Preparation of Biodegradable Insulin Nanocapsules from Biocompatible Microemulsions

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Purpose. To prepare poly(ethyl 2-cyanoacrylate) nanocapsules containing insulin by interfacial polymerization of spontaneously forming, biocompatible microemulsions.

Methods. A pseudo-ternary phase diagram of a mixture of medium chain glycerides (caprylic/capric triglycerides and mono-/diglycerides), a mixture of surfactants (polysorbate 80 and sorbitan mono-oleate) and water was constructed. Polarizing light microscopy was used to identify combinations forming microemulsions. Microemulsions were characterized by conductivity and viscosity to select systems suitable for the preparation of poly(ethyl 2-cyanoacrylate) nanocapsules by interfacial polymerization. Nanocapsules were prepared by addition of 100 mg of ethyl 2-cyanoacrylate to a stirred water-in-oil microemulsion containing 1 g of water, 7.6 g of oil, and 1.4 g of surfactant. The nanocapsules formed were characterized by photon correlation spectroscopy, freeze fracture transmission and scanning electron microscopy. Insulin nanocapsules were prepared by using an aqueous solution of insulin (100 units/ml) as the dispersed phase of the microemulsion. The entrapment and the release of insulin from the nanocapsules were determined.

Results. Three regions were identified in the pseudo-ternary phase diagram; a microemulsion region, a region in which liquid crystalline structures were present and a coarse emulsion region. All systems in the microemulsion region were water-in-oil dispersions. Poly(ethyl 2-cyanoacrylate) nanocapsules having a mean particle size of 150.9 nm were formed upon interfacial polymerization of the microemulsion. Nanocapsules were found to have a central cavity surrounded by a polymer wall. In excess of 80% of the insulin present in the microemulsion was encapsulated upon interfacial polymerization.

Conclusions. Interfacial polymerization of spontaneously forming water-in-oil microemulsions represents a convenient method for the preparation of poly(alkylcyanoacrylate) nanocapsules suitable for the entrapment of bioactive peptides.

KEY WORDS: nanocapsules; microemulsions; interfacial polymerization; insulin; peptides.

INTRODUCTION

The convenience and acceptability of the oral route for drug administration has meant that it has received much attention for the delivery of macromolecules, such as proteins and peptides. However, oral delivery of these bioactives is associated with low bioavailability due to proteolytic degradation and poor membrane permeability (1).

Various approaches have been used in an attempt to overcome these barriers and increase the oral bioavailability of such bioactives including the use of polymeric particulates (2). It has been demonstrated that encapsulation within particulate delivery systems can protect peptides from proteolytic enzymes (3,4). Further, it has been suggested that if the size of the particles is sufficiently small (<1 μm), they may pass across the intestinal mucosa and hence facilitate the absorption of peptide drugs from the gut lumen (5–7). Under such circumstances, the polymer used for the preparation of the particulate carrier must be biodegradable.

Biodegradable, aqueous cored nanocapsules suitable for the entrapment of proteins and peptides can be prepared by interfacial polymerization of water-in-oil dispersions using alkylcyanoacrylates. However, to obtain a dispersed aqueous phase having a suitably small particle size from kinetically stabilised water-in-oil coarse emulsions, a high input of energy in the form of either ultrasonication (8) or vigorous stirring (9) is required. Even then, it is difficult to obtain a disperse phase having a uniform particle size below 200 nm.

The requirement of a high input of energy may be overcome by the use of microemulsions. Microemulsions are spontaneously forming, thermodynamically stable, dispersed systems having a uniform droplet size of less than 200 nm. As such, they represent a novel system that may be exploited for the preparation of poly(alkylcyanoacrylate) nanocapsules by interfacial polymerization. If biocompatible oils and surfactants are used to formulate the microemulsions, the necessity of isolating the nanocapsules from the reaction matrix following polymerization is removed.

The *in situ* formation of nanocapsules in an oily microemulsion matrix may also prove beneficial for the delivery of the encapsulated bioactive as the oral bioavailability of a number of peptides has been increased by administration in lipid-based microemulsions (10–14). The mechanism by which microemulsions enhance the absorption of peptides may be a result of their effect on membrane structure and fluidity (11–14). Lipid-based delivery systems may also promote uptake by lymph thereby overcoming hepatic first-pass metabolism (14–17).

The aim of the current study was to investigate whether dispersions of poly(ethyl 2-cyanoacrylate) nanocapsules having a narrow size distribution and capable of efficiently encapsulating a model peptide can be prepared from biocompatible microemulsions in a simple one step process that requires only minimal input of energy and no need for subsequent separation procedures.

MATERIALS AND METHODS

Materials

Caprylic/capric triglycerides (Crodamol GTCC™), polysorbate 80 (Crillet 4™) and sorbitan mono-oleate (Crill 4™) were supplied by Croda Surfactants NZ (Auckland, NZ). Caprylic/capric mono-/diglycerides (Capmul MCM™) was a gift from Abitec Corp. (Columbus, OH). Ethyl 2-cyanoacrylate was purchased from Sigma (St. Louis, MO). Human insulin (Humulin R™) was obtained from Eli Lilly (Auckland, NZ). Acetonitride (HPLC grade) and chloroform (AR grade) were

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ABBREVIATIONS: PCS, photon correlation spectroscopy; TEM, transmission electron microscopy; SEM, scanning electron microscopy.

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purchased from BDH (Poole, Dorset), UK. Distilled water was used throughout.

Methods

Construction of Pseudo-Ternary Phase Diagram

Pseudo-ternary systems of oil (Crodamol GTCC and Capmul MCM, 3:1 weight ratio), surfactant (Crillet 4 and Crill 4, 3:2 weight ratio) and water of various weight ratios were prepared and left overnight at room temperature to equilibrate. The weight ratio of the two surfactants used was chosen according to the combination which solubilized the greatest amount of water in an oil system containing 20% w/w total surfactant. Visual observation, phase-contrast and polarizing light microscopy (Optiphot Nikon PFX microscope) were used to identify combinations forming microemulsions, liquid crystalline structures or coarse emulsions. Colloidal systems showing birefringence when viewed by cross-polarized light microscopy were designated liquid crystalline whereas clear, non-birefringent, isotropic systems were designated microemulsions. A pseudo-ternary phase diagram for the components was constructed and the phase boundaries identified.

Characterization of Microemulsions

Oil/surfactant/water combinations forming microemulsions were characterized with regards to viscosity (Brookfield DVIII viscometer fitted with a CP-42 cone and plate spindle) and conductivity (YSI 3418 conductivity cell, Yellow Spring Instruments, Yellow Springs, OH having a cell constant (K) of 0.1/cm). Samples for conductivity measurement were prepared using 0.1 molar sodium chloride as the aqueous phase. Viscosity and conductivity measurements were carried out in duplicate at 25°C.

Preparation of Nanocapsules

A microemulsion was prepared at 4°C by mixing 1.4 g of the surfactant blend, 7.6 g of the oil mixture and 1.0 g of water (buffered to pH 7.4 with isotonic phosphate buffer). A solution containing 100 mg of ethyl 2-cyanoacrylate monomer in 300 mg of chloroform was slowly added to the microemulsion under mechanical stirring. The system was left for at least 4 hours at 4°C for polymerization. Nanocapsules were collected for characterization by centrifugation at 51500 g for 60 min at 25°C (Beckman J2/MC Centrifuge, JA20.1 rotor). For SEM and PCS, the product was redispersed by ultrasonication in ethanol followed by centrifugation at 18,500 g for 10 minutes at 25°C to remove residual oil and surfactant. This process was repeated to ensure complete removal of residues.

For the preparation of nanocapsules containing insulin, the water was substituted with an aqueous solution of insulin having a concentration of 100 units/ml and a pH of 7.4 (Humulin R®).

Characterization of Nanocapsules

The external and internal structure of the nanocapsules was visualized by SEM (Cambridge Stereoscan 360) and freeze fracture TEM (Philips 410LS) respectively. Samples for SEM were dried by using a graded series of ethanol and then critical point dried using a Bal-Tec 030 critical point dryer. Samples were then sputter coated with gold/palladium (BioRad coating system) prior to viewing. For freeze fracture TEM, nanocapsules were sandwiched between two copper grids, snap-frozen by immersion in liquid propane (−180°C) and freeze fractured using a Balzers BAF 300. Fractured samples were shadowed with platinum (45°) and carbon (90°). The replica was then washed in chloroform, methanol and finally distilled water. Dried replicas were viewed at an accelerating voltage of 80 kV.

The particle size and distribution of the nanocapsules was measured by PCS (Zetasizer 3000, Malvern Instruments Ltd.). For analysis, nanocapsules were washed repeatedly following synthesis and dispersed in a 0.2% w/w polysorbate 80 solution in ethanol. Measurements were carried out at 25°C.

Determination of Encapsulated Insulin

1.6 g of the polymerized insulin microemulsion was diluted to 10 ml with water adjusted to pH 2.5 by addition of hydrochloric acid. An acidic medium was chosen for dilution to inhibit polymer hydrolysis and thus prevent release of entrapped insulin. 300 μl of this dispersion was thoroughly mixed with 300 μl of 80% v/v methanol in water (pH 2.5). Nanocapsules and oil were separated from the methanolic aqueous phase by centrifugation (12,000 g for 12 minutes at room temperature). The concentration of insulin in the aqueous supernatant representing the insulin not associated with the nanocapsules was determined by HPLC using a C18 column (Luna™ 5 μ C18 (2), 250 × 4.6 mm; Phenomenex) and a mobile phase of 23.5% w/w acetonitrile in a 0.125 molar solution of sodium dihydrogen phosphate adjusted to pH 2.5 with orthophosphoric acid. The column was maintained at 50°C and the flow rate at 1.4 ml/min. 200 μl of sample was injected and the eluent monitored at a wavelength of 212 nm (Spectra-Physics UV-2000). The amount of insulin encapsulated was estimated from the difference in concentration of insulin detected in the supernatant of a polymerized microemulsions following processing and that in the supernatant of an unpolymerized microemulsions to which an equivalent concentration of insulin had been added. The extraction efficiency of insulin from an unpolymerized microemulsion by this method was 95.19 ± 2.58% (n = 3).

The release of insulin from nanocapsules was carried out by diluting 4 g of polymerized microemulsion containing insulin to 25 ml with water (pH 2.5) which was subsequently stirred at 200 rpm in a water-jacketed beaker (45°C). These conditions were chosen to obtain a time-release profile suitable for the investigation of the reservoir nature of the nanocapsules. The concentration of insulin in the release medium as a function of time monitored. Samples were processed and insulin analyzed as for determination of encapsulated insulin within nanocapsules. Under these conditions, it was found that when free insulin was added to the release medium containing polymerized microemulsions, 8% degraded in a first-order fashion over a five hour period. The results for the release of insulin from the nanocapsules were thus compensated for this degradation.

RESULTS

Pseudo-Ternary Phase Diagram

The choice of components used for the preparation of the pseudo-ternary phase diagram was based on the work of

Constantinides *et al.* (13). They demonstrated that a mixture of medium chain glycerides similar to those used in the present investigation formed water-in-oil microemulsions when mixed with certain weight ratios of polysorbate 80 and water. Further, these researchers demonstrated that a microemulsion prepared from these components significantly increased the oral bioavailability of both a water-soluble marker and a water-soluble peptide. Thus, if nanocapsules could be prepared from such a microemulsion, then a combination of nanocapsules dispersed in the microemulsion matrix may be beneficial in the oral delivery of water-soluble peptides. To maximize the peptide loading and yield of nanocapsules prepared from such systems, we investigated whether the fraction of the aqueous dispersed phase of the microemulsions reported by Constantinides *et al.* (13) could be increased. The maximum water solubilized by a 20% surfactant in oil system was therefore determined for a series polysorbate 80/sorbitan mono-oleate mixtures (Fig. 1). The maximum percentage water solubilized was achieved for systems containing polysorbate 80:sorbitan mono-oleate having weight ratios of between 3:2 and 1:1, being approximately 14% w/w compared with 8% w/w when only polysorbate 80 was used.

The pseudo-ternary phase diagram for the mixture of medium chain triglycerides and mono-/diglycerides having a weight ratio of 3:1 and a mixture of polysorbate 80 and sorbitan mono-oleate having a weight ratio of 3:2 and water is shown in Fig. 2. The nature of the phase diagram was not altered if the water was replaced with a 0.1 molar aqueous solution of sodium chloride. The maximum amount of water which could be incorporated into systems forming microemulsions following an adequate equilibration period was found to be only 14% w/w. In preliminary studies, the microemulsions obtained by Constantinides *et al.* (13) at high surfactant concentrations (50–80% w/w) and water concentrations of between 15 and 40% w/w were only observed immediately following mixing of the components. However, these microemulsions were found to transform to systems containing liquid crystalline structures upon overnight equilibration.

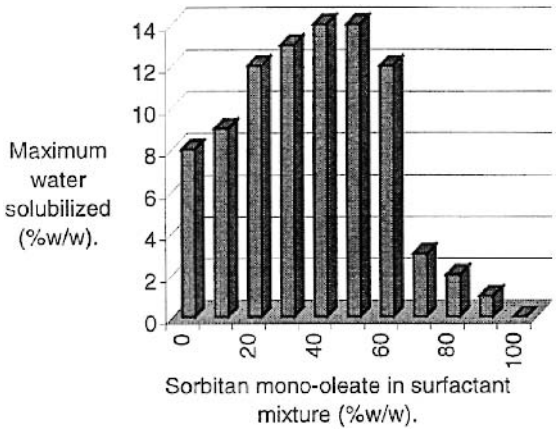

Fig. 1. Maximum percentage water solubilized by a system containing medium chain glycerides and mixtures of polysorbate 80/sorbitan mono-oleate having different weight ratios. Ratio of medium chain glycerides to surfactant mixture is 4:1.

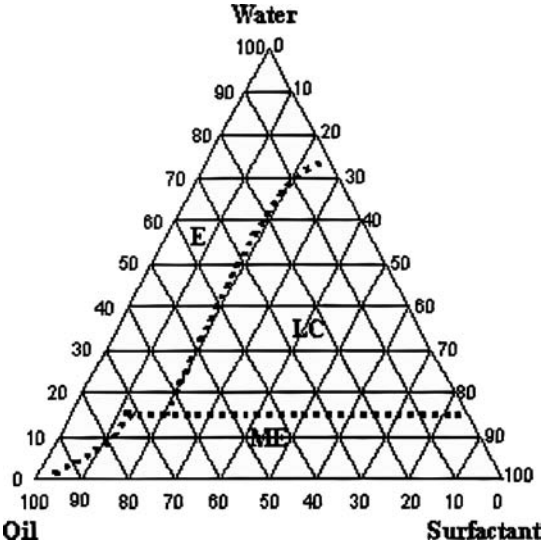

Fig. 2. Pseudo-ternary phase diagram for a mixture of medium chain glycerides (Crodamol GTCC:Capmul MCM, 3:1), a mixture of surfactants (polysorbate 80:sorbitan mono-oleate, 3:2) and water. ME: microemulsion, LC: systems containing liquid crystals and E: coarse emulsions.

Characterization of Microemulsions

All microemulsions were poor conductors having specific conductances in the range 0.35–5.05 μmhos/cm as compared to 10,420 μmhos/cm for a 0.1 molar aqueous solution of sodium chloride. Thus all systems in the microemulsion region of the phase diagram are water-in-oil dispersions.

Microemulsions demonstrated Newtonian flow behavior. The viscosity was found to be dependent on both the concentration of water and surfactant (Fig. 3). Systems having a viscosity of greater than 50 cps were considered too viscous to allow for good mixing upon addition of the ethyl 2-cyanoacrylate monomer. A system containing 10% water, 76% glycerides and 14% surfactant (3:2 ratio of polysorbate 80:sorbitan mono-oleate) having a viscosity of 39 cps was therefore used for the preparation of aqueous cored poly(ethyl 2-cyanoacrylate) nanocapsules.

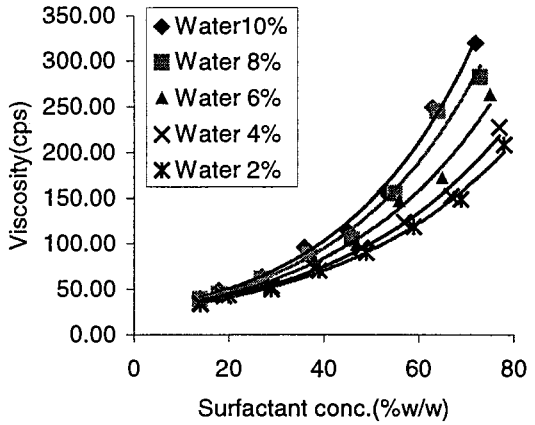

Fig. 3. Viscosity of microemulsions as a function of surfactant and water content.

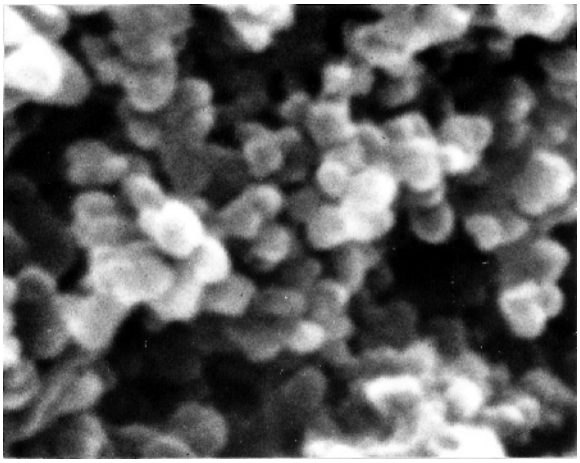

Fig. 4. Scanning electron micrograph of poly(ethyl 2-cyanoacrylate) nanocapsules.

Characterization of Nanocapsules

Particles isolated following addition of ethyl 2-cyanoacrylate to the microemulsion were observed to be spherical with a smooth surface when viewed by SEM (Fig. 4). TEM of freeze-fractured samples showed that the particles had a central cavity surrounded by a polymer wall (Fig 5). The particle size of the nanocapsules as determined by PCS was 150.9 nm with a polydispersity of 0.101 representative of the narrow distribution of the microemulsion droplets.

Nanoencapsulation of Insulin

The concentration of insulin in the supernatant of processed samples of polymerized microemulsions was found to be 13.9 ± 2.7% (n = 4) of that of unpolymerized microemulsions containing an equivalent amount of insulin. Thus, 86% of the insulin was associated with the nanocapsules.

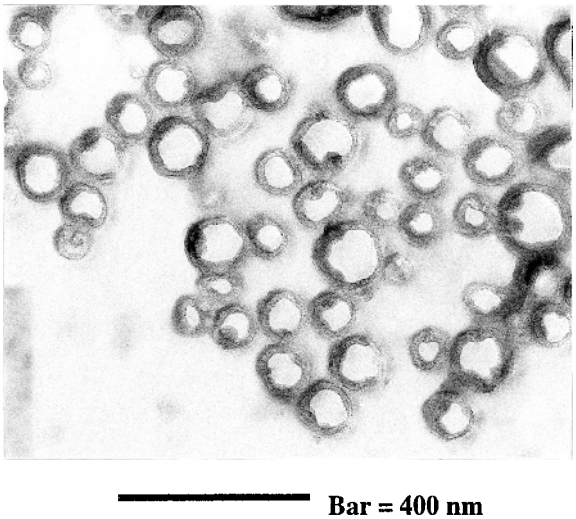

Fig. 5. Freeze-fracture transmission electron micrograph of poly(ethyl 2-cyanoacrylate) nanocapsules.

When the rate of release of insulin from polymerized microemulsions was measured in water (pH 2.5), an initial rapid rate of release was observed over the first 30 minutes which was followed by a period during which the rate remained constant (60–180 mins) before a decline in the rate was observed after 180 minutes (Fig. 6). The initial concentration of insulin in the release medium was found to be equivalent to 14% of the total insulin which was in agreement with the concentration of unencapsulated insulin present in the formulation. The concentration of insulin in the medium when release was complete was equivalent to around 80% of the total insulin added to the microemulsion prior to polymerization.

When the release medium was maintained at 4°C, retarding the hydrolytic degradation of the polymer, no release of associated insulin was noted over a 10 hour period. Thus the method used for processing the samples did not cause disruption of the nanocapsules and suggests that the associated insulin is encapsulated within the nanoparticles.

DISCUSSION

Poly(alkylcyanoacrylates) constitute a group of biocompatible, biodegradable polymers, which can be prepared by a base-catalyzed anionic polymerization of the appropriate alkylcyanoacrylate monomer (18). Aqueous cored poly(alkylcyanoacrylate) particulates suitable for the encapsulation of peptides can therefore be prepared by the interfacial polymerization of the monomer using water-in-oil dispersions (9). However, to achieve a dispersed phase having a suitably small particle size to form nanocapsules, a high input of energy in the form of either ultrasonication (8) or vigorous stirring (9) is usually required. This requirement can be overcome by the use of thermodynamically stable colloidal systems, such as micelles or microemulsions. The use of such systems offers further advantages over kinetically stabilised dispersions in that their particle size is small and uniform, and they form spontaneously and reproducibly. These properties render them amenable to industrial scale-up.

It is however unlikely that micellar polymerization can be used to efficiently nanoencapsulate hydrophilic bioactives such as proteins and peptides due to the aqueous nature of the continuous phase, although a high association has been reported when

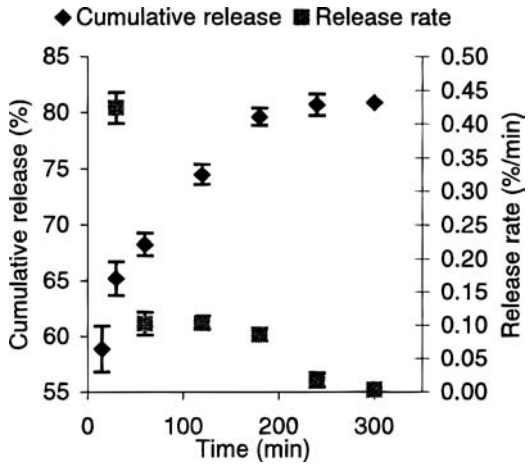

Fig. 6. Release of insulin from nanocapsules in water (pH 2.5). Values represent means ± SD, n = 4.

insulin is adsorbed onto pre-formed nanoparticles (4). The preparation of nanocapsules from water-in-oil microemulsions as described in the present study results in a high encapsulation efficiency of insulin since the water-soluble peptide is confined to the aqueous phase which is in the form of nanodroplets of the water-in-oil microemulsions. Polymerization at the oil/water interface can therefore encapsulate a large percentage of the water-soluble peptide. A drawback of this technique, however, could lie in the difficulty of isolating the nanoparticles from the polymerization vehicle, requiring extensive washing to remove residual oil and surfactant with associated potential for particle aggregation (19). However, if non-toxic components, as used in the present study are used as the polymerization vehicle, isolation of the nanoparticles from the microemulsions may not be necessary. In fact, the use of a microemulsion as a matrix for polymerization may be beneficial for the oral delivery of peptides. Microemulsions in themselves can protect incorporated peptides from proteolytic degradation and enhance absorption either by inducing membrane fluidity (11–14,20,21) or by promoting lymphatic uptake (14–17). The use of medium chain glycerides, as used in the formulation of the microemulsion in the present study, has shown to be particularly beneficial in enhancing drug absorption (22,23).

The use of nanocapsules for the oral delivery of insulin has also been shown to have beneficial effects (7). Nanoparticles can protect encapsulated peptides from proteolytic enzymes and facilitate absorption, since uptake and translocation of nanoparticles can occur following oral administration (5,24,25). Nanoparticulates prepared from poly(alkylcyanoacrylate) again appear to be particularly useful in this respect. It has been demonstrated that the efficacy of insulin loaded poly(alkylcyanoacrylate) nanoparticles can be further enhanced by their administration in a solution of medium chain triglycerides containing surfactant (4). Preparation of nanocapsules from water-in-oil microemulsions by interfacial polymerization of alkylcyanoacrylate monomers can form such a system *in situ*. Further requirements of microemulsions for use in the preparation of such systems are that the dispersed droplets must be aqueous and the microemulsion must be sufficiently fluid to enable good mixing following addition of the monomer.

Interfacial polymerization of water-in-oil microemulsions would be expected to result in reservoir rather than matrix type nanoparticles. Freeze fracture TEM confirms the capsule nature of the nanoparticles prepared and the release rate profile is in agreement with what would be expected from such a system. Nanoencapsulation of insulin by this technique results in a high entrapment, although not complete. When the release of insulin is evaluated, it is noted that not all the insulin originally added to the system is recovered in the release medium. This may suggest that under the conditions used, some of the insulin may have been involved in the base-catalyzed polymerization reaction.

This study has shown that aqueous cored nanoparticles capable of efficient entrapment of peptides can be prepared from microemulsions. The use of biocompatible oils and surfactants can eliminate the requirement of isolating the nanoparticles from the polymerization vehicle to produce a formulation in which peptide-containing nanocapsules are dispersed in a vehicle which may have absorption enhancing effects. The potential of this system for the delivery of the model peptide, insulin will be evaluated in a future study.

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