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Influence of post-emulsification drying processes on the microencapsulation of Human Serum Albumin

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Abstract

In the present work, methods used to microencapsulate Human Serum Albumin (HSA) in a biodegradable polymer were compared for their effects on the physicochemical characteristics of HSA-loaded microparticles and on the release and integrity of encapsulated HSA. The polymer used was poly(D,L-lactide-co-glycolide) (75:25) (PLGA) (Boehringer Ingelheim, Resomer RG 752, MW 20,900). Microparticles were formulated by (i) w/o/w emulsification and freeze-drying (EFD) or (ii) w/o/w emulsification and spray-drying (ESD). Particle morphology and size were evaluated by scanning electron microscopy and by laser diffraction analysis. Loading, encapsulation efficiency and protein release were determined using a commercial protein assay kit. Protein integrity was evaluated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Particles produced by emulsification/spray-drying exhibited greater diversity in shape than those produced by emulsification/freeze-drying. Additionally, protein loading values were significantly higher for particles produced by emulsification/spray-drying rather than particles produced by emulsification/freeze-drying. The structural integrity of encapsulated protein was confirmed for particles produced by both processes. The fraction of HSA released was similar for both formulations. The emulsification/spray-drying technique described appears to be a rapid and efficient method for the preparation of PLGA microparticles loaded with a model protein.
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Keywords: PLGA; Human Serum Albumin; Water-in-oil-in-water emulsion; Freeze-drying; Spray-drying

1. Introduction

The number of peptide and protein-based drugs approved by regulatory authorities for human use has increased significantly in recent years. In general these drugs are more potent and have poorer solubility then traditional agents and are generally not effective after oral administration because of their low bioavailability. The application of biodegradable and non-biodegradable polymers for the design of controlled-release delivery systems for peptides and proteins is well documented in the literature (Alonso et al., 1993; Johnson et al., 1996; Yamaguchi et al., 2002).

The commonly used polymers in the area of sustained delivery systems include poly-lactide/glycolide at ratios of 50:50 (Capan et al., 1999; Chen et al., 2001; Jiang et al., 2002), 65:35 (Yang et al., 2000), 75:25 (Mehta et al., 1996) and 85:15 (McGee et al., 1997). The choice of polymer is dependent on several factors including desired degradation rate, polymer molecular weight, desired microsphere particle size and crystallinity.

The most commonly employed method to achieve protein encapsulation into the matrix of bioerodible polymers is the so-called water-in-oil-in-water (w/o/w) technique (Ogawa et al., 1988; Boury et al., 1997; Bouissou et al., 2004); other techniques such as spray-drying (Blanco-Príeto et al., 1999) and phase inversion nanoencapsulation (Sandor et al., 2001) have also been examined.

The aim of this work was to investigate the influence of post-emulsification drying processes on the physicochemical characteristics of microparticles fabricated from PLGA 75:25 encapsulating a model protein, Human Serum Albumin (HSA). In the current study the protein was formulated in a water-in-oil-in-water emulsion formulation followed by freeze- or spray-drying. The effect of the microencapsulation technique on the morphology of HSA-loaded microparticles and on the release and structural integrity of the encapsulated protein

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was subsequently evaluated. The PLGA polymer selected was PLGA 75:25 as PLGA polymers of this grade have previously been shown to sustain protein release over extended periods (Chen et al., 1997; Cleland et al., 1997).

2. Materials and methods

The PLGA copolymer used in this study comprised of lactic and glycolic acids in the ratio 75:25 lactide/glycolide (Resomer RG 752, MW 20,900, Boehringer-Ingelheim, Germany). Human Serum Albumin (HSA) protein and all other chemicals were obtained from Sigma (Poole, UK) unless otherwise indicated.

2.1. Formulation of protein microparticles

HSA loaded microparticles were manufactured using (i) emulsification/freeze-drying (EFD) and (ii) emulsification/spray-drying (ESD):

(i) *EFD particles*: a modified w/o/w double emulsion technique as described elsewhere (Jeffery et al., 1993; Uchida et al., 1995; McGee et al., 1997) was used. Briefly, 2 ml of HSA (1 mg/ml) was dissolved in deionised water and emulsified with a 5% (w/v) solution of PLGA in dichloromethane. The resulting mixture was slowly added to 1 l of 1% PVA and further emulsified using a Silverson L4R Mixer (Silverson, Bucks, UK). After solvent evaporation by stirring at a controlled temperature for 12 h, microparticles were recovered by centrifugation and were subsequently washed three times in deionised water. Microparticles were then lyophilised by immersion in a Hetofrig cooling bath (Heto) for 15 min, transferred to a Hetosicc CD52 freeze-drier (Heto) and left overnight (O'Connor and Corrigan, 2001).

(ii) *ESD particles*: w/o/w emulsions were prepared in the same manner as for the EFD particles. Dispersions were spray dried in a Buchi mini Spray Dryer Model 191 (Buchi Laboritorium-Technik, AG, Flawil, Switzerland) using an inlet temperature of 78/79 °C, an outlet temperature of 50 °C, an aspirator rate of 75%, a pump rate of 10% and an airflow rate of 600 NL h^{-1} (Corrigan et al., 2003).

2.2. Microparticle characterisation

For morphological examination, the microparticles were viewed using a Hitachi S-7000 scanning electron microscope (Hitachi, Japan). The particle size of the microparticles was determined by laser diffraction using a Malvern 2600 particle size analyser (Malvern, UK).

2.3. Differential scanning calorimetry

Microparticles containing protein and empty microparticles were characterised by DSC (Differential Scanning Calorimeter 821^{e}, Mettler Toledo) in the range of 0–250 °C at a heating rate of 10 °C per minute with average sample weights of 6 mg.

2.4. HSA loading

Determination of loading and encapsulation efficiency was carried out after HSA extraction from microparticles. HSA was extracted from microparticles using a modified alkaline hydrolysis extraction method reported previously (Hora et al., 1990; Jeffery et al., 1993; Sharif and O'Hagan, 1995). In brief, 10–15 mg of microparticles accurately weighed, were shaken overnight with 3 ml of 5% (w/v) sodium dodecyl sulphate (SDS) in 0.1 M sodium hydroxide solution (NaOH/SDS). Following centrifugation at 4000 × *g* for 10 min, 1 ml was removed and assayed to determine HSA concentration which was measured using a Micro-BCA assay (Pierce, Rockford, IL). All determinations were conducted in triplicate.

2.5. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of extracted protein

Aqueous samples of protein extracted from microparticles were analysed using (SDS–PAGE) as described by Laemmli (1970), using an electrophoresis unit (Atto, Tokyo) with 5% stacking gels and 10% resolving gels for the detection of the HSA protein. Polyacrylamide gels were run for approximately 2 h at 80 V and protein bands were detected using a silver staining technique (Pandey et al., 2000).

2.6. In vitro release of HSA

In vitro protein release studies were conducted as previously described (Panyam et al., 2003). Release studies were carried out in a phosphate buffer solution (Sørensons PBS, pH 7.4, Pharmaceutical Handbook). A quantity of protein-loaded microspheres (50 mg) was suspended in 50 ml of PBS buffer in 100 ml conical flasks (Sah et al., 1994; Coombes et al., 1998). The samples were incubated at 37 °C in a shaking water bath. The triplicate release tests were sampled at predetermined intervals. An amount of 2 ml samples were removed and centrifuged at each sampling time. An amount of 1 ml of the buffer was removed and assayed for protein release using a protein assay kit (Pierce) and samples analysed with a Genesys 5 spectrophotometer. After each sample collection, the sample PBS was replaced with fresh buffer. Analyses were conducted in triplicate.

2.7. Statistical analysis

Differences in particle size, loading and encapsulation efficiency were analysed by the Mann–Whitney *U*-test. The effects of incubation time on % recovery were analysed using the Kruskal–Wallis test followed by Dunn's test to determine individual differences. Differences in cumulative amounts of protein released were evaluated by the Mann–Whitney *U*-test. Differences were considered significant at $p < 0.05$.

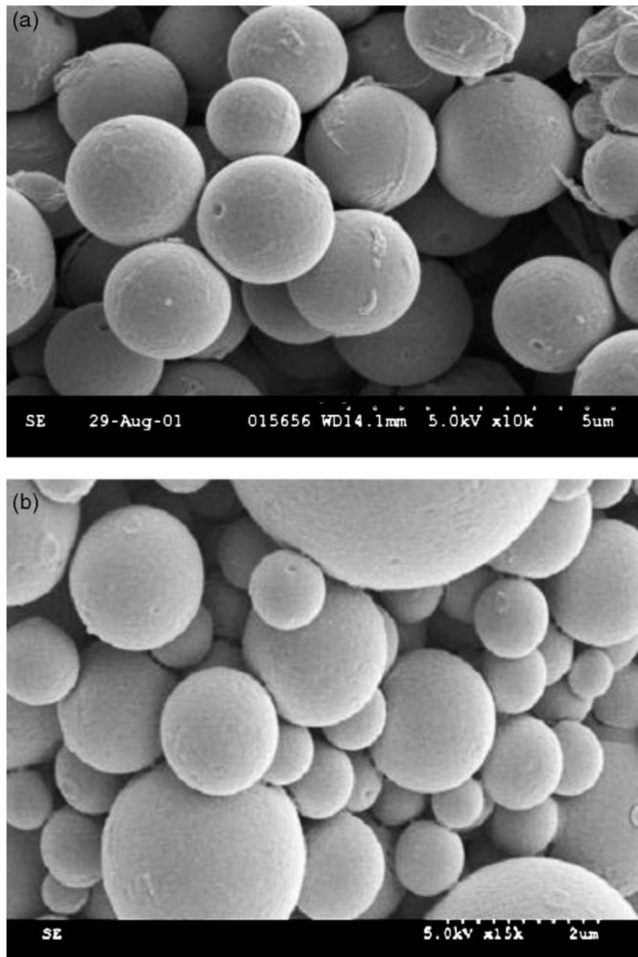

Fig. 1. (a) Scanning electron micrographs of empty microparticles produced by EFD. (b) Scanning electron micrographs of protein-loaded microparticles produced by EFD.

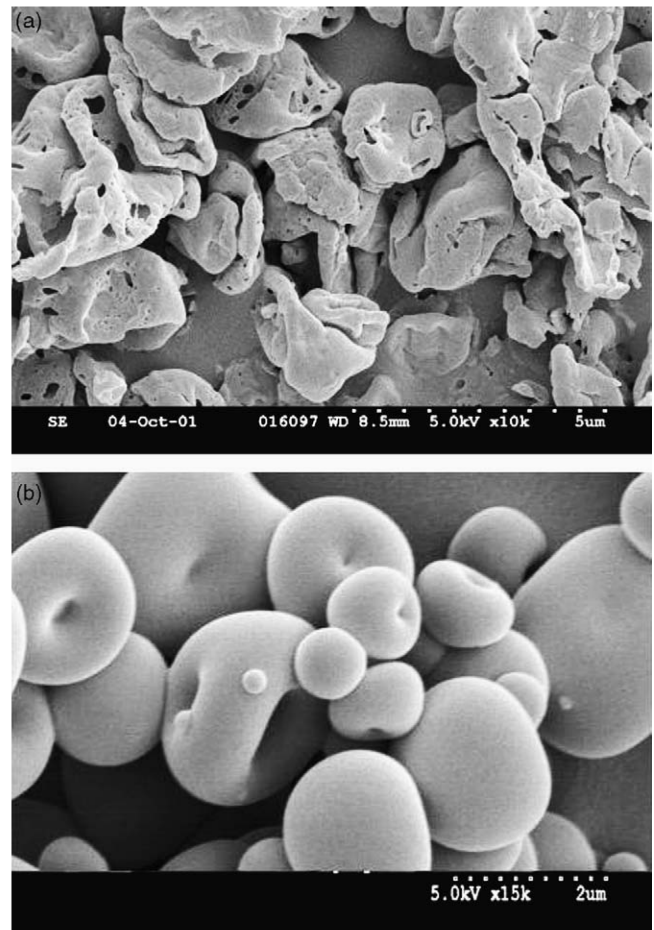

Fig. 2. (a) Scanning electron micrograph of typical empty microparticles produced by ESD. (b) Scanning electron micrograph of typical protein-loaded microparticles produced by ESD.

3. Results and discussion

3.1. Microparticle morphology and particle size

The morphology of empty microparticles formed via EFD is illustrated in Fig. 1a and b. Empty microparticles manufactured by EFD were spherical and similar in shape and morphology to protein-loaded microparticles. Micrographs revealed that protein-loaded microparticles produced by EFD were manufactured with relatively smooth surfaces (Fig. 1b). Unloaded particles produced by ESD are illustrated in Fig. 2a and appear fragmented with no characteristic morphology. Micrographs of protein-loaded spray dried microparticles (Fig. 2b) show toroidal shaped microparticles with a greater diversity in shape.

Particle size will influence polymer degradation (Dunne et al., 2000) as well as the potential for uptake of the microparticles by phagocytosis (Ahsan et al., 2002). The average median particle size values [$D(v, 0.5)$] for EFD particles were determined as 6.65 ± 0.21 μm in agreement with the SEM images. The [$D(v, 0.5)$] values for the ESD particles were determined as 3.46 ± 0.51 μm and were significantly different from the values for the EFD particles ($\alpha = 0.05$, Table 1). In all of the microparticle batches at least 90% of the microparticles were less than 10 μm in diameter, in line with the findings of Baras et al. (2000) where similar particle dimensions for protein-loaded PLGA microparticles produced by spray-drying of a w/o emulsion were reported.

3.2. Differential scanning calorimetry

Fig. 3a illustrates the thermal characteristics of the polymer RG 752, empty microparticles manufactured via EFD, protein-loaded microparticles manufactured via EFD and a physical mix of Human Serum Albumin and polymer blend in the same ratio as the loading of protein in particles.

Table 1
Particle size data, loading and encapsulation efficiency for microparticles produced by (i) emulsification/freeze-drying (EFD) and (ii) emulsification/spray-drying (ESD)

Process	Particle size, $D(v, 0.5)$ (μm)	Loading (μg HSA/mg microparticles)	Encapsulation efficiency (%)
EFD	6.65 ± 0.20	11.64 ± 1.58	58.20 ± 7.92
ESD	3.46 ± 0.31	16.89 ± 0.36	84.45 ± 1.85

Data are means ± S.E.M. for three batches.

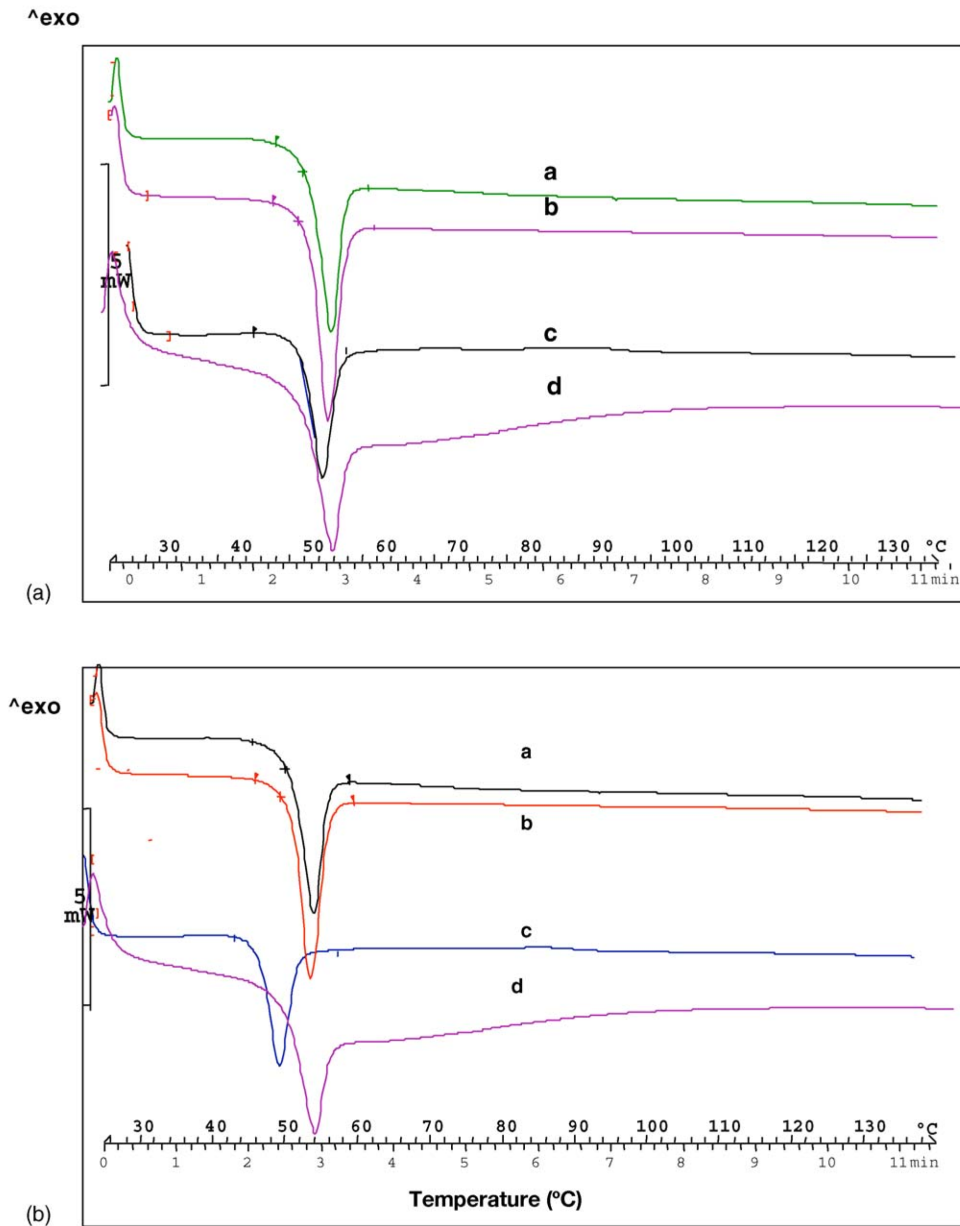

Fig. 3. (a) DSC scans of: (a) RG 752, (b) empty microspheres, (c) protein-loaded microspheres manufactured by EFD, (d) physical mix of protein and polymer. (b) DSC scans of: (a) PLGA 752, (b) empty microspheres, (c) protein-loaded microspheres manufactured by ESD, (d) physical mix of protein and polymer.

Representative DSC traces showed a glass transition temperature for RG 752 at 52.00 °C. For empty microparticles and protein-loaded microparticles manufactured via EFD endotherms at 51.19 and 49.43 °C were observed. The blend of protein and polymer exhibited an endotherm at 52.13 °C. Encapsulation of protein in polymer resulted in a slight reduction of the T_g of the polymer which might be attributed to the dispersion of the protein in the polymer matrix. This protein Human Serum Albumin exhibits a broad endotherm with a glass transition of 78.6 °C (not shown). There was no evidence of a similar broad endotherm in any of the protein polymer blend possibly because of the relatively low amounts of protein relative to polymer (<2%, w/w).

The thermal characteristics of the polymer RG 752, empty microparticles manufactured via ESD, protein-loaded microparticles manufactured via ESD and a physical blend of polymer and protein were further examined by DSC analysis (Fig. 3b). The glass transition temperatures for PLGA 75:25, empty microparticles, and the protein polymer blend were 51.94, 52.31 and 51.84 °C, respectively. Protein-loaded microparticles manufactured via ESD showed an endotherm of 48.25 °C, the lower glass transition temperature again possibly reflecting the presence of the encapsulated protein in the polymer.

3.3. Loading and encapsulation efficiency

Analysis of loading and encapsulation efficiency (Table 1) indicated that the encapsulation efficiency for particles produced by ESD was significantly higher ($84.45 \pm 1.85\%$, $\alpha = 0.05$) as compared with particles produced by EFD ($58.20 \pm 7.92\%$). The

lower values for EFD microparticles may reflect a greater loss of protein to the external aqueous phase during the longer drying period relative to the more rapid spray-drying process.

For microparticles manufactured by formation of a double emulsion with solvent evaporation it has been reported that the progression of phase transition (solidification) will play a major role in the resulting microparticle properties (Li et al., 1995; Mehta et al., 1996). In these studies, a rapid solidification of polymer was observed to give rise to a denser skin layer, with consequent increases in loading efficiency. In the present study, the rate of solvent removal associated with the different drying processes (12 h for microparticles manufactured via EFD versus the much more rapid process for ESD) might be expected to influence protein encapsulation efficiencies for the different microparticle types. Microparticles manufactured via EFD are formed at lower temperatures (20 °C) relative to the temperatures employed by the ESD process (78/79 °C inlet temperature, 50 °C outlet temperature).

At temperatures close to or exceeding the normal boiling point of dichloromethane (39.8 °C) the evaporation of the solvent should be very rapid. For microparticles formed by emulsification followed by solvent evaporation and extraction this results in rapid microparticle formation and low shrinkage rates (Yang et al., 2000). Microparticles made at the higher temperature of the ESD process should solidify more rapidly than the microparticles formed by EFD, rapidly forming a dense coat, which should promote higher protein encapsulation efficiency relative to the lower temperatures of the EFD process.

3.4. Comparison of processing methods on protein integrity

The structural integrity of extracted protein was investigated via SDS–PAGE analysis. Fig. 4 illustrates a polyacrylamide gel of protein extracted from spray-dried microparticles manufactured by EFD (lane 3) and from microparticles manufactured by ESD (lane 4). Lane 1 contains a range of molecular weight markers (6.5–175 kDa). HSA is a 66 kDa protein and lanes 3 and 4 contain bands which, when compared with lane 1, correspond to the 66 kDa molecular weight marker for HSA. The structural integrity of the HSA within microparticles was retained as shown by SDS–PAGE of the protein before and after microencapsulation. Results for EFD and ESD microparticles indicated that the encapsulation processes used did not appreciably affect the integrity of the encapsulated protein when compared with the HSA standard as smearing was not evident in the protein extracted from microparticles. The lower encapsulation for the EFD process relative to the ESD process resulted in a less intense band in lane 3 than in lane 4.

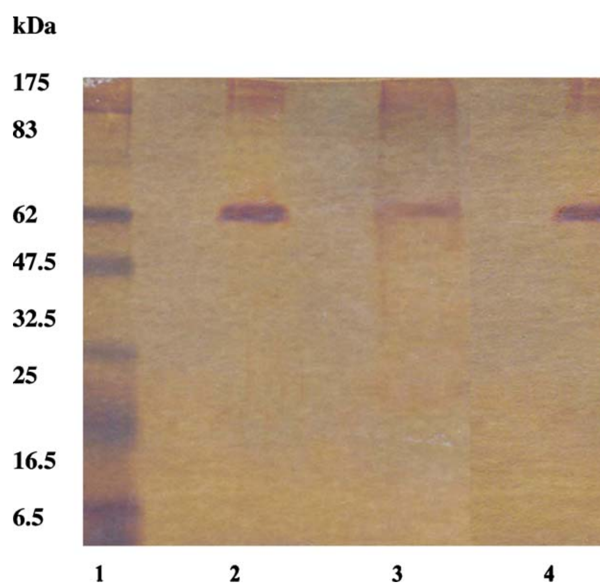

Fig. 4. SDS–PAGE of extracted protein. Lane 1: molecular weight markers (MW range: 6.5–175 kDa); lane 2: HSA standard (MW 66 kDa); lane 3: HSA extracted from EFD microparticles; lane 4: HSA extracted from ESD microparticles.

3.5. Protein release

Cumulative release profiles of HSA under in vitro sink conditions from different particles are shown in Fig. 5. The amount of protein released from the ESD particles was significantly greater than for the EFD particles ($p < 0.05$; $\alpha = 0.05$) with 118.2 μg of protein per mg of particles being released over 100 days for the ESD particles and 66.1 μg protein per mg of particles being released for EFD particles.

If the protein was uniformly distributed in an inert matrix and diffusion was the sole mechanism of release, the amount of protein released would be expected to be linear with the square root of time. Fig. 5 clearly demonstrates that the release profiles do not conform to this type of release. While a polymer layer or skin may represent a possible barrier or "skin" through which diffusion will take place, diffusion cannot be the sole mechanism governing protein release. For this to take place there would be a lag time followed by pseudo steady state (zero order) release culminating in a slower release as the driving concentration depletes (Guy et al., 1982).

For the ESD particles the rate of release appears to be close to zero order over the time course studied (Fig. 5). For this to occur under the experimental conditions it appears that the protein is being released as a result of slow diffusion and dissolution. Separation of the dissolution and diffusion process would be

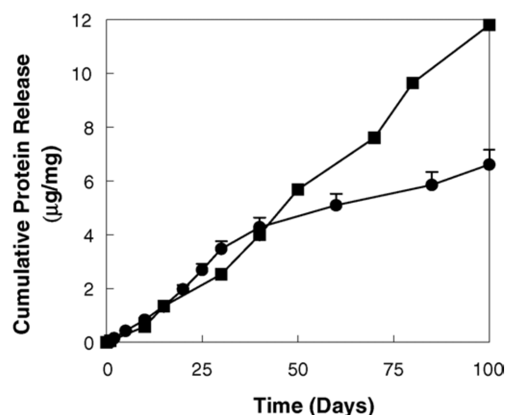

Fig. 5. Cumulative release of protein (μg HSA/mg microparticles) EFD Δ, ESD X; data represent the mean values for three batches.

complex given the variables that are involved. On the other hand the EFD particles appear to release the protein at a slightly faster rate during the first half of the experiment, thereafter the release profiles fall. This may be a result of slower dissolution and the drug at the core of the particles having to diffuse further to the outside of the particle.

4. Conclusions

The manufacture of microparticles by spray-drying of oil-in-water (o/w) formulations for a wide range of drugs and bioactive molecules is well documented in the literature. However, spray-drying of water-in-oil-water (w/o/w) emulsions for microencapsulation of proteins has not been reported. This study was conducted to compare physicochemical characteristics of microparticles manufactured by freeze- or spray-drying of a double emulsion formulation of HSA.

Particles formed following solvent evaporation and freeze-drying (EFD) were spherical in shape in comparison to the folding and indentation observed for microparticles produced from spray-drying of w/o/w emulsions. The EFD particles exhibited greater diversity in size than ESD particles, which also exhibited a narrower particle size distribution. For both processes the dimensions of 90% of the particles were less than 10 μm.

Spray-drying w/o/w emulsion formulations of HSA resulted in particles with significantly higher encapsulation efficiencies than particles produced by freeze-drying double emulsion formulations. Moreover, the structural integrity of the protein does not appear to be compromised by the spray-drying process as determined by gel electrophoresis analysis.

Relative to the release profile for the EFD particles, the HSA release kinetics from ESD particles are quasi-linear over 100 days. The generation of a dosage form with uniform, controlled and sustained drug release characteristics constitutes a desirable element in the design of protein delivery systems.

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