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Microencapsulation within crosslinked polyethyleneimine membranes

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A microencapsulation technique is proposed involving the formation of a polyethyleneimine (PEI) membrane crosslinked by an acid dichloride. The membranes were formed at pH 8 in a non-polar solvent, conditions which are better suited for the encapsulation of biocatalysts or fragile biochemicals than those using polyamide membranes. The mean diameter and size distribution of the PEI microcapsules were similar to that observed with nylon membranes. The resultant microcapsules were spherical, free-flowing with a strong membrane. The mass of membrane was seen to be independent of the reaction time (1–4 min), insensitive to the PEI concentration and proportional to the concentration of crosslinking agent.

Introduction

Microencapsulation is a promising immobilization technique for biocatalysts, widely used in a variety of commercial applications in the fields of agriculture (Powel 1968), pharmacology (Chang 1984), medicine (Chang 1964) and biotechnology (Dueck *et al.* 1986, King *et al.* 1987).

The entrapment of a biocatalyst within gel structures such as calcium alginates (Champagne and Boyaval 1986) is one of the least disruptive methods of immobilization, yielding resilient beads with a high retention of activity. However, low diffusion constants of substrates in the gel have been reported (Tanaka *et al.* 1984). In addition, biocatalyst leakage may lower the performance and complicate control of the process. Cell release contaminates the product and makes downstream processing more laborious. Cell release is particularly undesirable when the product is directly destined for consumption, as in denitrification of drinking water, or in dairy applications (Champagne and Boyaval 1986). Dissolution of the gel may also occur when ions in the substrate solution complex calcium (Birnbaum *et al.* 1981). Finally, the technique of bead formation by needle extrusion (Matsumoto *et al.* 1986), although simple and convenient for small-scale preparations, is not suitable for large-scale applications. Moreover extrusion methods result in large particles which increase mass transfer limitation.

Encapsulation of biocatalysts in thin, semipermeable membranes provides an attractive alternative to bead entrapment. Encapsulation is a unique form of immobilization in that the encapsulating membrane controls the chemical species

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exchange with the medium, and prevents biocatalyst leakage or phage contamination. Polyamide membranes formed by interfacial polycondensation (Chang 1964) are generally strong and stable. The dispersion of the biocatalyst solution before encapsulation is performed by emulsification which is well suited to industrial scale. The small microcapsule size and thin membrane minimize mass transfer limitations. The main disadvantage with interfacial polymerization involves a reduction in biocatalyst activity due to the reactive nature of the membrane formation process, elevated levels of pH and the toxic nature of solvents and reagents used in the process (Chang 1964). Polyamide membranes, for example, are formed by the polymerization of a strong diamine base solubilized in an aqueous solution of high pH, ionic strength and osmotic pressure with a dichloride dispersed in a polar solvent mixture.

The objective of the present study was to develop a method of microencapsulation via interfacial polymerization which has the potential for a minimal loss of biocatalyst activity or biochemical integrity. The use of toxic solvents, extremes of pH and osmotic pressure were eliminated. Polyethyleneimine can be crosslinked at an interface, under mild conditions of pH, at a low ionic strength, and in the absence of toxic organic solvents.

Materials and methods

Materials

Polyethyleneimine (PEI, 50% in water), 1,6-hexanediamine (HDA), sebacoyl chloride (SC), terephthaloyl chloride (TC), trimesoyl chloride (TMC; 1,3,5-benzene tricarboxylic acid chloride), and Tween 20, were purchased from Aldrich; chloroform and cyclohexane were obtained from A&C Chemical; Span 85 from Atkemix and Bromothymol blue from Sigma.

Preparation of PEI microcapsules

PEI membranes were formed by a polycondensation reaction between the PEI in buffered aqueous solution at an initial pH of 8·0–9·5, and di- or trichloride in cyclohexane, the organic solvent. The optimum procedure for PEI membrane formation involved emulsifying 50 ml of cyclohexane containing 2% v/v Span 80 as emulsifier, with 10 ml of the buffered biocatalyst preparation containing 5% w/w polyethyleneimine. Mixing in a 200 ml beaker with a frame lattice type impeller at 200 rpm for 2 min provided a stable emulsion. Membrane formation was then initiated at the droplet interface by adding 0·94 mmol sebacoyl chloride in a small volume of cyclohexane. After 3 min the reaction was stopped by dilution with 50 ml of cyclohexane. The suspension was then allowed to settle after 1 min, the supernatant discarded, and the microcapsules rinsed with 50 ml cyclohexane. Transfer into the aqueous phase was achieved by dispersing the capsules in 50 ml of Tween 20 (50% v/v) and gradually adding 250 ml of distilled water. The microcapsules were finally recovered on a Buchner filter and rinsed several times with distilled water to remove traces of organic solvent and surfactant. The microcapsules were washed twice with cyclohexane before final transfer into water to remove solvent adhering to the membrane surface.

The dispersion of the aqueous PEI solution within the the solvent was performed with a sheet lattice-type impeller as described elsewhere (Poncelet De Smet *et al.* 1990). The volume of organic and aqueous phases and dichloride organic solutions were 50, 10 and 10 ml respectively. Some experiments were performed in a system scaled up to 133% (235% in volume).

Microcapsule size distribution

Mean diameter and size distribution were determined by analysing the microcapsule aqueous suspension with a Malvern 2600 LC Particle Size Analyzer (Malvern Instruments), using the volume distribution (Poncelet De Smet *et al.* 1990). The reported values are means of two or three sample results with a variation of generally less then 5 per cent.

Membrane weight

Microcapsule membranes were isolated by sonicating the microcapsules (Artek Sonic 300 Dismembrator) to release their soluble core contents, and then washing with water to remove residual soluble PEI. Membrane fragments were filtered (Whatman no. 4), dried at 100°C, and weighed. Mass of membrane reported is per batch of microcapsule preparation.

Measurements of pH

Bromothymol blue, introduced prior to encapsulation, served as a pH probe. Titration showed that Bromothymol blue–PEI solution is blue at a pH higher than 7·6, green between 7·6 and 6·0 and yellow at pH less than 6·0.

Results

Spherical PEI membrane-bound microcapsules were formed with a mean diameter of 100 μm in a log-normal distribution (figure 1) with a standard deviation of 64 μm. The smooth thin membranes appeared rigid when examined microscopically with micromanipulators. An increase in the concentration of sebacoyl chloride from 0·47 to 1·4 mmol produced microcapsules which resisted washing and filtration (table 1). Higher concentrations of TC were required in comparison to the SC for microcapsule formulation. The branching of the crosslinked PEI by use of a trifunctional crosslinking agent such as TMC both with or without SC, results in the formation of intact microcapsules with less rigid membranes. Introduction of dextran, albumin or hexanediamine did not enhance the strength of the microcapsules, nor did the addition of various buffers (tris, carbonate, borax) affect membrane formation.

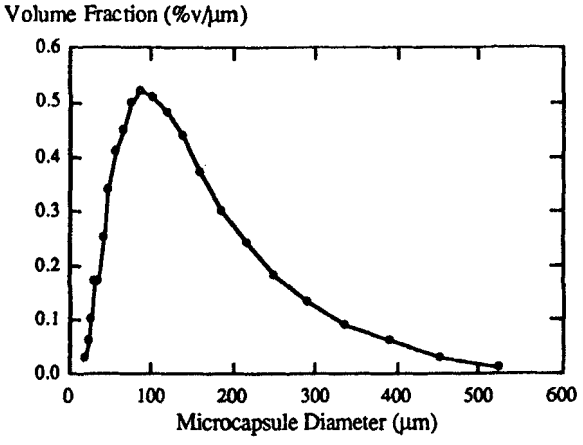

Figure 1. Size distribution of crosslinked polyethyleneimine microcapsules (5 per cent PEI, 224 mg SC, pH 8·5, 200 rpm).

Table 1. Selection of a crosslinking agent for the preparation of PEI microcapsules.

Amount of crosslinker (mmol)	Observations	pH	Classification
Sebacoyl chloride			
0·47	Many broken, spherical, pliable, smooth	>7·6	+ +
0·94	Some broken, spherical, rigid	>7·6	+ + +
1·40	Intact, spherical, rigid, strong	7·6	+ + +
Trimesoyl chloride			
0·38	Many broken, fragile membrane	<6·0	−
0·76	Many broken, non-spherical, fragile	<6·0	−
1·50	Some broken, non-spherical, pliable	<6·0	+
3·20	Some broken, spherical	7·6	+ +
Terephthaloyl chloride			
3·9	Some broken, irregular	<6·0	+
2·0	Aggregation, irregular	>7·6	+
Combination			
TMC 0·38, SC 0·47	Some broken, spherical	6·0–7·6	+
TMC 0·38, SC 0·94	Some broken, strong	7·6	+ +
TMC 0·76, SC 0·47	Some broken, fragile, aggregation	—	+ +

† Qualitative evaluation of microcapsules in degrees of desirable (+) or undesirable (−) qualities.

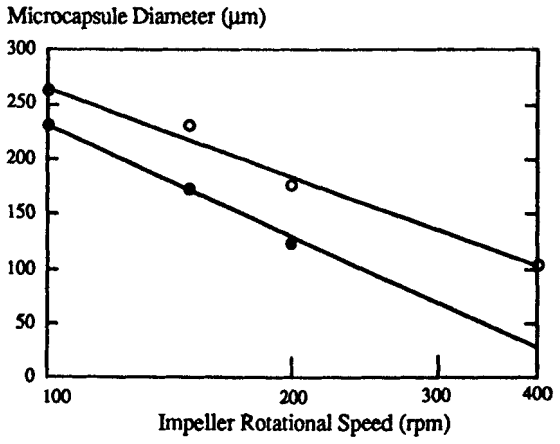

Figure 2. Effect of impeller rotational speed and reactor scale on mean diameter of crosslinked PEI microcapsules (5 per cent PEI, 224 mg SC, pH 8·5, 200 rpm, 3 min reaction time).

Bromothymol blue was used to monitor pH during membrane formation. For an initial pH higher than 8·0, with 0·94 mmol of SC, the core pH remained above 6 during formulation, even in the absence of an acid buffer. When using TC and TMC the pH reduction was more significant (table 1), leading to pH values lower than 6·0 even when 0·45 M of tris was used as buffer.

The mean diameter of the microcapsules decreased as the rotational speed of the impeller was increased, as shown in figure 2. The size was also affected by doubling

the scale of the system (reactor, impeller, solution volume; figure 2). Furthermore, microcapsule size decreased as emulsifier concentration was increased (figure 3). However, strong, intact and spherical microcapsules were formed only in the presence of an emulsifier at concentrations from 1 to 2 per cent. At lower concentrations, weak membranes were obtained with a high proportion of ruptured capsules. At higher concentrations, microcapsules tended to aggregate and the membrane was weak and wrinkled. The size of the microcapsules decreased as the pH of the initial PEI solution was increased (figure 3). The microcapsule size was not affected by the nature or concentration of the cross-linking agent.

Figures 4 and 5 present the impact of reaction time as well as sebacoyl chloride and PEI concentrations on the membrane mass. Membrane formation was primarily influenced by the concentration of crosslinking agent (figure 5), while both PEI concentration (figure 5) and reaction time (figure 4) appeared to have little effect on membrane weight.

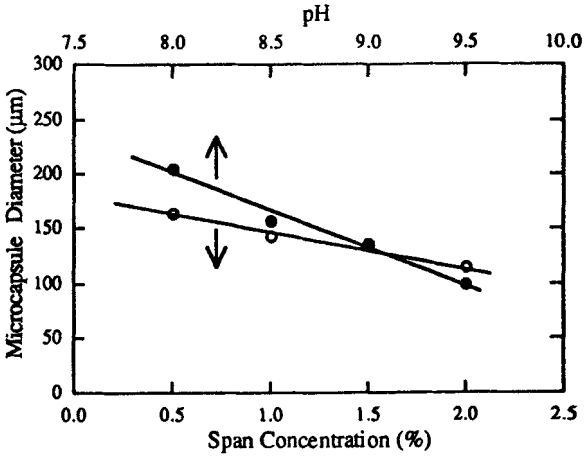

Figure 3. Effect of emulsifier concentration (Span 85) and pH on mean diameter of crosslinked PEI microcapsules (5 per cent PEI, 224 mg SC, pH 8·5, 200 rpm, 3 min reaction time).

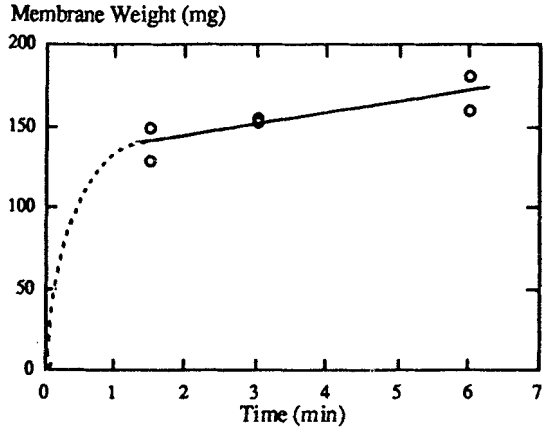

Figure 4. Effect of reaction time on membrane weight of crosslinked PEI microcapsules (5 per cent, 224 mg SC, pH 8·5, 200 rpm).

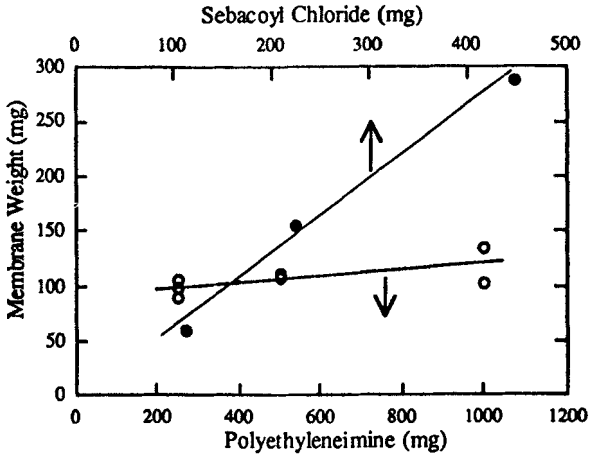

Figure 5. Effect of SC and PEI concentrations on microcapsule membrane weight (224 mg SC, pH 8·5, 200 rpm, 3 min reaction time).

Discussion

Microencapsulation by interfacial polymerization was first developed for nylon membranes in 1964 (Chang 1964). Nylon-6,10 polymerization is a result of the polycondensation reaction between 1,6-hexanediamine (HDA) and sebacoyl chloride (SC) and is limited by the diffusion of diamine through the forming membrane to the organic side of the interface. This transfer rate is reduced when the polarity of an organic solvent and the pH of the diamine solution are decreased (Morgan and Kwolek 1959, Poncelet *et al.* 1990). Although a mixture of chloroform and cyclohexane is generally used as the organic solvent phase in nylon membrane preparation, the activity of *Streptococcus cremoris* was negatively affected when contacted with both solvents, which may be replaced with butylacetate (Larisch 1990, Larisch *et al.* 1992), mineral oils or silicon oils (Hyndeman *et al.* 1992). The use of less polar solvents have been shown to be suitable for cell manipulation (Brink and Tramper 1985).

Ndong-Nkoume *et al.* (1981) advised that the pH of the diamine solution in nylon membrane formation should be approximately 11 to obtain strong microcapsules. A high pH may denature enzymes and destroy living cells. However, several authors have produced microcapsules at a lower pH (9·5) by using pyridine instead of hexanediamine (Takamura *et al.* 1973) or PEI as a cofiller (Grunwald and Chang 1978).

Povey *et al.* (1987), in studying the entrapment of carcinogens in magnetic polyhexamethyleneterephthalamide microcapsules containing PEI found that PEI was incorporated within a range of 16–30 per cent w/w into the membrane during encapsulation. Also, a more uniform formation of the microcapsule membrane occurred at lower concentrations of HDA used in preparation. The same authors noted that PEI membranes could be formed in the absence of diamine. On the other hand, the preparation of polyamide microcapsules in the absence of PEI filler at pH 9·5 results in fragile membranes (unpublished data). When PEI was used as filler, the pH inside the microcapsules dropped to a value lower than that predicted from a physicochemical model (Poncelet *et al.* 1985). This same model predicted that the membrane thickness must be proportional to the HDA concentration. However,

a reduction of the HDA concentration by a factor of 4 did not appear to affect the membrane characteristics (unpublished data). It may be that there is a chemical incorporation of PEI into the membrane during formation, and not merely physical inclusion.

PEI membranes are produced by the crosslinking of the PEI polymer. PEI is insoluble in organic solvents, thus the reaction would tend towards the aqueous side of the interface. Under these conditions the pH in the aqueous phase, and the polarity of the organic solvent, should not affect the reaction kinetics or membrane characteristics to the same extent as was the case with nylon membranes. Thus PEI microcapsules may be prepared in a variety of solvents of different polarity including biocompatible solvents such as mineral, silicon or perfluorocarbon oils. The second advantage of PEI microcapsules is that preparation is possible at an initial pH lower than that required for the formulation of polyamide membranes. The pH drop due to the release of acid chloride during the crosslinking reactions, imposes a limit on the extent to which the initial pH can be reduced. An initial buffered pH between 8·0 and 8·5 will maintain the final pH between 6·5 and 7·0, compatible with most enzymes, biological cells and natural compounds.

Polyamide membrane formation requires high concentrations of diamine (0·45 M). Taking into account the high concentration of counterions necessary to maintain the pH at 9·5 (about 0·9 M Cl^-), the corresponding ionic strength and osmotic pressure in the microcapsule core will be greater than 1·35 M and 30 atm. In the PEI membrane system the ionic strength and osmotic pressure are much lower, permitting the encapsulation of sensitive biological cells.

Membrane strength

Nylon and PEI membranes have very different characteristics. Nylon is an elastic and deformable membrane (Jay and Edwards 1968), whereas the PEI membrane appears more rigid. In a previous study (Poncelet and Neufeld 1989), it was observed that the mechanical resistance of nylon membranes decreased strongly when the temperature was lowered below the glass transition, since the membrane became more rigid. The rigidity of the PEI membrane could then be a limiting factor for applications requiring high shear hydrodynamic conditions.

Polyethyleneimine includes a large spectrum of water-soluble polyamines of variable molecular weight with varying degrees of modification. All PEIs produced by the ring-opening cationic polymerization of ethyleneimine are believed to be highly branched, containing primary, secondary and tertiary amine groups in the ratio of approximately 1 : 2 : 1 (Horn 1980). Crosslinking of PEI forms a three-dimensional network. In contrast, nylon is formed by the polymerization reaction between a diamine and a dichloride, forming mostly linear chains, most likely interlaced to form a net. At high pH values (11), the nylon membrane thickness is sufficiently large (approximately 1 μm) to ensure good mechanical resistance. However, at lower values of pH the membrane is sufficiently thin (200 nm) that compounds such as proteins or PEI are needed to ensure good mechanical resistance. The membrane is then composed of a network of pure nylon chains, crosslinked PEI and PEI linked by nylon bridges. This structure ensures a high resistance and elasticity of the membrane. At lower pH levels (8·5), the contribution of the nylon to the membrane becomes negligible, and the membrane becomes brittle. The resistance of the membrane may be improved by appropriate selection of cross-linking agent, use of lower molecular weight PEI or introduction of preformed linear

chains. Present results show that strong microcapsules may be prepared by using SC as the crosslinker and a 40 000 dalton PEI.

Size distribution

The present results concerning the control of size distribution are similar to that of previous results obtained with collodion and nylon microcapsules (Poncelet De Smet *et al.* 1989, 1990). Size distribution curves follow the log-normal law. The mean diameter may be controlled by adjusting the emulsifier concentration and the rotational speed of the turbine.

The pH of the PEI aqueous phase affects microcapsule size distribution. PEI is a positively charged polymer, the charge increasing by protonation of the amine groups with lowered pH (figure 5). The increased charge on the polymer results in a change in the solution viscosity, affecting the size of droplets formed during the emulsification step.

Control of pH

Assuming that the acid release is proportional to the microcapsule surface area during formulation, the drop of pH is more important in small microcapsules than in large, as confirmed by experimental observations. Increasing the size of the microcapsules will then help maintain the pH at a higher level. However, expecting a relatively constant thickness of membrane (Poncelet *et al.* 1990) as a function of the size, the resistance to shear will drop quickly (Poncelet and Neufeld 1989). A smaller size dispersion would be an important improvement in pH control.

The initial PEI concentration can be decreased without affecting membrane formation. However, the buffer capacity is lowered and the addition of an external buffer becomes indispensable.

Membrane formation process

Nylon membrane formation was first described in 1959 (Wittbecker and Morgan 1959). The process involves the transfer of diamine to the organic side of the membrane, reaction with the dichloride and precipitation of the nylon polymer thus formed. PEI membrane formation is slightly different. Firstly, PEI is a polar compound, insoluble in the organic phase (Horn 1980). Acid dichloride, on the other hand, is hydrolysed in water. The reaction takes place at the organic/aqueous interface, probably more extensively on the aqueous side due to the hydrophilic properties of crosslinked PEI.

Figure 4 shows that most of the membrane mass is formed after 1 min or less, indicating a very fast reaction. However, this does not exclude a persistent reaction inside the membrane beyond this initial period. This maturation may lead to stronger microcapsules.

Figure 5 also shows that membrane formation is a stronger function of SC concentration than that of PEI concentration. It can be concluded that both components are in excess as the membrane weight is always lower than the weight of each reactant. This was partially confirmed by titration in which only 20–40 per cent of the SC was shown to be consumed.

PEI membrane formation probably proceeds due to the penetration of the crosslinker into a PEI layer which forms near the organic/aqueous interface. The crosslinker either reacts with the PEI or is hydrolysed. For thicker membranes the crosslinking agent must diffuse further through the membrane in order to reach a

reactive site. Since the probability of hydrolysis is greater, the resulting membrane thickness should be the smallest value between the maximum distance from the interface that the crosslinker can diffuse without being hydrolysed, and the thickness of the layer formed by PEI accumulation.

Under the conditions tested, the diffusion of the crosslinker seems to be the limiting factor. The PEI concentration may then be lowered. Use of a higher concentration of crosslinker or a crosslinker that is more stable in water will lead to stronger membranes. However, a decrease of reactivity with water may also result in a lower reactivity with PEI.

Selection of crosslinker

Results show that the selection of a crosslinker has a very strong impact on the final product. Membrane properties, final pH, and settling characteristics are all affected by this selection. Sebacoyl chloride appears to be the most appropriate crosslinker among those tested, since it yields strong, individual microcapsules which settle quickly in water, while maintaining a pH between 6·5 and 8·5 during formulation.

Conclusions

The present work is the first report of microencapsulation involving interfacial crosslinking at a pH of 8 in a non-polar solvent. The resultant microcapsules were spherical, with smooth membranes. The membrane mass was found to be independent of the reaction time and PEI concentration, and proportional to the concentration of crosslinking agent.

The proposed method of encapsulation demonstrates that interfacial polymerization may be conducted under conditions compatible with cells, enzymes and biochemicals. Microencapsulation within crosslinked PEI membranes is being evaluated for encapsulation of lactic bacteria (Larisch 1990, Larisch *et al.* 1992) and DNA (Alexakis 1992). Other membrane-forming polymers such as gelatine (Hyndeman *et al.* 1992) and chitosan (unpublished data), crosslinking agents such as diisocyanate (Hyndeman *et al.* 1992) or anhydride (unpublished data), and solvents (Hyndeman *et al.* 1992) are being tested to achieve a gentle, biocompatible procedure for membrane encapsulation.

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