Hydrophilic Microspheres from Water-in-Oil Emulsions by the Water Diffusion Technique

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Purpose. In this study, we developed and evaluated a novel method to produce insulin-loaded hydrophilic microspheres allowing high encapsulation efficiency and the preservation of peptide stability during particle processing. The preparation method used the diffusion of water by an excess of solvent starting from a water-in-solvent emulsion.

Methods. The water dispersed phase containing albumin or lactose, or albumin-lactose in different weight ratios, and insulin was emulsified in water-saturated triacetin with and without emulsifiers, producing a water-in-triacetin emulsion. An excess of triacetin was added to the emulsion so that water could be extracted into the continuous phase, allowing the insulin-loaded microsphere precipitation. Insulin stability within the microspheres after processing was evaluated by reverse-phase and size-exclusion high-performance liquid chromatography.

Results. The water diffusion extraction process provided spherical microparticles of albumin or albumin-lactose. The mean diameter of the microspheres prepared with or without emulsifiers ranged from 2 to 10 μm, and the encapsulation efficiency of insulin was between 60% and 75%, respectively. The analysis of microsphere content after processing showed that insulin did not undergo any chemical modification within microspheres. The use of lactose alone led to the formation of highly viscous droplets that coalesced during the purification step.

Conclusions. The water extraction procedures successfully produced insulin-loaded hydrophilic microspheres allowing the preservation of peptide stability. The type of excipient and the size of the disperse phase of the primary w/o emulsion were crucial determinants of microsphere characteristics.

KEY WORDS: albumin microspheres; insulin; water extraction; water-in-triacetin emulsion.

INTRODUCTION

The preparation methods for hydrophilic microparticle carriers, such as albumin, lactose, and gelatin, can be classified in three main categories: precipitation of the carrier from a water-in-oil emulsion, coacervation, and spray-drying.

The first method (1) involves the formation of small droplets of aqueous carrier solution in an immiscible liquid (e.g., soya oil); acetone is then normally added to solidify the droplets of the disperse phase. Once formed and eventually hardened, the microperes are filtered, washed, and dried. It is difficult to produce microperes less than 1 μm in size, and it may also be problematic to remove all traces of the oil. In the coacervation (2,3) method, hydrophilic microperes are produced by controlled desolvation using an organic nonsolvent, such as ethanol, and acetone leading to very small particles but with low drug entrapment efficiency. Microparticles can also be produced using a spray-dryer (4). Solutions are pumped into the drying chamber and pneumatically atomized through a two-fluids external mixing nozzle using compressed air. The inlet temperature is generally set at about 100°C; the outlet temperature depends on the inlet temperature and the liquid and gas flow rates and varies between 50 and 70°C. Yields are between 10% and 30%, with the highest yields achieved with the densest powders. The main advantages of spray drying are that the product is free of oil or organic solvent residues and the process is useful for continuous operation. One concern, however, is the instability of heat-labile drugs during spray drying.

The study investigated the feasibility of producing hydrophilic microparticles via the solvent diffusion process.

The solvent diffusion technique is widely described in the literature. The method uses a transient oil-in-water (o/w) emulsion containing a partially water-soluble solvent as internal phase. Upon transferring the emulsion into an excess of water, the oil droplets, containing a polymer, a lipid, or a drug, solidify instantly due to the almost-complete diffusion of the organic solvent from the droplets to the continuous phase. Using ethyl formate, benzyl alchohol, triacetin, or propylene carbonate, this technique has led to the successful fabrication of good-quality drug-loaded polymeric microspheres such as poly (lactic acid) (PLA) and its copolymers with poly (glycolic acid) (PLGA) (5,6), drug microparticles (7), and lipid microspheres (8).

Many studies have been conducted on albumin and lactose microspheres for drug delivery to the lung (9,10). Albumin and lactose microspheres can be prepared over a wide range of particle size and are able to entrap a variety of drugs. It is also been demonstrated that these excipients are biocompatible and biodegradable. Albumin and lactose microspheres may therefore provide suitable carriers for the delivery of drugs to the airways.

This study looks at the possibility of applying the solvent diffusion procedure starting from a w/o emulsion to prepare hydrophilic microparticles using albumin and lactose. The goal was to prepare microspheres of about 3–6 μm containing insulin as model drug. The preservation of its native conformation is a condition for the maintenance of insulin's biological activity, both for use in traditional therapy as well as for novel delivery methods (11,12). Insulin aggregation induced by a simple water-organic solvent interface was also monitored *in situ*, to guide the choice of solvent to prepare the w/o emulsion.

MATERIALS AND METHODS

Materials

Ovoalbumin (albumin), lactose, bovine insulin, polyoxyethylensorbitan monooleate (Tween 80), sorbitan monolaurate (Span 20), trifluoroacetic acid (TFA), and all regents were obtained from Sigma Chemicals Co (St. Louis, MO, USA). Water was freshly bidistilled.

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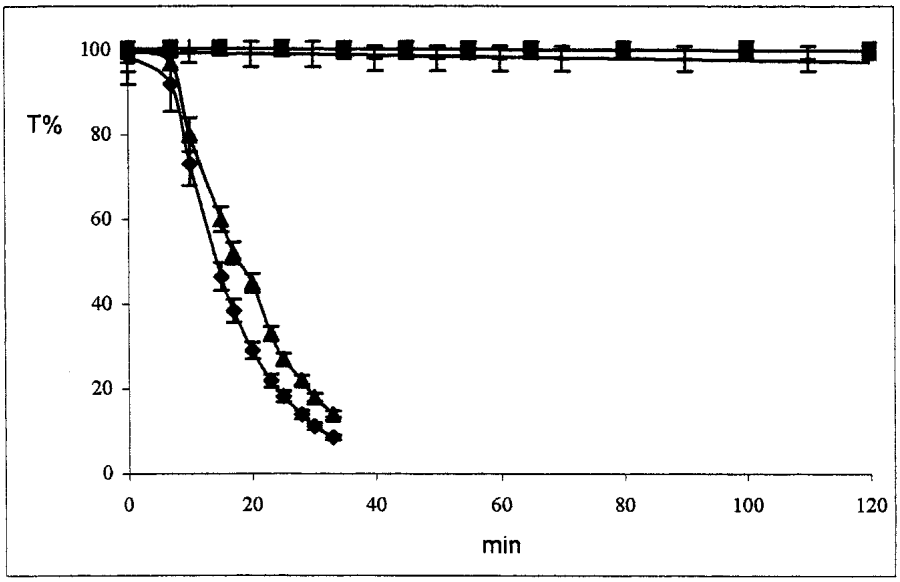

Fig. 1. The effect of triacetin (+), triacetin-Span (■), methylene chloride (♦), and methylene chloride-Span (▲) on insulin aggregation is shown. Data points are presented as mean ± SD (n = 3).

In Situ Study of Insulin Aggregation

Insulin aggregation at a simple water-solvent interface was monitored spectrophometrically by detecting the percentage of turbidity change (%T) at 350 nm, as described by Kwon (13). Briefly, a Zn-free insulin solution was prepared by dissolving an appropriate amount of Zn-crystalline insulin powder in 0.01 mol/L HCl. Phosphate saline buffer containing 0.06% EDTA was then added dropwise and the Zn-free insulin solution filtered through a 0.22-μm low protein-binding syringe filter. The insulin concentration was determined by high-performance liquid chromatography (HPLC) and was 1.5 mg/ml for all experiments. This solution was then gently placed in the top of UV quartz cell containing triacetin (density = 1.158 g/ml) or methylene chloride (density = 1.325 g/ml); if used, emulsifier (10 mg/ml) was in the bottom. The height of the cuvette was adjusted so that the light beam did not touch the interface, and the percentage turbidity change (%T) under unstirred condition was recorded as a function of time. Decreased transmittance was associated with increased aggregation at the solvent-aqueous solution interface. Appropriate blanks were used in all experiments (i.e., the same components except for the protein).

Preparation of Microspheres

Triacetin and 0.01 mol/L HCl were mutually saturated at room temperature for 10 min in order to ensure initial thermodynamic equilibrium of both liquids. Typically, 80 mg albumin or lactose or albumin:lactose at different weight ratio (4:1, 1:1, 1:4) were dissolved in 400 μl of triacetin-saturated 0.01 mol/L HCl containing 8 mg/ml insulin. This solution was emulsified at room temperature with 1.5 ml of 0.01 mol/L HCl -saturated triacetin, using an Ultra Turrax (IKA, Staufen, Germany) at 12,000 rpm for 2 min. When emulsifier was used, 20 mg Span 20 or 20 mg Tween 80 or 10 mg Span 20 and 10 mg Tween 80 were dissolved in the organic solvent prior to the emulsification step. Aliquots of emulsion were collected and observed under light microscope to investigate stability. The w/o emulsion (1 ml) was immediately transferred into 3

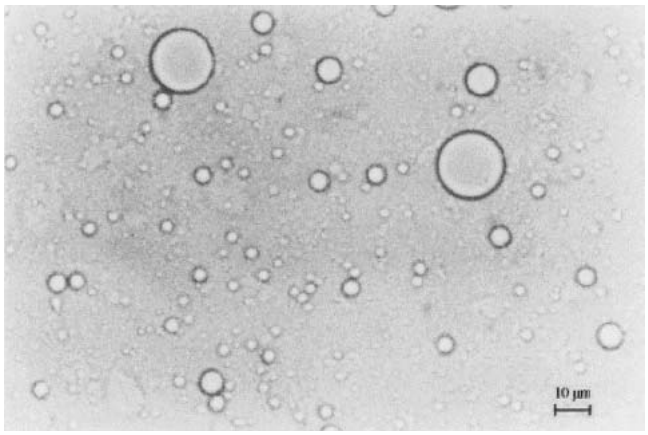

Fig. 2. Light microscope photograph of water-in-triacetin emulsion.

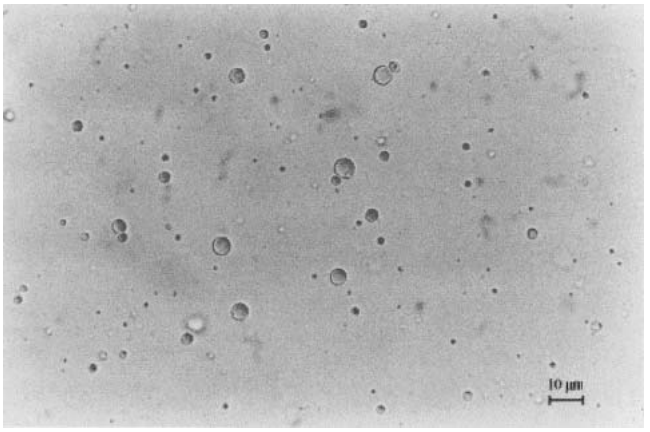

Fig. 3. Light microscope photograph of water-in-triacetin emulsion with emulsifier.

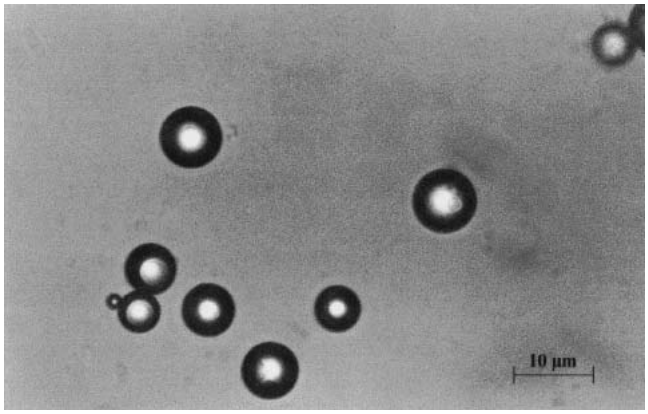

Fig. 4. Light microscope photograph of albumin-lactose (4:1 w/w) microsphere suspension.

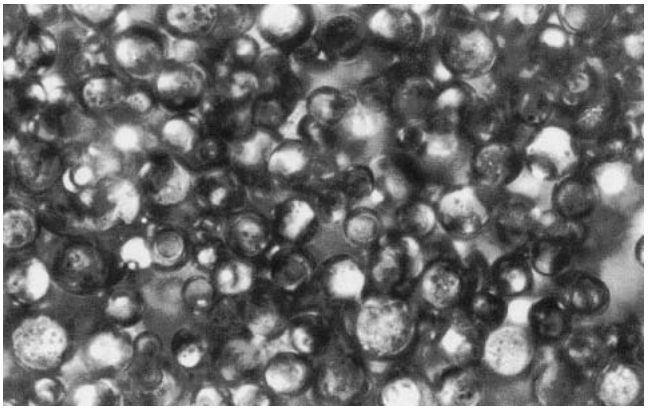

Fig. 6. Light microscope photograph of albumin-lactose (4:1 w/w) dry microspheres.

ml of triacetin and stirred at 500 rpm with a magnetic stirrer for 10 min. An aliquot of microsphere suspensions was observed under light microscope and analyzed by image analysis (Motic Inc., Hong Kong). The microspheres were then collected by centrifugation, washed with ethanol, and dried under vacuum for at least 24 h. At least three batches were prepared for each sample.

Insulin Recovery and Insulin Stability

The insulin was analyzed by HPLC after dissolution of a weighed amount of dry microspheres in 0.01 mol/L HCl. Reverse-phase (RP-HPLC) and size-exclusion (SE-HPLC) chromatography were performed in order to detect the different degradation products of insulin. The cromatograph was equipped with a Shimadzu (Shimadzu, Kyoto, Japan) HPLC system with a UV detector at 220 nm. For the RP-HPLC, a Kromasil C18 column (5 µm, 4.6 × 250 mm, Teknokroma, Barcelona, Spain) was used. The mobile phase was a mixture of water containing 0.1% v/v TFA and acetonitrile containing 0.1% v/v TFA. The solutions were mixed in a ratio of 69.1: 30.9 v/v. The flow rate was 1 ml/min and the retention time was 11 min.

For the SE-HPLC, a TSK gel column (7.8 × 300 mm, Tosoh Bioscience, Amsterdam, NL) was used. The mobile phase was an aqueous solution of potassium phosphate monobasic 50 mmol/L adjusted to pH 6.8 with potassium hydroxide. Flow rate was 1 ml/min and the retention time was 9.2 min.

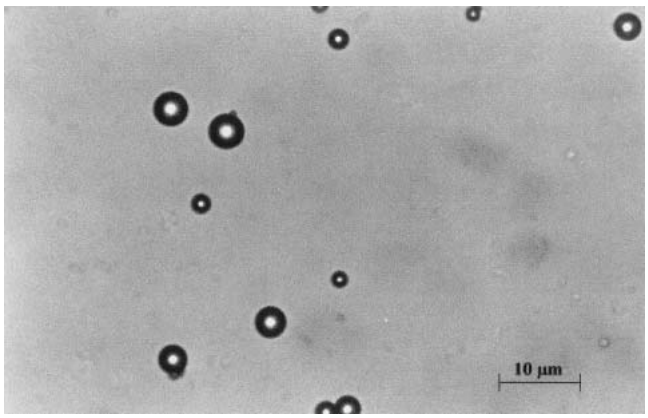

Fig. 5. Light microscope photograph of albumin-lactose (4:1 w/w) microsphere suspension obtained in the presence of emulsifier.

RESULTS AND DISCUSSION

The production of hydrophilic microparticles by the diffusion technique required, as first step, the formulation of a water-in-solvent emulsion (w/o emulsion), containing the drug and the hydrophilic carrier in the inner phase, with a solvent having the capacity to solubilize water. After preliminary experiments, triacetin, a solvent possessing low toxicity (oral-rat LD_{50} = 3100 mg/kg), was used as external phase to prepare the w/o emulsion. The solubility (w/w) of water in triacetin is about 6%, and the water solubility of triacetin is 6.5%.

Albumin, lactose, and a mixture of the two substances were used as hydrophilic excipients.

Insulin was chosen as model drug, being an unstable, water-soluble protein for which the physical stability issues have been study in detail (14). It is known that the presence of an air-water or a water-organic solvent interface leads to rapid aggregation and fibril formation of an aqueous solution of insulin (15,16). Because protein molecules in the inner-water phase of an emulsion may maintain contact with the organic solvent until the fabrication process is complete, it was preferred to check the stability of insulin at the water-triacetin interface.

The water-in-solvent-in-water double emulsion solvent evaporation technique, using a volatile and immiscible organic solvent such as methylene chloride (17), is often used to prepare microspheres containing water-soluble protein drugs,

Table I. Insulin Encapsulation Efficiency (% ± SD) by RP- and SE-HPLC

Microsphere composition	RP-HPLC	SE-HPLC
Albumin	70.4 ± 3.2	72.8 ± 2.8
Albumin Tween 80-Span 20	60.3 ± 3.6	63.6 ± 2.2
Albumin-lactose (80–20)	75.7 ± 2.8	78.3 ± 2.4
Albumin-lactose (80–20) Tween 80 Span 20	64.4 ± 4.1	65.9 ± 1.9

The values are the mean of three measurements ± SD calculated on three batches of microspheres. RP-HPLC, reverse-phase high-performance liquid chromatography; SE-HPLC, size-exclusion high performance liquid chromatography.

so this solvent was used as reference in the *in situ* study of insulin aggregation.

Figure 1 shows the effect of triacetin or methylene chloride on the insulin aggregation rate. As has been reported (13), rapid aggregation was observed with methylene chloride. The %T values decreased to 20% T within 20 min in the presence of methylene chloride alone, and within 30 min in the presence of 10 mg/ml Span 20. Essentially, no aggregation was observed for 2 h at the triacetin-water interface with or without emulsifiers.

When 400 μl of triacetin-saturated water containing albumin or albumin-lactose were emulsified in 1.5 ml water-saturated triacetin, with or without emulsifiers, the water phase was well broken into microdroplets. The emulsification time was set at 2 min, which was sufficient to break down the excipient containing the dispersed phase into small droplets.

Figures 2 and 3 show examples of light microscope photographs of the water-in-triacetin emulsions examined immediately after homogenisation.

Optical microscopy was utilised to determine emulsion droplet size. It was not possible to use laser light scattering technique, because of the impossibility of diluting the emulsions (e.g., with water-saturated triacetin) without changing their original droplet size.

As expected, finer water droplets were obtained using emulsifiers (Fig. 3) than without (Fig. 2), and no appreciable differences were observed using Tween, Span, or mixtures of the two emulsifiers. Without mechanical stirring, all emulsions visibly started to segregate after 15–20 min. The subsequent dilution under standard conditions with an excess of triacetin was therefore done immediately after homogenisation.

Upon transferring a transient w/o emulsion into an excess of triacetin, the droplets solidify instantly due to the almost complete diffusion of water from the droplets to the continuous phase. The amount of triacetin used was calculated such that it was sufficient to extract all the water from the aqueous phase. The fast dissipation of water caused rapid excipient precipitation.

Light micrographs were taken to observe the status of microparticle suspensions and image analysis was used to determine a coarse mean particle size. Using albumin or 4:1 or 4:2 albumin:lactose mixtures, spherical particles of about 6.8 μm ($n \sim 500$) were obtained in the absence of emulsifiers (Fig. 4); the use of emulsifiers led to smaller particles (Fig. 5) of about 3.2 μm ($n \sim 500$). This result suggests that the use of emulsifiers breaks up the embryonic water droplets into smaller ones and that, in consequence, smaller microspheres can be obtained. This is in agreement with previous reports (5) concerning the influence of the onset of solvent quenching on the size distribution pattern of PLGA microspheres.

The suspensions were then centrifuged, the microspheres washed three times with ethanol and then dried under vacuum. Figure 6 shows as an example a light micrograph of the microspheres prepared from albumin:lactose 4:1 suspension after purification and drying; the microspheres are relatively monodispersed and smooth.

The water diffusion technique did not give satisfactory results with 1:1 or 1:2 albumin:lactose mixtures because not only microcapsules but also large irregular aggregates formed as soon as the w/o emulsion was added to the triacetin. Also, the use of lactose alone led to the formation of highly viscous droplets as confirmed by the coalescence that occurred during the subsequent centrifugation step. This behavior was also observed when increasing the volume (up to 5 ml) of triacetin added to the primary w/o emulsion to extract the water from the emulsion.

The analysis of insulin content and insulin stability within microspheres after freeze-drying by both RP- and SE-HPLC are reported in Table I. It should be noted that in this paper, the term stability referred to insulin is defined as the ability of this peptide not to undergo transformation into side-products, namely A21 desamido-insulin assessed by RP-HPLC and water soluble aggregates assessed by SE-HPLC. Recoveries of 70% and 75% were obtained by RP-HPLC from microspheres of pure albumin and albumin-lactose (4:1), respectively. In the presence of emulsifiers, the recovery was of about 60% regardless of the excipient. The chromatograms of insulin from the microspheres also had identical retention times to the insulin standard and no transformation products were detected; this provided supportive evidence that insulin did not degrade to products of a different chemical nature. These results are in agreement with literature data that describe the multiple emulsion method as the most effective to limit the formation of the desamido-insulin inside the device (18).

There were little differences between the data obtained with the two chromatographic techniques. This implies that the process used to prepare the microspheres did not significantly affect the stability of insulin.

CONCLUSIONS

The study has shown that it is possible to produce albumin-based microparticles with the emulsification-diffusion process starting from w/o emulsions using triacetin as external phase. Due to the partial miscibility of water in this solvent, salting-out agents are no longer required. The particles prepared are spherical with relatively smooth surfaces and size ranging from 2 to 8 μm.

Insulin was successfully encapsulated into the microspheres, its integrity being maintained.

The results suggest the potential application of this method to prepare adequate systems for protein and peptide delivery.

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