumin present.

Microencapsulated enzymes have frequently been regarded in a simplified manner as soluble enzyme in aqueous droplets which are enveloped by a semipermeable ultrathin membrane. While some of the important experimental considerations for the successful preparation of microcapsules containing enzymes are well known from an empirical point of view, there is little information concerning the proportion of enzyme (active or inactive) which is retained when the microcapsules are isolated in the form of an aqueous suspension. Similarly, little is known about the proportion of enzyme which may become incorporated into the membrane by chemical reaction or physical entrappment. For this reason two model proteins, ^{125}I-labelled albumin and fibrinogen, with different adsorption characteristics have been employed in this study.

In addition to simple losses of enzymic activity due to the efficiency of the process or denaturation, the 'apparent' enzymic activity may be lowered due to diffusional restriction caused by the membrane and to shifts in the pH-activity curves. These 'apparent' values are met in other immobilized enzyme systems and have been the subject of many studies (Zaborsky 1973).

MATERIALS AND METHODS

1,6-Diaminohexane and sebacoyl chloride (redistilled before use) were obtained from BDH. Piperazine hexahydrate, terephthaloyl chloride, Span 85 (sorbitan trioleate) and Tween 20 (polysorbate 20) were used as received from Koch Light. Organic solvents and inorganic salts were of Analar grade. Chloroform had the stabilizer (ethanol) removed before use.

α-Chymotrypsin (EC 3.4.21.1) (Bovine pancreas, 3 times crystallized, lyophilized), histidase (EC 4.3.1.3)(H-7500, *Ps. fluorescens* cells), purified histidase (H-8754) and the substrates for enzyme assays were obtained from Sigma Chemical Co. Histidase (H-7500), used in most experiments, was partially purified using a modification (Wood 1979) of the technique of Tabor & Mehler (1955) which involved grinding, heat-treatment, centrifugation and concentration. Haemoglobin, included as protective protein, was substrate grade powder from Cambrian Chemicals.

^{125}I-labelled human fibrinogen injection (ca 1 mg fibrinogen and ca 22 mg human serum albumin with buffer salts was reconstituted to 2·2 ml with water to give a solution of 50 μCi ml^{-1}) and ^{125}I-labelled human serum albumin injection (in 0·9% NaCl with 0·9% benzyl alcohol, 50 μCi ml^{-1}, 20 mg albumin ml^{-1}) were obtained from the Radiochemical Centre, Amersham, England.

Preparation of microcapsules

α-Chymotrypsin was microencapsulated with nylon 6,10 using the method of Chang (1972) with minor modifications. An aqueous phase volume of 5 ml was used and 4 mg of α-chymotrypsin was included in most preparations. In some cases the microcapsules were subsequently treated with glutaraldehyde (Chang 1972).

The conditions for the preparation of poly(piperazine terephthalamide) microcapsules were studied in some detail and optimized (Wood 1979). This type of microcapsule was used to encapsulate histidase extracts and ^{125}I-labelled proteins. Briefly, the aqueous phase (2 ml) was emulsified (1 min) in an organic phase (10 ml, chloroform–cyclohexane 1:4 containing 5% v/v sorbitan trioleate). A further 10 ml of organic phase containing terephthaloyl chloride (0·04 M) was added. The reaction was allowed to proceed for 3 min and quenched with a further 20 ml of organic solvent. The microcapsules, in a minimum volume of organic solvent, were resuspended in a 30% v/v solution of polysorbate 20 (20 ml), and water (20 ml) followed by ethanol (30 ml) was added before stirring was discontinued. The microcapsules were repeatedly washed with

buffer solution on a centrifuge. All solutions used during the preparation were maintained at about 4 °C.

The aqueous phase contained piperazine (0·1 M) in a carbonate buffer and haemoglobin (5% w/v). The solutions were prepared in double and four times the strengths stated above to allow the addition of the solutions of histidase or ^{125}I-labelled protein to maintain the 2 ml aqueous volume. 5 μCi of ^{125}I-labelled protein were added (0·1 ml of stock solution) in each batch of microcapsules.

Titrimetric assay of α-chymotrypsin activity

The hydrolysis of acetyl tyrosine ethyl ester (ATEE, 1 mM with 0·05 M NaCl, 25 °C) was followed in a stirred vessel using a pH-stat (Radiometer TTT1c) where the rate of the addition of 0·05 M NaOH solution to maintain the desired pH was measured. Small volumes of uniform suspensions of microcapsules were assayed in this way with good reproducibility.

Spectrophotometric assay of histidase activity

Unit definition: one unit of histidase will deaminate one nanomole of L-histidine to urocanic acid per minute at pH 9·2 at 25 °C. The assay method recommended by the suppliers of histidase was used; in this the appearance of the product, urocanic acid, was measured spectrophotometrically at 277 nm. The assay mixtures were composed of 0·01 M $MgCl_2$ (2·0 ml), 0·1 M Tris-HCl (pH 9·2, 1·0 ml), 0·1 M glutathione (adjusted to pH 9·2 with NaOH and prepared fresh daily, 0·2 ml), a suitable volume of histidase or microcapsules and water to a volume of 5·8 ml. The suspensions of microcapsules were mixed by 'end-over-end' motion, provided by placing the reaction tubes onto a rotating wheel, and incubated for 25 min at 25 °C. The reaction was started by adding 0·1 M L-histidine solution (pH 9·2, prepared fresh daily, 0·2 ml) and the reaction tubes replaced onto the mixing wheel. At this stage samples (duplicates) were removed, centrifuged, filtered and spectrophotometrically assayed to obtain an initial absorbance value. The final absorbance value was taken from other samples 60 min later. Two sets of controls (in duplicate) were measured to correct for self-deamination of histidine during the reaction period and for any protein which may have been released from mechanically weak microcapsules during assay. In most cases these corrections were negligible. Other checks were carried out on the supernatants resulting from the assays after a further period to confirm that histidase had not diffused out of the microcapsules. The activity (units) present in an aliquot was calculated using a value of 0·0188 for the absorbance (1 cm, at 277 nm) of one nanomole of urocanic acid in one ml.

RESULTS AND DISCUSSION

Microcapsule formation and yields of enzymic activity

The procedures used to prepare poly(piperazine terephthalamide) microcapsules resulted in spherical microcapsules with mean diameters in the range 20–30 μm. The variables which are involved in microencapsulation are numerous and frequently interdependent, and the processes occurring at the interface of the water-in-oil emulsion are still little understood. The major variables include the size of the aqueous droplets, the stability of the emulsion, the concentration of the reactants and the amount and type of 'protective' protein added. By the nature of the procedure used to prepare microcapsules some losses of enzyme molecules are inevitable.

When α-chymotrypsin was encapsulated with nylon 6,10 about 15% of the activity was retained with the microcapsules when isolated as an aqueous suspension, and enzyme which had leaked from the capsules during the 10 min assay could be detected after the assay procedures. Treatment with glutaraldehyde (Zaborsky 1973), which is believed to crosslink both the enzyme and the membrane, considerably reduced the loss due to leakage during storage and assay. When histidase was microencapsulated with poly(piperazine terephthalamide) membranes, yields were typically around 30% (mean 29%, mean deviation 13%, n = 8) and no detectable enzyme leaked from the microcapsules during assays or storage for several weeks. These low yields are typical of others in the literature (e.g. Chang & Poznansky 1968; Boguslaski & Janik 1971; Mori et al 1972, 1973; Kondo & Muramatsu 1976; O'Grady & Joyce 1981).

Loss of protein during the microencapsulation process

Microencapsulation of ^{125}I-labelled human serum albumin (HSA) and fibrinogen revealed that about 65% of these proteins was retained with poly(piperazine terephthalamide) microcapsules when they were isolated as aqueous suspensions. Tables 1 and 2 show the proportion of the activity which was recovered in the organic phase which includes small polymeric particles and a relatively high concentration of surfactant. The aqueous washing medium was found to remove a further proportion of the proteins. In this case microcapsules with poor mechanical

Table 1. Incorporation of ^{125}I-labelled human serum albumin in poly(piperazine terephthalamide) microcapsules.

System	^{125}I-activity (%)	Mean with s.d.
Organic phase	(6·4, 6·8, 8·0)	7·1 (0·8)
Aqueous washings	(31·6, 24·4, 30·3)	28·8 (3·8)
Microcapsules	(60·9, 69·0, 62·5)	64·1 (4·3)
	total	100·0

strength were broken, thereby releasing their contents. During this procedure excessive centrifugation causes the collapse and fragmentation of the microcapsules while inadequate centrifugation does not bring the microcapsules down completely. Both extremes result in losses of protein. One similar study (Boguslaski & Janik 1971) in which the kinetic properties of bovine carbonic anhydrase containing microcapsules (nylon 6,10) were examined, employed ^{125}I-labelled enzyme and only 10% of the enzyme was retained with the microcapsules.

Adsorption and denaturation of protein

The microcapsules which contained ^{125}I-labelled proteins were broken into very small fragments by ultrasonication and the radioactivity retained with the membranes was isolated by centrifugation and counted. The two proteins behaved differently. The ^{125}I-activity of the albumin initially 69% fell after sonication to 35% the respective figures for fibrinogen were 76 and 71%. These two proteins are known to have different adsorption characteristics which are related to the biocompatibility of synthetic polymeric implant materials (Brash & Lyman 1971; Bagnall 1978; Bagnall & Annis 1977). There still remains some doubt as to their exact role in biocompatibility and it has been suggested that ^{125}I-labelled proteins could have different adsorption characteristics compared with the unlabelled protein (Van der Scheer et al 1978). However, as a 'rule of thumb' HSA adsorbs onto biocompatible (hydrophilic) surfaces while fibrinogen preferentially adsorbs onto poorly biocompatible (hydrophobic) surfaces. Table 3 shows the proportion of each protein adsorbed when labelled protein was added to a suspension of unlabelled microcapsules. It is evident that fibrinogen adsorbed rapidly and the extent of adsorption was almost complete. This implies that polyamide microcapsules in their present form may not be entirely biocompatible, despite several successful animal studies.

Table 2. Incorporation of ^{125}I-labelled fibrinogen in poly(piperazine terephthalamide) microcapsules.

System	^{125}I-activity (%)	Mean with s.d.
Organic phase	(9·9, 12·3, 10·5)	10·9 (1·2)
Aqueous washings	(15·2, 9·5, 13·3)	12·7 (2·9)
Microcapsules	(65·4, 75·8, 57·6)	66·3 (9·1)
	total	89·9

HSA was found not to adsorb to any great extent (Table 3). Therefore, when this protein was microencapsulated, about one third was lost due to the microencapsulation procedure, a further third was chemically or physically entrapped in the membrane while the remaining one third was free in the internal aqueous solution.

Table 3. Adsorption of ^{125}I-labelled proteins onto poly(piperazine terephthalamide) microcapsules.

Contact time	Albumin % adsorbed	Fibrinogen %
5 min	1·2	92
3 h	1·7	93
24 h	3·2	95
	0·9% w/v NaCl, pH 7·4	

The adsorption of haemoglobin onto polymeric surfaces has been studied (Horbett et al 1977, 1978) and it has been found to have a high affinity for diverse surfaces; a re-evaluation of the role of fibrinogen in thrombus formation has been suggested. In this study haemoglobin, included at relatively high concentration to maintain osmotic pressure and to protect the enzyme, must also compete to be adsorbed at the water-oil interface.

It is clear that competitive adsorption of proteins occurs on different surfaces and will be related to the pH of the solution and the isoelectric point of the particular protein (Brash & Lyman 1971). During microencapsulation the surface presented to the aqueous phase may change from an organic solvent saturated with surfactant to an insoluble growing polymeric membrane. Hydrochloric acid is formed as the polymerization reaction proceeds and a concentration gradient will be set up as the acid diffuses into the aqueous droplets where it is neutralized by the alkaline buffer.

If enzyme molecules adsorb at the interface they may be denatured or even become incorporated into the membrane by reaction with the diacid chloride. This problem was emphasized when attempts were made to microencapsulate a purified sample of histidase (Sigma H-8754) instead of the partially purified extracts from *Ps. fluorescens* cells. When the water-in-oil emulsion was formed in the usual way

and then broken by centrifugation, no significant loss of the activity was found. However, when the diacid chloride was added to the emulsion and the microcapsules formed, there was a complete loss of activity. A reverse behaviour was found by Sundaram (1973) who found that when a low concentration of crude urease was microencapsulated in the presence of haemoglobin, 35% of the activity was retained while the encapsulation of a high concentration of purified urease, with protecting protein, resulted in a product with 90% of the initial activity.

In a study by Kondo & Muramatsu (1976), the microencapsulation of arginase with poly(piperazine terephthalamide) resulted in a 60% loss in activity while the formation of the emulsion without addition of the diacid chloride produced a 40% loss of activity. Suspecting that the alkaline diamine solution was the major cause of the activity loss, these workers prepared poly(γ-benzyl-L-glutamate) microcapsules and found a 20% loss due to the emulsification step while the microcapsules retained 60% activity under optimized conditions.

pH-activity and diffusional restriction effects

In addition to the losses of enzymic activity discussed above there are the classical apparent activity losses due to the different conditions under which the activity of the immobilized enzyme is measured. Fig. 1 shows that the peak of the pH-activity curve of α-chymotrypsin was shifted to one unit higher when the enzyme was microencapsulated and that the profile was broadened. This 'apparent' pH-optimum value is generally ascribed to the fact that the pH of the microenvironment surrounding the active site of the enzyme is different from that measured in the bulk solution. Generally, negatively-charged supports displace the pH-optimum towards more alkaline values and this effect has been detected when trypsin (at low concentration) was adsorbed onto negatively-charged glass vessels (Johnson & Whateley 1972). When chymotrypsin was immobilized by chemical attachment to dextran, the pH-optimum was shifted to 9·8, but subsequent degradation of the support by dextranase returned the pH-optimum to its original value of 7·8 (Axen et al 1970). This phenomenon of the microenvironment of the microcapsules may in some cases be responsible for an apparent loss of enzymic activity. However, it should be possible to incorporate suitably charged polyelectrolytes into microcapsules to provide the optimal pH for a particular enzyme while the pH of the surrounding medium (e.g. extracellular fluid) may be different.

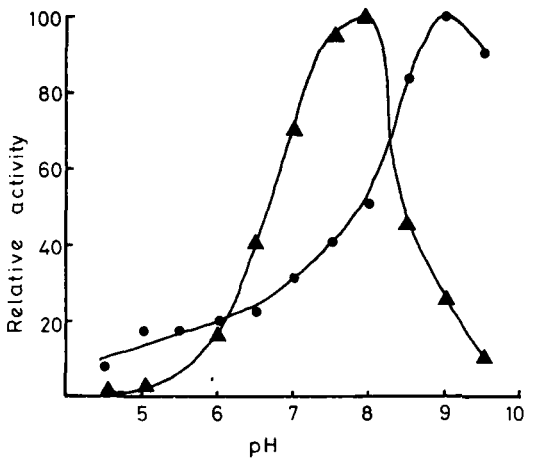

FIG. 1. pH-activity curves for microencapsulated α-chymotrypsin (●) and α-chymotrypsin in solution (▲). (1mM ATEE, 50mM NaCl at 25 °C).

While kinetic studies on soluble α-chymotrypsin resulted in a K_m value of 0·75 (±0·05) mM, which compared well with literature values (Berezin et al 1971), a satisfactory value could not be obtained for the microencapsulated enzyme. The Lineweaver-Burk plot for the encapsulated enzyme showed curvature, concave to the 1/[s] axis. With histidase a significant effect, presumed to be due to a diffusional restriction, was found when microcapsules were broken, resulting in ca 25% increase in the activity compared with the unbroken microcapsules. Mori et al (1973) reported a one hundred times increase in the K_m of asparaginase encapsulated in nylon 6, 10 and an increase in the K_m value of urease which was dependent upon the size of the microcapsules. O'Grady & Joyce (1981) found little difference between the K_m and the pH-activity of soluble and encapsulated arginase while retaining up to 32% of the original activity of the enzyme.

CONCLUSIONS

Losses of enzymic activity after microencapsulation appear to be due to a complex series of additive separate factors involving protein loss, denaturation and apparent activity losses. The precise preparation conditions are important and even when optimized a considerable loss of protein may be expected. Percentage activity yields can be significantly dependent upon the concentration of any particular enzyme, its purity and the presence of added protective protein. A balance must be struck between the leakage of enzyme from the semipermeable membrane and the restriction of diffusion of substrate and products. Apparent activity losses due

to shifts in the pH-activity curve of some enzymes may be expected to further reduce the apparent activity of the microencapsulated enzyme.

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REFERENCES

Axen, R., Myrin, P., Janson, J. (1970) Biopolymers 9: 401–413

Bagnall, R. D. (1978) J. Biomed. Mater. Res. 12: 203–217

Bagnall, R. D., Annis, J. A. D. (1977) J. Colloid Interface Sci. 61: 396–397

Berezin, I. V., Kazanskaya, N. F., Klyosov, A. A. (1971) FEBS Lett. 15: 121–124

Boguslaski, R. C., Janik, A. M. (1971) Biochim. Biophys. Acta 250: 266–269

Brash, J. L., Lyman, D. J. (1971) in: Hair, M. L. (ed.) The Chemistry of Biosurfaces, Vol. 1, Marcel Dekker, New York, p 177

Chang, T. M. S. (1964) Science 146: 524–525

Chang, T. M. S. (1972) Artificial Cells, C. C. Thomas, Springfield, Ill

Chang, T. M. S. (1977a) in: Chang, T. M. S. (ed.) Biomedical Applications of Immobilized Enzymes, Vol. 1, Plenum, New York, pp 69–90

Chang, T. M. S. (1977b) in: Chang, T. M. S. (ed.) Biomedical Applications of Immobilized Enzymes, Vol. 1, Plenum, New York, pp 93–104

Chang, T. M. S., Poznansky, M. J. (1968) Nautre (London) 218: 243–245

Edman, P., Sjoholm, I. (1981) J. Pharm. Sci. 70: 684–685

Fiddler, M. B., Hudson, L. D. S., Desnick, R. J. (1977) Biochem. J. 168: 141–145

Ghadimi, H., Partington, M. W., Hunter, A. (1961) N. Eng. J. Med. 265: 221–224

Horbett, T. A., Weathersby, P. K., Hoffman, A. S. (1977) J. Bioeng. 1: 61–78

Horbett, T. A., Weathersby, P. K., Hoffman, A. S. (1978) Thrombosis Res. 12: 319–329

Johnson, P., Whateley, T. L. (1972) Biochim. Biophys. Acta 276: 323–327

Kondo, T., Muramatsu, N. (1976) in: Nixon, J. R. (ed.) Drugs Pharm. Sci. Vol. 3 (Microencapsulation), Marcel Dekker, New York, pp 67–75

Kurosaki, Y., Kimura, T., Muranishi, S., Sezaki, H. (1981) Chem. Pharm. Bull. 29: 1175–1179

LaDu, B. (1978) in: Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S. (eds) Metabolic Basis of Inherited Diseases. 4th edn, McGraw-Hill, New York, pp 317–327

Mori, T., Sato, Y., Matuo, Y., Tosa, T., Chibata, I. (1972) Biotechnol. Bioeng. 14: 663–673

Mori, T., Tosa, T., Chibata, I. (1973) Biochim. Biophys. Acta 321: 653–661

O'Grady, P., Joyce, P. (1981) Enzyme Microb. Technol. 3: 149–152

Raivio, K. O., Seegmiller, J. E. (1972) Annu. Rev. Biochem. 41: 543–554

Roggendorf, E. (1976) J. Biomed. Mater. Res. 10: 123–143

Sundaram, P. V. (1973) Biochim Biophys. Acta 321: 319–328

Tabor, H., Mehler, A. H. (1955) in: Colowick, S. P., Kaplan, N. O. (eds) Methods in Enzymology, Vol. 2, Academic Press, New York, pp 228–233

Van der Scheer, A., Feijen, J., Elhorst, J. K., Krugers Dagneaux, P. G. L. C., Smolders, C. A. (1978) J. Colloid Interface Sci. 66: 136–145

Wood, D. A. (1979) Some Studies of Microencapsulation by Interfacial Polymerisation, Ph.D. Thesis, University of Strathclyde

Wood, D. A., Whateley, T. L., Florence, A. T. (1979) J. Pharm. Pharmacol. 31: 79P

Wood, D. A., Whateley, T. L., Florence, A. T. (1981) Int. J. Pharm. 8: 35–43

Zaborsky, O. R. (1973) Immobilized Enzymes, CRC Press, Cleveland, Ohio, pp 66–68