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Preparation of non-porous microspheres with high entrapment efficiency of proteins by a (water-in-oil)-in-oil emulsion technique

N. Badri Viswanathan[a], P.A. Thomas[a], J.K. Pandit[b], M.G. Kulkarni[a,*], R.A. Mashelkar[c]

[a]*Polymer Science and Engineering Group, Chemical Engineering Division, National Chemical Laboratory, Pune 411 008, India*
[b]*Department of Pharmaceutics, Institute of Technology, Benaras Hindu University, Varanasi 221 005, India*
[c]*Council of Scientific and Industrial Research, Anusandhan Bhavan, 2 Rafi Marg, New Delhi 110 001, India*

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Abstract

Emulsification–solvent removal methods have been widely used for encapsulating bioactive macromolecules like proteins and polypeptides in biodegradable polymers. We report, a (water-in-oil)-in-oil emulsion technique wherein proteins and polypeptides differing in molecular weight and shape were encapsulated in polymers of current biomedical interest. When an oil was used as the processing medium in combination with a carefully selected mixed solvent system such that a stable ($w/o_1/o_2$ emulsion is formed and solvents are removed by a combination of extraction and evaporation, the entrapment efficiency was high and the product nonporous. The entrapment efficiency of globular proteins exceeded 90% while that of fibrous proteins was around 70%. Fracture studies revealed that the polymer matrix was dense. The mechanism of entrapment involved solvent-induced precipitation of the protein as the microspheres were being formed. The principle of the method will find use in preparation of non-porous polymer microparticles with reduced burst effect.

Keywords: Biodegradable polymers; Poly(lactic-co-glycolic acid); Microencapsulation techniques; Emulsification–solvent removal; Protein/polypeptide encapsulation; Entrapment efficiency; Mechanism of encapsulation

1. Introduction

Encapsulation of bioactive macromolecules, especially of peptides and proteins, has received immense attention in recent years. Injectable drug delivery systems providing sustained release of such bioactive macromolecules have been designed for various applications viz., suppression of gonadotropic activity [1–3]; immunization [4–6]; supplementing or providing hormones like insulin, growth hormone etc.

Microencapsulation of proteins present a unique problem attributable to their delicate structural conformation, which may be disturbed or destroyed by slight changes in temperature, ionic concentration, pH, solvent composition etc. Often these changes are irreversible. Most of these molecules need to retain their native conformation to be biologically active. These considerations restrict the choice of conditions for designing a microencapsulation process. Further, due to the high cost of these macromolecules, limited availability (if obtained from a natural source) and

*Corresponding author. Tel.: +91 212 342784, Ext. 2178; fax: +91 212 333941; e-mail: mgk@ems.ncl.res.in

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tedious methods of isolation, the method must ensure high efficiency of entrapment. The challenge is, thus, to develop a process that would ensure high efficiency of entrapment, possess the desirable product characteristics and ensure retention of biological activity of the entrapped molecule.

Emulsification–solvent evaporation processes, where the processing medium is aqueous, are widely used for entrapment of various active ingredients in polymers of lactic and glycolic acids [7,8]. Many modifications of the process have been made to entrap proteins and polypeptides [2,9–11]. In these processes, an aqueous solution of the protein or polypeptide is first emulsified into the polymer solution in dichloromethane to form the $water_1$-in-oil emulsion. This $water_1$-in-oil emulsion is further emulsified into an aqueous processing medium to form a ($water_1$-in-oil)-in-$water_2$ emulsion. Dichloromethane is removed from this emulsion to harden the droplets and obtain solid microspheres. Entrapment efficiency of water-soluble drugs is likely to be low [11] unless protein diffusion into the aqueous processing medium is minimized. For example, entrapment efficiency of polypeptides was increased by enhancing the viscosity of inner aqueous phase by incorporating viscosity builders [2]. Such additives are usually biocompatible proteins like gelatin. It is desirable to avoid such inclusions in parenteral delivery systems since these materials could elicit undesirable immune responses. Recently Crotts and Park [12] showed that the products made by a (water-in-oil)-in-water emulsion technique result in a morphology that has a hollow interior with an outer shell due to coalescence and escape of the inner aqueous phase during microsphere formation. Synthesis of dense microparticles by such methods has not been reported so far.

One method of ensuring high entrapment efficiency of water-soluble active ingredients is to use a hydrophobic processing medium into which the hydrophilic macromolecule is unlikely to migrate out. Acetonitrile–liquid paraffin combination has been used by Jalil and Nixon [13] for small molecules, while acetonitrile–vegetable oil combination has been used for small proteins like insulin by Wada et al. [14]. In this process, insulin was suspended in an acetonitrile solution of lactic acid oligomers, and aliquots of water were added until the protein dissolved to give a clear solution. This homogenous solution was later processed by an emulsification–solvent evaporation method using vegetable oil as the medium. This process is not applicable to large proteins and polymers of molecular weights greater than the oligomeric range, approximately above ten thousand, because the non-solvent effects of water would precipitate the polymer while the non-solvent effects of acetonitrile would precipitate the protein, destroying homogeneity.

Our aim was to develop a microencapsulation method using a non-aqueous processing medium to ensure high efficiency of entrapment of macromolecules from their aqueous solution that would be applicable for proteins and polymers of all types and molecular weight. The process described in this paper ensures high entrapment efficiency. It also yielded a non-porous matrix, which may be desirable to suppress initial burst from a microparticulate delivery system for proteins. The development of the process and its applicability to various proteins and polymers are described using model proteins in polymers of lactic and glycolic acids. The method can be used to encapsulate both small and large molecules alike, by appropriate selection of processing conditions like solvent system and processing medium to produce matrix systems. Investigation of the process under optical microscope offered valuable insight into the mechanism of encapsulation. A brief description of the in vitro release performance of the microspheres is also included.

2. Experimental

2.1. Materials

Glycolide (prepared in-house from glycolic acid according to Gilding and Reed [15]) was melt copolymerized with lactide (Aldrich) using tetraphenyl tin (Aldrich, recrystallized from dichloromethane) at 150°C under vacuum in a sealed ampoule. Polymers were characterized by GPC and viscometry for molecular weight determination and by NMR for determining their copolymer composition. Proteins bovine serum albumin (BSA fraction V), lysozyme (chicken egg) and gelatin (type A, 300 bloom) were procured from Sigma Chemical Co. Liquid paraffin

was of pharmacopoeial grade (~60 centistokes) and Span 80 (sorbitan monooleate) was a gift from M/s HICO Products, Bombay. All solvents were distilled before use.

2.2. The microencapsulation process

Microencapsulation was carried out in an all-glass assembly fabricated in-house and consisted of a jacketed round bottom flask stirred by a hemispherical blade. A glass syringe was used to fabricate a vacuum tight seal for the stirrer shaft which held at least 0.1 mm of Hg vacuum. The procedure involved emulsification of an aqueous solution of protein in a polymer solution by sonication to form a water-in-oil_1 emulsion, which was further emulsified into a stirred non-aqueous processing medium containing surfactant to form a [(water-in-oil_1)-in-oil_2] emulsion. The solvents for the polymer were removed by a combination of extraction and evaporation. The most important feature of this process was the use of a carefully selected mixture of two. solvents (termed as ''mixed solvent system'' or MSS here) and a suitable non-aqueous processing medium to enable (a) formation of [(water-in-oil_1)-in-oil_2] emulsion and (b) solvent removal by a combination of extraction and evaporation.

A typical example of the encapsulation process is a MSS comprising a 1:1 mixture of acetonitrile (AN) and dichloromethane (DCM) for polymers of lactic and glycolic acids. The aqueous protein solution (20 mg/ml) was emulsified into this polymer solution by sonication to form the (water-in-oil_1) emulsion. This emulsion was further emulsified into liquid paraffin containing 4% Span-80 to form the [(water-in-oil_1)-in-oil_2] emulsion. The volume ratios of water:oil_1:oil_2 phases were typically 1:8:100. The polymer solution concentration was 12.5% w/v. Up to one gram polymer was used.

In the procedure described above, DCM was extracted off by liquid paraffin while AN was removed by evaporation under vacuum. Droplet sizes were allowed to stabilize after introduction of (water-in-oil_1) emulsion into liquid paraffin, for 10 minutes. Thereafter, solvent removal by evaporation was effected by the following sequence: filtered air-sweep for ten minutes followed by reduction in pressure to 300 mm of Hg and then to about 50 mm of Hg at 10 minute intervals. The pressure was maintained at about 50 mm of Hg for 2 h after which the solid microspheres were separated by diluting the oil, decanting, washing and sieving. The progress of formation of microspheres was studied by observing under microscope few drops of the [(water-in-oil_1)-in-oil_2] emulsion, removed at various stages of the process. The product was stored at 4°C in a desiccator.

2.3. Product characterization

2.3.1. Actual loading determination

Actual loading of proteins in polymers was determined by estimating the nitrogen content of microsphere product. Since the polymers used here do not contain any nitrogen in their chemical structure, the nitrogen content of product is a direct indication of protein entrapped in it. Nitrogen was first converted quantitatively to ammonium sulphate by micro-Kjeldahl method of digestion of microspheres in sulfuric acid using sequential addition of 30% hydrogen peroxide at high temperatures. The ammonium sulphate was then determined by developing a color with Nessler's reagent and the colour intensity was read spectrophotometrically at 400 nm. Extrapolations were done on a linear best-fit equation obtained by processing several dilutions of standard ammonium chloride solution by a similar procedure. This method is fairly rapid and can detect 10 to 100 microgram of nitrogen and therefore requires only about 20 mg of microsphere containing approximately 100 microgram protein per reading.

2.3.2. Microscopy

Samples of the emulsion in processing medium were removed at different times of preparation and observed under an optical microscope.

3. Results and discussion

Existing methods of encapsulation of proteins or polypeptides in polymers are emulsification–solvent removal techniques [8], where an aqueous medium is used for processing. Due to its hydrophilicity, proteins and polypeptide active ingredients are likely to preferentially partition out into the aqueous process-

ing medium leading to low efficiency of entrapment. Depending upon the processing conditions, as much as 80% of the added protein can partition out into the outer processing medium [12]. We wanted an encapsulation method that ensured high entrapment efficiency when the attempted loading are low (less than 5% and frequently less than 2%), as is usually required for vaccine and hormone delivery systems. Encapsulation using an oil as the processing medium was chosen in the expectation that the hydrophilic proteins, would find it unfavorable to diffuse out of the microspheres before they harden.

3.1. The microencapsulation process

The principle of the present method is one of emulsification–solvent removal (or in-oil drying method [8]) and has the following characteristic features:

- use of a carefully selected ''mixed-solvent system'' (MSS) for wall-forming polymer.
- use of a non-aqueous processing medium
- use of a combination of extraction and evaporation for solvent removal.

3.1.1. Influence of polymer solution concentration and polymer molecular weight on particle size distribution

Exploratory experiments were carried out to establish some of the process parameters for further study. These included establishing the conditions needed to produce microspheres of approximately 100–200 microns diameter. This size is easy to administer through a normal 20 gauge syringe needle. Some parameters were kept constant for all preparations and are summarized in Table 1. Table 2 summarizes the results of exploratory experiments carried out. Comparing the particle size distributions of G-1, G-2 and G-3 (all of same polymer composition and molecular weight), it is seen that the average particle size increases with rise in polymer solution concentration. While majority of the particles were less than 53 microns (mean 36 μm) when the polymer solution concentration is 6.25% (G-1), the particle sizes tend to be greater than 210 microns (mean 208 μm) for a polymer solution concentration of 25% (G-3). A polymer solution concentration of 12.5% (G-2) yielded most particles in the desired range of 100–200 microns (mean 115 μm). The particle size distribution of a typical batch was unimodal (Fig. 1). This was determined by measuring the size of about 300 particles under an optical microscope and grouping them into different particle size ranges. The mean of this range was plotted against the percent of particles in this size range.

For the same polymer solution concentration of 12.5%, a polymer of molecular weight of 74 500 (G-14) yielded a greater fraction (51%) of particles in the size range of 210-150 microns. For a still higher molecular weight (1.7×10^5) polymer (G-15) there were greater proportion of large (>210 microns) particles (15%) in spite of reducing the polymer solution concentration to 7.8%. For a very high molecular weight polymer (P-7) large deformed comma and ribbon shaped particles and aggregates

Table 1
Processing conditions that were fixed parameters throughout the study

Parameter	Processing condition
Sonication	Sonication in a Branson's 450 Watts Ultrasonic Processor using titanium microtip. Sonication in pulse mode (at 50% duty cycle) for 75 s at 20% output.
Stirring speed	220±20 rpm
Vacuum application sequence	Ten min after formation of [(water-in-oil$_1$)-in-oil$_2$] emulsion filtered air-sweep for ten min, followed by reduction in pressure to 300 mm of Hg and then to about 50 mm of Hg at 10 min intervals. The pressure was maintained at about 50 mm of Hg for 2 h.
Product recovery	The solid microspheres were separated by diluting the oil, decanting, washing and sieving.
Processing medium	Liquid Paraffin (~60 centistokes) containing 4% Span-80.

Table 2
Influence of polymer solution concentration on the particle size distribution of microspheres

Batch code no	Polymer mol wt M_w	Polymer compn L:G	Polymer soln conc %	Particle size distribution (wt %) >210 μm	210/150 μm	150/105 μm	105/50 μm	<53 μm	Mean[a] μm	Remarks
G-1	45 000	80:20	6.25			2	15	75	36	
G-2	45 000	80:20	12.5		17	37	40	1	115	
G-3	45 000	80:20	25	43	35	15			208	
G-14	74,500	70:30	12.5	2	51	35	5	1	155	
G-15	1.7×10^5	80:20	7.8	15	40	35	10	2	178	
P-7	3.78×10^5	100:0	5.1							b
P-9	3500	100:0	12.5							c
P-10	3500	100:0	31		7	31	44	15	93	

All batches were made with 2% BSA loading; 1:1 AN:DCM as MSS.

[a] Mean particle size was calculated as follows: $\frac{\sum(\text{mean particle size of the fraction}\times\text{weight fraction})}{\sum(\text{weight fraction})}$

[b] Large comma and ribbon shaped particles and aggregates.
[c] Very fine particles $\ll$53 microns.

were obtained even when the polymer solution concentration was lowered to 5.1%. For very low molecular weight polymers (P-9 and P-10) the solution concentration had to be increased to 31% to achieve higher particle size and a significant portion of the particles above 100 microns.

It is clear that the particle sizes obtained are directly proportional to the polymer solution viscosity. For the polymers studied here, the influence of polymer composition on the particle sizes was not evident. Thus, the polymer solution concentration could be fixed at 12.5% for PLGA and P(DL)LA of molecular weight ranging from ~30 000 to 1×10^5 while approximate doubling or halving the concentrations for lower or higher molecular weight polymers respectively, would yield significant proportion of microspheres in the range of 100–200 microns.

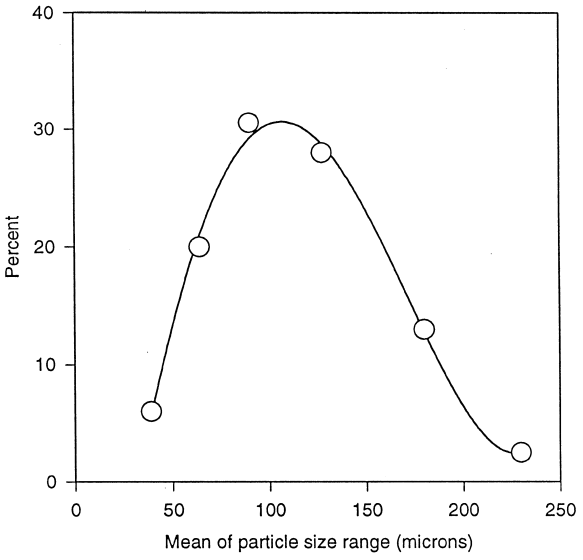

Fig. 1. Particle size distribution of a typical product (sample code G-2). Average particle size 107 microns.

3.1.2. The choice of solvent system and processing medium

The primary requirement to obtain microspheres is the preparation of a stable emulsion of the polymer solution in a processing medium. Having chosen an oil as the processing medium, it is imperative that the solvent for polymer be immiscible with oil. Acetonitrile (AN) is a unique organic solvent which is polar, water-miscible and oil-immiscible. All other polar organic solvents like methanol, ethyl alcohol, ethyl acetate, acetone, dimethylsulfoxide, tetrahydrofuran are oil-miscible and will not form emulsions of the polymer solution in an oil. Other non-aqueous liquids like polyhydric alcohols (low molecular weight polyethylene glycol, glycerol etc.) or organic solvents can also be used instead of oil as the processing medium.

With oil as the processing medium, use of AN alone as a solvent did not ensure formation of

emulsion of the aqueous protein in the polymer solution. Immediately on mixing, the water-miscibility of AN brought about the precipitation of protein (S-l, Table 3). Hence, a small proportion of a non-polar solvent, namely, dichloromethane (DCM) was included with AN to decrease polarity of the polymer solution. Additionally, it was also desirable that the second solvent be oil-miscible so that solvent removal is facilitated by extraction by processing medium. Inclusion of an oil-extractable solvent would bring about rapid extraction immediately on introduction into processing medium leaving behind a viscous polymer solution. This would prevent the migration of aqueous protein phase thereby enhancing the entrapment efficiency.

As expected, incorporation of 10% v/v of DCM in the solvent mixture led to an emulsion with aqueous phase (batch S-2). However, the microsphere formation in the oil presented problems of aggregation and the particle sizes were small. This was due to the low viscosity of the polymer solution after DCM was extracted off. When the proportion of AN was decreased, satisfactory product characteristics resulted (S-3 and S-4). When the AN proportion was decreased to only 30%, the particle sizes became very large due to high viscosities of polymer solution after the DCM was extracted off (S-5). Decreasing the AN proportion further led to lump formation (S-6 and S-7). It was, thus, concluded that a *mixed solvent system (MSS)* comprising 1:1 proportion of AN and DCM (Table 3) enabled emulsion formation as well as yielded the desired particle sizes. The process and its features are discussed in greater detail in subsequent sections with reference to batch code G-4 (Table 4) unless indicated otherwise.

From the above experiments, it was clear that an oil in combination with a MSS yields the desired product characteristics. The MSS essentially is a mixture of two organic solvents in which the polymer dissolves. Each component of the MSS individually is a good solvent for the polymer. One of the components of the MSS (DCM) is miscible with processing medium (to enable solvent removal by extraction) and the other (AN) immiscible with processing medium (to enable solvent removal by evaporation). The MSS, as a whole, is immiscible with the aqueous solution of protein, so as to prevent miscibility of organic solvents and aqueous protein. Components of the MSS can be selected from any of the commonly available organic solvents like DCM, chloroform, ethyl acetate, acetone, acetonitrile, ethanol, etc. The solvent choice, in turn, depends on the choice of processing medium.

The processing medium is immiscible with the aqueous medium so that the aqueous protein droplets. do not readily partition out during processing. The processing medium must be a non-solvent for the polymer. Vegetable oils such as arachis, cottonseed, sunflower, corn, olive, castor oil etc. are best preferred as they are hydrophobic. An added advantage is their biocompatibility allowing their use in pharmaceuticals.

Table 3
Influence of solvent composition on the microencapsulation process and product characteristics

Batch code No	Solvent Compn (AN:DCM)	Property of w/o_1 emulsion	Properties of $(w/o_1)o_2$ emulsion	Product characteristics
S-1	100:0	Heterogeneous: Protein Precipitated		
S-2	90:10	Homogenous	Aggregation tendency very high	Very fine particles; Spherical.
S-3	70:30	Homogenous	Satisfactory	Spherical; Particles in 100–200 micron range; High entrapment efficiency
S-4	50:50	Homogenous	Satisfactory	Spherical; Particles in 100–200 microns range; High entrapment efficiency
S-5	30:70	Homogenous	Stable	Spherical; Large particles; High entrapment efficiency
S-6	10:90	Homogenous	Lumps and ribbons formation	
S-7	0:100	Homogenous	Lumps formed	

The batches were all made form PLGA (80:20) of M_w 45 000 with 2% w/w BSA loading.

Table 4
Influence of composition and molecular weight on efficiency of entrapment

Batch code no	Polymer type	Polymer composition	Polymer mol wt	Polymer soln conc %w/v	Loading efficiency %
G-6	PLGA	80:20	1.7×10^5	7.8	91.6
G-4	PLGA	80:20	45 000	12.5	92
G-7	PLGA	70:30	74 500	12.5	100
G-8	PLGA	55:45	90 500	12.5	97.5
P-7	P(DL)LA	100:0	3.78×10^5	5.1	a
P-4[a]	P(DL)LA	100:0	90 000	12.5	92
P-5[a]	P(DL)LA	100:0	90 000	12.5	95
P-6[a]	P(DL)LA	100:0	90 000	12.5	96
P-10	P(DL)LA	100:0	3500	31	70.5

Protein used for entrapment-bovine serum albumin.
Attempted loading-2% w/w.
Acetonitrile:Dichloromethane ratio used-1:1.
[a] The three batches had a particle size distribution of around 165 μm.
a-Aggregates, comma and ribbon shaped particles formed.

3.1.3. The process

The process, as described under Experimental, essentially comprises forming a [(water-in-oil$_1$)-in-oil$_2$] emulsion from which the components of MSS are removed by extraction and evaporation. DCM is rapidly extracted off by liquid paraffin while AN is removed by slow evaporation under reduced pressure. Immediately on introduction of the water-in-oil$_1$ emulsion into the processing medium two simultaneous events take place-one, breakdown of the water-in-oil$_1$ emulsion into tiny droplets due to shear between viscous water-in-oil$_1$ emulsion and processing medium and two, the rapid extraction of DCM by liquid paraffin leads to an increase in polymer solution concentration resulting in increase in viscosity of the polymer solution.

A rapid solvent-extraction step immediately on introduction into processing medium is essential to increase viscosity of the polymer solution droplets and trap aqueous globules within. This increased viscosity of polymer solution droplets helps in immobilizing aqueous globules within the microparticles and prevents it from moving out into processing medium. Such a rapid extraction process also leaves a polymer-rich film on the exterior of the droplets which acts as a barrier. This approach has been used to prevent formation of crystals of drug on the surface of microspheres [16]. Apart from increased viscosity due to solvent extraction, the shear between water-in-oil, emulsion and processing medium breaks the emulsion down to smaller droplets and deform the viscous droplets leading to loss of their spherical shape. Presence of a solvent component (AN) that is immiscible with processing medium is necessary here for the formation of spherical particles. In the absence of AN, these deformed droplets solidify into lumps and ribbons (S-7 of Table 3). On the other hand, excess of AN allows free movement of aqueous globules from within the polymer solution droplets into liquid paraffin leading to low efficiency of entrapment. AN is removed by evaporation, the rate of which affects product properties significantly. It is, thus, important to have solvent removal by a combination of extraction and evaporation at an optimum rate to enable high entrapment efficiency.

Table 4 shows entrapment efficiency for a large molecular weight globular protein, BSA, in various, polymers of lactic and glycolic acids. As shown, entrapment efficiency was generally higher than 90% for all copolymer ratios and molecular weights except when it was very low (~3500). The entrapment efficiency and the mean particle sizes were reproducible as seen with three different batches P-4, P-5 and P-6. The low entrapment efficiency in case of P-10 is due to the low viscosities of polymer solutions even at high concentrations and the resulting low resistance presented to migration of

Table 5
Influence of protein type and attempted loading on loading efficiency

Batch code no	Protein entrapped	Protein mol wt	Molecular shape	Radius of gyration	Attempted loading %	Loading efficiency %
G-4	BSA	66 000	Globular	26 A	2	92.6
G-17	BSA	-do-	-do-	-do-	5	
G-5	Lysozyme	14 500	Globular	18 A	2	91
G-9	Gelatin	>60 000	Fibrous	–	2	71.4
G-28-VA	Decapeptide	~1250	Fibrous	–	5	24.5
G-29-VA	-do-	-do-	-do-	–	5	46.2

Polymer used PLGA (80:20) M_w 45 000; Acetonitrile:Dichloromethane ratio 1:1.
Polymer solution concentration 12.5%.

water globules from yet-to-be-hardened microspheres. On the other hand, when the molecular weight is very high, the droplets of polymer are highly viscous and, hence, unable to regain their sphericality after deformation leading to hardening as ''ribbons'' and ''comma'' shaped products. This can be avoided by suitably diluting the polymer solution.

The process was used to encapsulate various kinds of proteins in a selected polymer viz., PLGA of 80:20 L:G ratio and molecular weight 45 000. Both globular and fibrous proteins of high and low molecular weight were entrapped (Table 5). Globular proteins are encapsulated with efficiency greater than 90%, while the efficiency was slightly lower (70%) for a high molecular weight fibrous protein (compare lysozyme and BSA with gelatin). Entrapment efficiency was still lower for low molecular weight fibrous molecule such as a polypeptide (only 25%) under comparable processing conditions. Efficiency for the polypeptide increased to only 45% even after doubling aqueous and polymer solution concentrations. There appears to be a strong dependence of entrapment efficiency on the molecular shape of protein. Although the shape of the molecule depends on numerous environmental conditions such as pH, ionic concentration, solvent composition, etc., the in-depth study of these parameters was not the scope of the present study with model molecules.

To extend the process to other biodegradable polymers like polyanhydrides and polycaprolactone, which are of current pharmaceutical interest, modifications were made taking into account their individual physicochemical characteristics (see Table 6). For example, poly(carboxy-phenoxyoctanoic acid) (a member of the polyanhydride family) is not soluble in AN, acetone etc. Hence, a MSS comprising 1:1 mixture of tetrahydrofuran (THF) and DCM was used in combination with glycerol and Tween-20 (polysorbate-20) as processing medium. In the encapsulation process, THF was removed by ex-

Table 6
Variation in encapsulation process parameters for various classes of polymers

Batch code no	Polymer type	Mol wt/ intr visc	Mixed solvent Used	Polymer soln conc %	Processing medium	Entrapment efficiency %
G-19	PLGA (80:20)	50 000	AN:DCM (1:1)	12.5	LP-4% Span8O	92
PCL-1	Polycaprolactone[a]	74 000	DCM:DMF(2:1)	16.7	Gly-10%Tween20	14.3
PCL-3	-do-	-do-	DCM:THF (1:2)	33	-do-	18.07
PCPO-2	Polyanhydride[b]	0.38 in $CHCl_3$	DCM:THF (1:2)	16.7	-do-	31.2
PCPO-3	-do-	at 30°C	-do-	33	-do-	39.4

Protein entrapped-lysozyme.
Attempted loading-5%.
[a] Polycaprolactone of Aldrich Chemical Co.
[b] Polycarboxy phenoxy octanoic acid.

traction while DCM was removed by evaporation. Entrapment efficiency here was low primarily due to use of a water-miscible processing medium which presents a favorable environment for aqueous protein globules to move out of the half-formed microspheres. Further modification of the polymer solution and processing medium is necessary to ensure higher efficiency of entrapment.

3.2. Mechanism of encapsulation

The results shown in Table 4 seem to indicate that the shape of protein influences its encapsulation efficiency-globular proteins being entrapped almost completely while it is low for fibrous proteins. When the progress of microsphere formation was followed under an optical microscope, it was observed that water-AN mixture is forced out of the half-formed microspheres. The mechanism of encapsulation inferred from these observations offered an explanation to the differences in encapsulation efficiency.

As discussed previously, immediately on introduction of the water-in-oil$_1$ emulsion into liquid paraffin, there is breakdown of the water-in-oil$_1$ emulsion into tiny droplets and rapid extraction of DCM. After all the DCM is extracted off, the MSS becomes a one component system, i.e., it comprises only AN, which is water-miscible. This mixing of AN with aqueous protein solution leads to the precipitation of the protein within the half-formed mIcrosphere. Precipitation of proteins by water-miscible organic solvents is reversible and is routinely used for protein purification by biochemists [17].

Simultaneously, the mixture of water and acetonitrile becomes a poor solvent for the polymer and is forced out of the droplet. The migration of water–AN mixture out of the droplet was captured under the microscope (Fig. 3) and occurs through pores/channels in the viscous half-formed microspheres. In absence of water, the AN was not observed as a separate phase (picture not shown). The polymer undergoes phase inversion leading to a microporous structure. The mechanism of phase inversion in this case is analogous to that experienced in the formation of microporous membranes, first proposed by Loeb and Sourirajan [18] and reviewed recently by Wienk et. al. [19]. The concept has also been recently used to explain the mechanism of formation of microparticles by the w/o/w emulsion technique [12]. The phase inversion theory as well as its extension to the elucidation of microsphere formation from w/o/w emulsion systems explain the formation of microporous or dense morphologies of the microspheres. Both have been observed in our case depending on experimental conditions.

Since the microspheres possess tendency to agglomerate until all AN is removed, the fluidity imparted to the polymer matrix by the water–AN mixture enables sealing-off of the pores/channels. The sealing-off of the pores/channels depends upon the rate of removal of AN by evaporation. A rapid evaporation under low pressure or high temperature immediately on introduction of water-in-oil$_1$, emulsion into liquid paraffin would result in pores remaining unsealed. These pores are seen under high magnification (above 6000×) in scanning electron microscope in some of our preparations. Slow evaporation of AN allows sufficient time for the viscous sphere to seal-off the pores and yield a dense internal morphology.

Based on these observations the process of encapsulation and the events that take place on introduction of the water-in-oil$_1$ emulsion into processing medium could be represented schematically as shown in Fig. 2.

3.2.1. Influence of mechanism of encapsulation on entrapment efficiency

Since the migration of water-AN mixture from the microsphere occurs *after* the protein has been precipitated within, the proteins do not have a chance to migrate from aqueous solutions, thereby resulting in successful entrapment. The entrapment efficiency is, however, low if the protein is not fully precipitated on mixing of AN with the aqueous protein solution and if the protein has considerable solubility in the resulting water–AN mixture. It was noted that the globular proteins used in this work were precipitated fully from their aqueous solutions on small additions of AN (~25% v/v) while fibrous proteins needed large additions (>80%) to precipitate.

Thus, it is clear that globular proteins used in this study precipitated fully resulting in almost 100% entrapment efficiency while fibrous proteins migrated along with the water–AN mixture leading to lower efficiency. Entrapment efficiency of the model de-

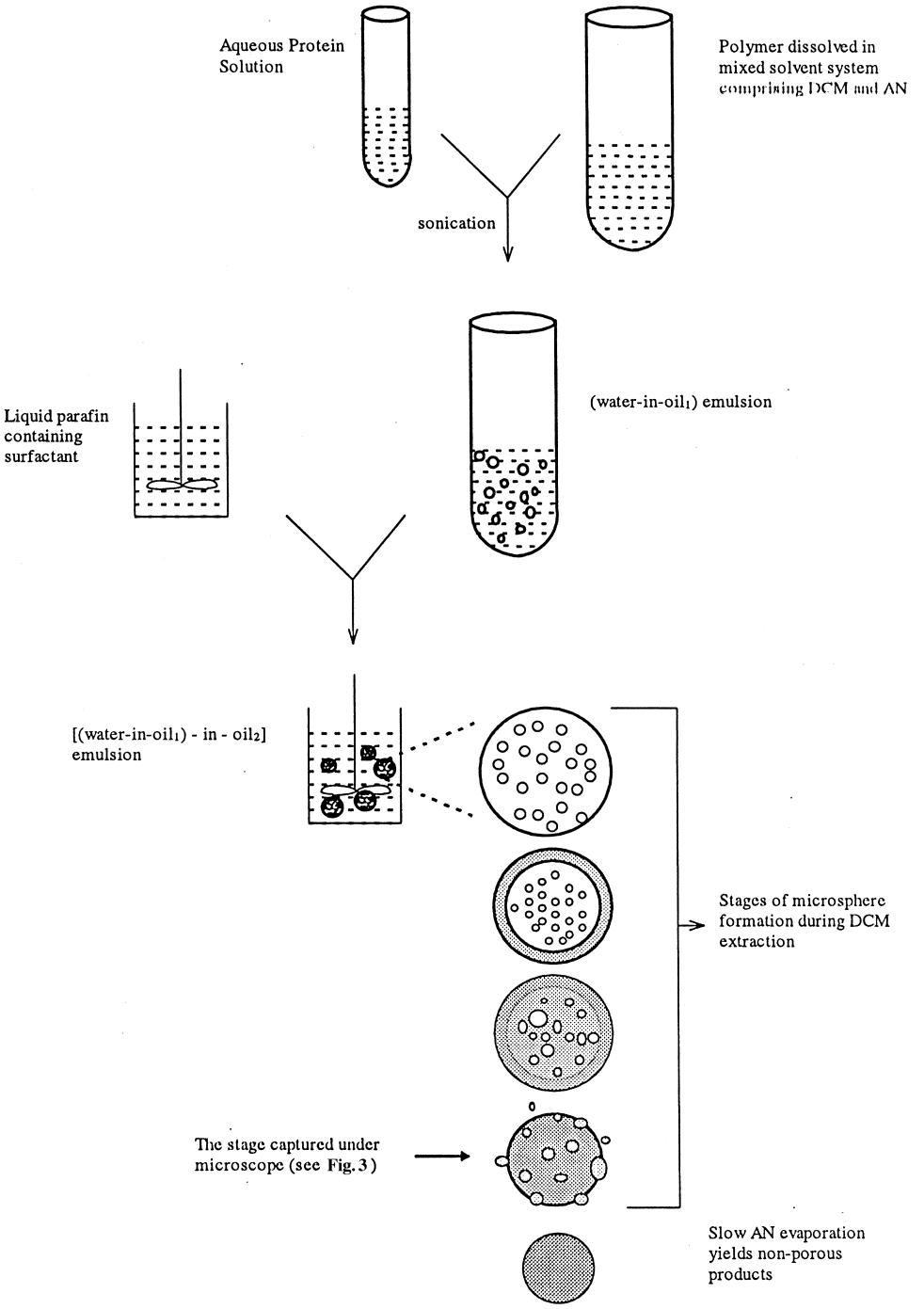

Fig. 2. Schematic representation of mechanism of microencapsulation by the new in-oil drying method.

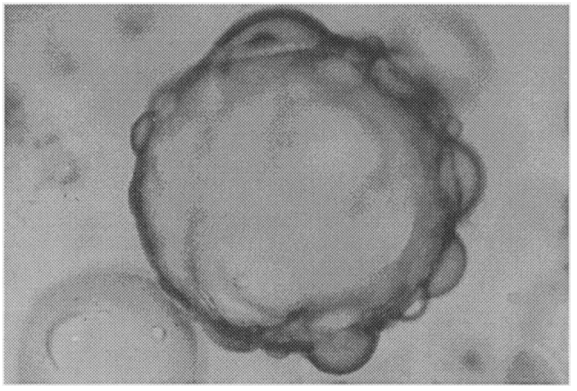

Fig. 3. Photomicrograph shows water-acetonitrile mixture being forced out of the all-formed microsphere during early stages of preparation (see also Fig. 2).

capeptide was low due to its high solubility in water–AN mixture. These observations satisfactorily explain the variation in entrapment efficiency of different proteins (Table 5). Entrapment efficiency of proteins and polypeptides in the in-oil drying method discussed in this work varied inversely with the solubility of proteins in the water–acetonitrile mixture formed during the process of encapsulation.

The shape and their solubility characteristics of proteins and polypeptides depend on factors such as pI, ionic concentration, solvent composition, etc. However, the influence of such parameters was not within the scope of our study with model molecules.

The above mechanism is applicable only when the component of MSS to be removed by evaporation, like AN, is water-miscible. If the component is not miscible with water, the loading efficiency will depend purely upon the barrier properties of polymer solution, i.e., the resistance presented to the migration of aqueous protein globules through this barrier. For example, in a situation where a MSS comprises DCM and THF while glycerin is the processing medium (see Table 6), the entrapment efficiency is low due to poor barrier properties and absence of any solvent aided precipitation within the droplet.

3.3. In vitro release profiles

The release of all globular proteins, from PLGA microspheres lysozyme and BSA, typically showed only a small release (of about 15%) within the first week of incubation which was followed by no further release. There was no burst release observed. The unreleased fraction of the entrapped protein was converted into a water-insoluble mass within the degrading polymer matrix. A similar behaviour was observed with tetanus toxoid loaded microspheres also. On the other hand, the fibrous protein, gelatin, showed a triphasic release and demonstrated satisfactory mass balance between the actual protein loading and cumulative release data. The fraction of lysozyme and tetanus toxoid that was released from the microspheres had retained their molecular weight (as tested by SDS–PAGE analysis) and their biological activity. Lysozyme was tested for its cell-lysis ability on a suspension of *Micrococcus lysodeikticus* while the tetanus toxoid was tested for its antigenicity by antigen–antibody reaction technique. The release behaviour and the stability of protein in the microsphere are dependent on the microsphere degradation mechanism and the microenvironment within the degrading microsphere. These issues are discussed in a separate communication [20].

4. Summary

The new in-oil drying method described involves the use of a combination of mixed solvent system for the polymer and an oil as processing medium to enable high entrapment efficiency. The product yields non-porous particles which could find use in the preparation of microparticles with reduced initial burst release. Mechanism of entrapment involves precipitation of proteins/polypeptides by water-miscible solvent within the microspheres being formed by phase inversion. In vitro release studies showed that the globular proteins were inactivated after a small initial release while the fibrous proteins showed triphasic release behaviour. Incomplete release and protein inactivation within PLGA microspheres has been the focus of research in recent literature [21,22]. In subsequent communications the results of in vitro and in vivo release studies and microsphere degradation will be presented and their influence on the mechanism of release and protein stability discussed.

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