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Polymeric microparticles containing protein prepared using a controllable combination of diffusion and emulsification steps as part of the salting out procedure

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The key to success of proteins as biopharmaceuticals is to have in place an efficient drug delivery system that allows in gaining access to their target sites. A novel procedure of preparing ovoalbumin-loaded Eudragit® S100 microparticles, based on combining salting out and double emulsion-evaporation steps was developed. The ratio of a water miscible solvent (acetone and isopropanol) to a non-water-miscible solvent (chloroform) and salt addition to aqueous phase external were shown to be the primary determinants of size, polydispersity index (PI) and encapsulation efficiency (EE). Once optimized, using an organic phase of 3:0.5:1.5 (acetone:isopropanol:chloroform, v/v/v), further control was exerted using modification of acetone diffusion by alterations in $MgCl_2$ concentration. Diffusion control, using 75% w/w $MgCl_2$ solution, produced microparticles with a mean size of 26.3 μm, a good PI of 0.36 and 56.5 ± 0.5% EE. Electron microscopy showed the particles to be smooth and spherical. Ovoalbumin release studies using different buffers demonstrated immediate release in the buffer at alkaline pH. Calorimetry studies suggested that ovoalbumin existed in the microparticle as a molecular dispersion. Thus, Eudragit® S100 microparticles have great potential as oral carriers for delivery of proteins to the intestines.

Key words: Double emulsion/solvent evaporation, emulsification-diffusion, microparticles, protein encapsulation.

INTRODUCTION

Proteins and peptides exhibit the widest structural and functional variation and are integral to the regulation and maintenance of all biological processes. The greater biochemical and structural complexity of proteins compared compared with conventional drug-based pharmaceuticals makes formulation design for delivery of therapeutic proteins a very challenging and difficult task.

During the last two decades, parenteral drug delivery

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systems based on polymeric microparticles (MP) have extensively been investigated (Liechty et al., 2010). Although the microencapsulation of peptides has led to several commercially available products (Hougaard et al., 2013), the encapsulation of proteins still presents considerably difficulties (Aamir and Ahmad, 2010; Wieber et al., 2010).

Polymeric drug-loaded MP are colloidal systems, typically 1 to 1000 µm in diameter, with a therapeutic payload entrapped, adsorbed or chemically coupled to an orbicular polymer matrix (Licciardi et al., 2012). Since the microencapsulation procedure offers a large number of possibilities for modifications, the success of this technology strongly depends on the adaptation of process variables, including polymer composition and concentration, rates of solvent diffusion, drug loading, phase ratio of emulsion system, emulsion stability, homogenization techniques and temperature (Chaisri et al., 2009; Sartori et al., 2013; Ogawa et al., 1988).

The multiple emulsion evaporation method differs fundamentally from diffusion in that the main phases stay immiscible at all times, being removed later by evaporation. This, biphasic emulsion systems (W_1/O/W_2) have been described and used to incorporate water-soluble drugs, such as proteins (Joshi et al., 2013; Xia et al., 2013) and propranolol HCl (Ubrich et al., 2004). However, several difficulties are still encountered when using this technique, such as poor encapsulation efficiency of the drug and high polydispersity index (PI) (Win and Fend, 2005). PI is a unit-less expression for the tightness of the particle size distribution. Theoretically, PI is zero for a monodisperse colloidal suspension, however standard latex particles with a polydispersity index of about 0.05 are practically monodisperse. The values greater than 0.5 indicate a very broad size distribution (Jain et al., 2004). In many respects, the salting out-diffusion procedure, as first introduced by Allemann et al. (1992) and modified by Konan et al. (2002), draws on aspects of the method described earlier. The addition of sufficient electrolyte is used for separating an aqueous phase from an organic phase (usually acetone), the two being fully miscible before any intervention or for at least to minimize such drug partitioning between the two miscible phases. Controlled dilution of the electrolyte content thereafter gives rise to particle formation, as acetone diffuses from the organic droplets.

The aims of the present study were to evaluate the influence of certain physicochemical properties of the aqueous and organic phases used during protein loaded-MP preparation, and their effect on the characteristics of polymeric microparticles produced by the combination of double emulsion solvent diffusion-evaporation and salting out methods, to obtain particles with a size > 20 µm to allow the encapsulation of biological interest proteins. In line with our laboratory internal focus, the release profiles were performed at different conditions, slightly acidic, neutral and alkaline pH as these are characteristic media for the release of insecticidal proteins.

MATERIALS AND METHODS

Chemicals

Microparticles were prepared from Eudragit[®] S100 (Helm) and stabilized using poly (vinyl alcohol) (PVA, Mowiol 4-88, Clariant®). Ovoalbumin (OA, lyophilized powder, ≥ 98% agarose gel electrophoresis, Sigma-Aldrich, St. Louis, MO). Magnesium chloride (Merck, Naucalpan de Juarez, Estado de México) was used as the salting out agent. All others reagents, such as acetone, isopropanol and chloroform were of appropriate laboratory grade (analytical grade, M TEDIA high purity solvents) and used without further purification.

Microparticles preparation and purification

Ovoalbumin (OA) was identified through the determination of ultra violet (UV) absorption maximum (Genesys 10UV scanning, *Thermo scientific,* Madison Wisconsin). Bradford based method was used for determination of OA, using protein estimation. The ovoalbumin-Eudragit[®] S100 microparticles (OA-MP) were prepared using controllable combination of diffusion and emulsification steps as part of the salting out procedure. Briefly, the inner aqueous phase (IAP) with 0.3 ml aqueous solution of OA (0.33%, w/v) was added to a 100 mg of Eudragit[®] S100 previously dissolved in 5 ml of an organic phase (OP), comprising different volume ratios of acetone (AC), chloroform (CH), and isopropanol (ISP) (Deepti et al., 2004: Liu et al., 2010). The IAP was then emulsified with the OP by mechanical stirring (EUROSTAR power-b, IKA® WERKE), for an optimized time period. The primary emulsion (W/O) was then immediately emulsified with 13 ml external aqueous phase (EAP) of PVA (optimized percentage (w/w) in MgCl2 aqueous solution) by mechanical stirring (EUROSTAR power-b, IKA® WERKE) to an optimized speed, on an ice bath. Later, the organic solvent was evaporated under reduced pressure, in order to obtain an aqueous suspension of OA-MP. OA-MP were then separated from the bulk aqueous phase by centrifugation at 1,630 g (rotor F1202, Beckman Coulter Allegra 64R Centrifuge, Fullerton, California) for 10 min at 4°C. After subsequent washing and centrifuged with cold distilled water, the pellet was lyophilized (LYPH-LOCK 6, Labconco, Kansas city, Missouri). Optimization encapsulation efficiency was determined along with the measurement of particle size, PI and particle shape of different formulations.

First, EAP volume and MgCl₂ 6H₂O concentration was modified in order to determine the effect on size. Further, variations in the organic phase composition where the ratio volume of AC:ISP:CH was 1:0.5:3.5; 2:0.5:2.5 and 3:0.5:1.5, in one-part incremental steps, were used. Further, stirring speed was needed to be optimized in order to obtain a sufficiently high yield of microparticles, with a desired size distribution and acceptable encapsulation efficiency. The stirring speeds used were 100, 300 and 500 rpm. The microparticles were produced at different PVA concentrations of 1, 2 and 3% (w/w) in order to determine the effect of PVA concentration on size and encapsulation efficiency.

Particle size measurement

The size and shape of OA-MP were estimated using optical microscopy (Micromaster[®] Fisher Scientific*,* with the software:

Table 1. Mathematical equations for the models used to describe release characteristics of OA from MP

Model	Equation
Zero order kinetics	$Q_t = Q_0 + K_0t$
First order kinetics	$Q_t = \log Q_0 + K_0t$
Higuchi	$Q_t = Q_0 + K_Ht^{1/2}$
Korsmeyer-Peppas	$Q_t = K_{MB}t^n e^{(-et)}$

Table 2. Initial conditions of polymeric microparticles formulation

Parameter	Conditions
Volume ratio of ACT:ISP:CH(mL)	2.0: 0.5: 3.5
Concentration of PVA in EAP (% w/w)	3.0
Concentration of $MgCl_2$ in EAP(% w/w)	15-75
Volume of EAP(mL)	10

Westover Digital MCD Model 2300). Formulation and dried microparticles re-dispersed in water were spread into a glass slide. Measurements of 100 consecutive particles were observed to microscope, were carried out at 25°C, each sizing determination done in triplicate and an average particle size (intensity average) expressed as the mean diameter ± standard deviation (SD). The PI was calculated by dividing standard deviation with average particle size (Jain et al., 2004). For external morphology, the OA-MP was coated with gold, using sputter coater, and the sample was randomly analyzed using a scanning electron microscope (SEM) (LEO-435VP, Cambridge, UK). The effect on particle size before and after drying was evaluated only by the optimized formulation.

Estimation of encapsulation efficiency

OA- MP were dissolved in AC and centrifuged to remove the supernatant because the acetone led to the OA precipitation. Then, the pellet was suspended in carbonate buffer 50 mM at pH 10.3 and analyzed using UV absorption spectroscopy at 583 nm, using appropriate calibration and blanking procedures with the Bradford method. Measurements were done in triplicate; the encapsulation efficiency was calculated using the ratio of the mass of protein determined analytically to the theoretical protein loading. Encapsulation efficiency was calculated as:

$$\text{Encapsulation efficiency } (\%) = \frac{\text{Actual protein loaded (mg)}}{\text{Theoretical protein loading (mg)}}$$

"In-vitro" release

The *in vitro* release profile of OA-MP was determined. Aliquots of 0.5 ml of the formulations were suspended separately in volumetric flasks containing dissolution media (pH 5.0, 7.4, or 10.3; acetate, phosphate or carbonate buffer 50 mM, respectively). Volumes were made up to 2 ml with each buffer and the flasks were incubated at 25°C under 100 cycles min^{-1} shaking. At various time intervals, flasks were removed, one at a time, and the contents were centrifuged at 33,250 g (rotor F1202, Allegra® 64R) for 25 min at

4°C. The supernatants were then used to determine the released OA by the Bradford method (n = 3; mean ± standard deviation). To analyze the *in vitro* release data, various kinetic models were used to describe the release kinetics. The zero order rate describes the systems where the drug release rate is independent of its concentration (Hadjiioanou et al., 1993). The first order describes the release rate is concentration dependent (Bourne, 2002). Higuchi (1963) described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion.

Table 1 shows the equations, where Qt is the amount of drug released in time, Q0 is the initial amount of the drug in table. The following equations were made: cumulative (%) protein release versus time (Zero order kinetic model); log cumulative of protein (%) release versus square root of time (Higuchi model) and log cumulative protein (%) release versus log time (Korsmeyer model) (Korsmeyer et al, 2010). Korsmeyer peppas et al. derived a simple relationship which described drug release from polymeric system (Peppas, 1983). To find out the mechanism of drug release, first 60% drug release data was fitted in Korsmeyer model (Table 1).

Then, the data of the dissolution profiles were summarized numerically and 95% confidence intervals for the differences between pHs in the mean dissolution profiles at each dissolution time point were evaluated using statistical package for social sciences (SPSS) Statistics 17.0 program.

Differential scanning calorimetry

The thermal characteristics of OA powder, Eudragit® S100, physical mixture of OA and Eudragit® S100 and OA-MP were determined using a differential scanning calorimeter (DSC-40, NETZSCH). Samples were crimped in a standard aluminum pan and heated from 40 to 330°C, at a heating rate of 10°C per minute, under constant purging of nitrogen at 30 ml min^{-1}.

RESULTS

Particle size measurement and determination of encapsulation efficiency

The effect of formulation variables on size, IP and encapsulation efficiency of OA-MP prepared by controllable combination of diffusion and emulsification steps as part of the salting out procedure was determined. Table 2 shows the initial conditions of OA-MP. EAP used was normal MgCl2 aqueous solution, to prevent the diffusion of any solvent to the aqueous phase. Different amounts of $MgCl_2$ $6H_2O$ (15, 35 and 75, w/w) increments were studied (Figure 1), while keeping the organic phase composition constant (AC: ISP: CH, 2.0:0.5:2.5, v/v/v). Therefore, the formulation with 75% w/w $MgCl_2$ $6H_2O$ was chosen to evaluate tensio-active concentration effect because it showed a PI of 0.38, indicating a less dispersion in particle size. Figure 2 shows the effect of PVA concentration on the particle size. It was observed that when PVA concentration was reduced, the particle size also decreased. Once determined that the $MgCl_2$ $6H_2O$ at 75% (w/w) and 3% (w/w) PVA are required to allow the formation of the MP, the influence of the composition of OP was assessed in the incorporation of the model protein through increases in the proportion of

Table 3. Effect of organic phase composition on mean particle size, polydispersity index and OA encapsulation. OA-MP were prepared using 3 % w/w PVA and 75% w/w MgCl$_2$ 6H$_2$O, using 300 rpm of mechanical stirring at and 10 mL of EAP.

Volume ratio of (AC:ISP:CH mL)	Particle size (µm ± SD)	Polydispersity Index	Aggregation	% EE
1.0:0.5:3.5	18.24 ± 11.3	0.62	+	14.1 ± 0.6
2.0:0.5:2.5	16.2 ± 6.1	0.38	--	32 ± 2.7
3.0:0.5:1.5	9.7 ± 3.4	0.35	--	19.3 ± 1.6

+ presence, -- without

Table 4. Effect of volume EAP on mean particles size, polydispersity index and OA encapsulation. OA-MP were prepared using 3% (w/w) PVA and 75% (w/w) MgCl$_2$ 6H$_2$O, using mechanical stirring at 300 rpm and the organic volume ratio of 3:0.5:1.5. (AC:ISP:CH).

Volume EAP (mL)	Particle size (µm ± ds)	Polydispersity Index	Aggregation	% E E
7	3.0 ± 1.2	0.38	---	15.45 ± 1.2
10	9.7 ± 3.4	0.35	---	19.3 ± 1.6
13	10.7 ± 3.8	0.35	---	57.5 ± 8.9

+ presence, -- without.

Table 5. Effect of mechanical stirring speed on mean particles size, polydispersity index and OA entrapment. MP were prepared using 13 mL of 1% (w/w) of PVA to 75 % (w/w) MgCl$_2$ 6H$_2$O in EAP and an organic volume ratio of AC:ISP:CH (3.0:0.5:1.5).

Stirring (rpm)	Particle size (µm)			Polydispersity Index	% EE
	Mean	Moda	Median		
100	8.60 ± 5.2	7.8	7.9	0.61*	9.3 ± 1.0
300	26.3 ± 9.7	22.8	25.5	0.36	56.5 ± 0.5
500	7.9 ± 3.9	4.6	7.1	0.49*	4.9 ± 0.3

* Polymer aggregation.

acetone. The results in Figure 3 show that MP properties, such as PI, can be improved by alterations in this diffusion rate once the solvent ratio in the organic phase has been optimized, as done in Table 3.

Variations in the ratio of solvents in OP were shown to have effect on the observed OA encapsulation efficiency, which was greater than 13 and less than 35%. The results from this part of the work indicated that a ratio of 3:0.5:1.5 (AC:ISP:CH) emulsified in 10 ml 3% (w/w) PVA and 75% (w/w) MgCl$_2$ 6H$_2$O of EAP with 300 rpm of mechanical stirring produced MP with a mean particle size of 9.7 ± 3.4 µm, PI of 0.35 and the encapsulation efficiency was 19.3 ± 1.6%. Although this formulation presents low encapsulation efficiency percentage, no presence of aggregates was shown (Table 3 and Figure 3). The volume of EAP was increased from 7 to 13 ml, the encapsulation efficiency and particle size also increased (Table 4). With the decreasing on PVA concentration from 3 to 1% (w/w), the surface tension increased and it was found that the particle size also increased (from 10 to 26 microns), whereas the encapsulation efficiency remained around 56% (Figure 4 and Table 5) with a speed of 300 rpm.

Clearly, some measure of control is needed to improve the particle size distribution, probably due to affording more speed for the mechanical stirring process to produce a narrower size distribution of emulsified droplets, as observed in Table 5.

Morphology of polymeric microparticles

The external morphology and shape of the optimized formulation were studied by scanning electron microscopy (SEM) (Figure 5), which revealed fairly spherical OA-MP with a smooth surface. A student t-test was made to analyze the influence of drying on particle size of an optimized formulation. The drying process has no effect on the morphology and size of OA-MP (n = 3; p < 0.05). It is clear that the shape of OA-MP is obtained and can be assumed to be spherical with smooth surfaces (Figure 5).

In vitro release profile

The *in vitro* release profile of OA-MP was performed to

Table 6. Mathematical Model used to describe the drug release

pH buffer	Zero order kinetics	First order kinetics	Higuchi model	Korsmeyer-Peppas model		Type of transport
	Regression co-efficient (R^2)				n	
5.0	0.807	0.715	0.886	0.897	0.703	Anomalous (non-Fickian)
7.4	0.720	0.639	0.742	0.987	0.682	Anomalous (non-Fickian)
10.3	0.835	0.833	0.841	0.814	0.038	----
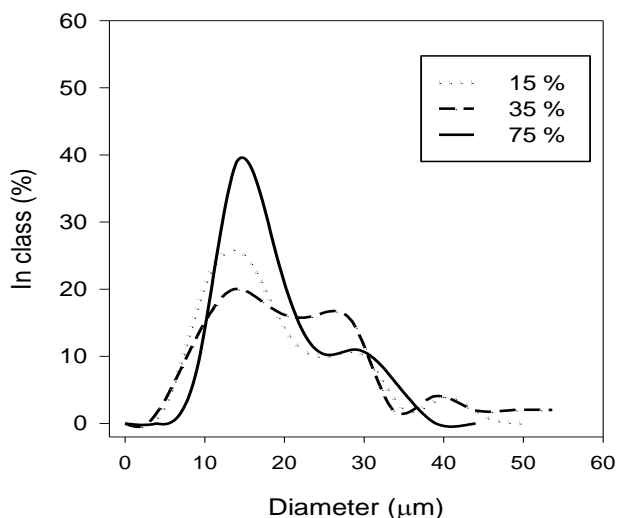
Figure 1. Effect on microparticle size distribution profiles by $MgCl_2$ $6H_2O$ in EPA.
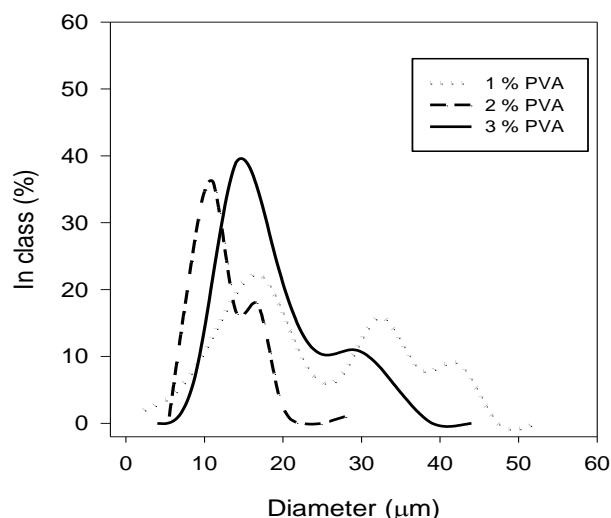
Figure 2. Effect on microparticle size distribution profiles by changes in PVA concentration of EPA.

determine the effect of pH on the release of the protein. Figure 6 shows that the formulation released about 95% of the protein model after 10 min in the dissolution medium having pH of 10.3. In contrast, at pH 5, it showed a gradual release, exhibiting 21% of release at 10 min. The kinetic analysis of the *in vitro* release data of OA from the prepared Eudragit® S100 MP are presented in Table 6. According to the determination coefficients (R2), the *in vitro* release data were in favor of Korsmeyer Peppas model (pH 7). Fast kinetic release of OA from MP at alkaline pH was followed by optical microscopy which showed the dissolution of the particles (Figure 7). Finally, we performed a statistical analysis to compare the releases at different values between pH 5.0 and 10.3.

Analysis of variance (ANOVA) showed that there was a significant difference (p-value < 0.05), thus confirming that the release depends on the environment in which the MP are exposed.

Differential scanning calorimeter (DSC) analysis

The DSC technique is one of the most convenient methods for investigating the compatibility of polymer
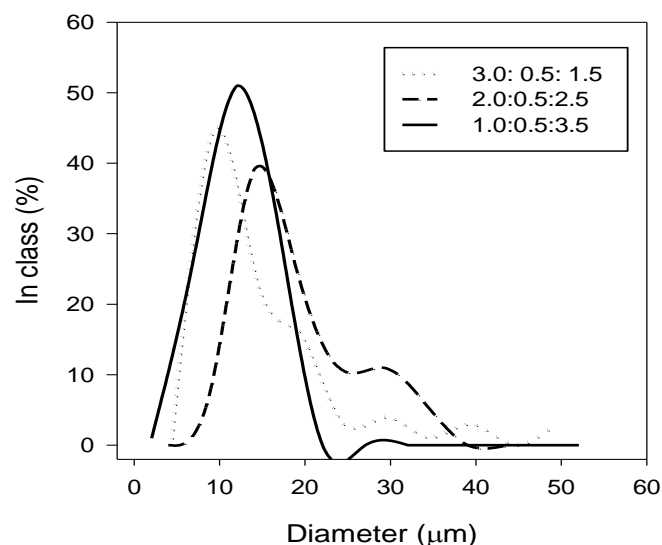
Figure 3. Effect on microparticle size distribution profiles by changes in organic phase composition (AC:ISP:CH).

blends; therefore it was used to evaluate thermodynamic compatibility between ovoalbumin and Eudragit® S100 in
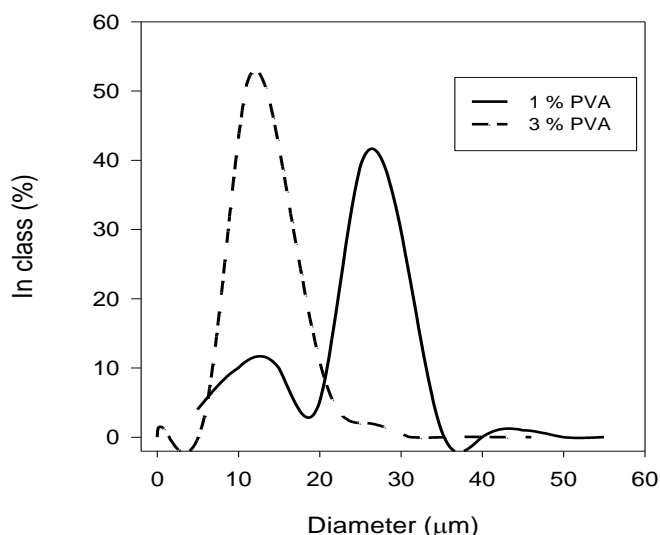
Figure 4. Effect on particle size by PVA. MP were prepared using 13 mL to 75 % w/w MgCl$_2$ 6H$_2$O in EPA, with 300 rpm of mechanical stirring speed and an organic volume ratio 3: 0.5: 1.5 AC: ISP: CH.
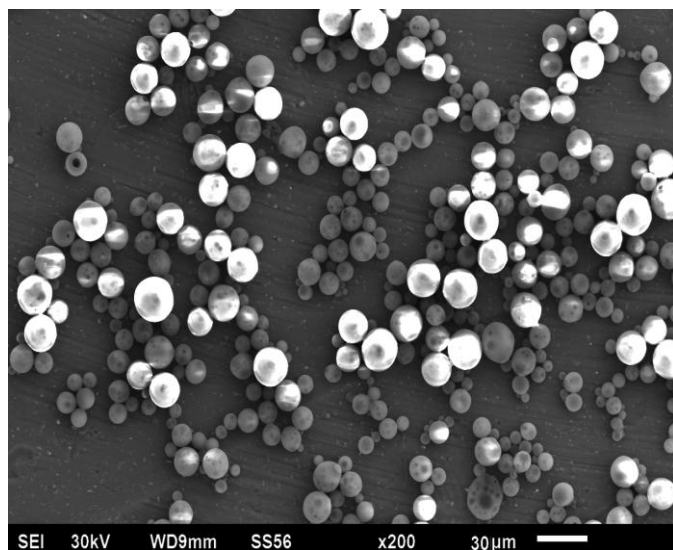

Figure 5. SEM of ovoalbumin-loaded MP. MP were prepared using 13 mL of 1 % w/w PVA and 75 % w/w MgCl2 6H2O in EPA, with 300 rpm of mechanical stirring speed and an organic volume ratio 3: 0.5: 1.5 AC: ISP: CH.

the OA-MP, based on crystalline melting temperature and the glass transition temperature. Figure 8 depicts the obtained DSC thermograms. In the Eudragit® S100; an endothermic peak at 217.9°C was observed as a melting point. The raw material OA showed a sharp endothermic peak at 92.5°C that corresponded to its melting point, indicating its crystalline nature. A physical mixture of the protein with polymer resulted in the decrease in such a
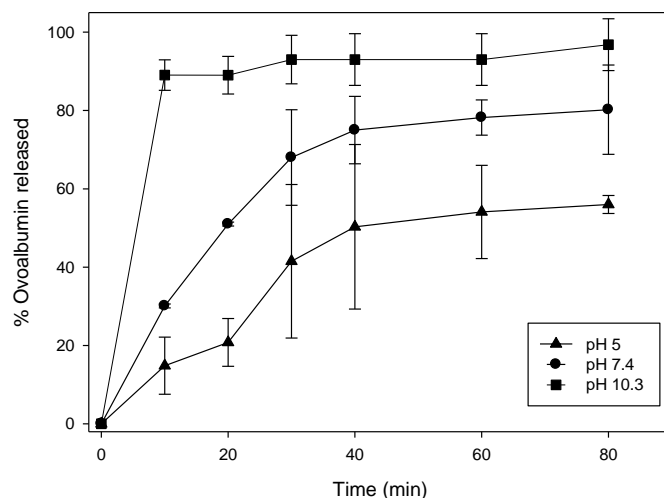

Figure 6. Ovoalbumin release profiles. Error bars show mean + SD; n=3. MP were prepared using 13 mL of 1 % w/w PVA to 75 % w/ MgCl$_2$ 6H$_2$O in EPA, with 300 rpm of mechanical stirring speed and an organic volume ratio 3: 0.5: 1.5 AC: ISP: CH.

fusion peak.

DISCUSSION

Particle size measurement and determination of encapsulation efficiency

The effect of formulation variables on size, IP and encapsulation efficiency of OA-MP prepared by controllable combination of diffusion and emulsification steps as part of the salting out procedure was determined. Maintaining some diffusion control is advantageous as at all concentrations of MgCl$_2$ 6H$_2$O, low polydispersity is evident with 75% (w/w) MgCl$_2$ 6H$_2$O (Figure 1). With no modulating control over acetone diffusion (15 and 35% w/w MgCl$_2$ 6H$_2$O) the polydispersity was found to be excessive (> 0.5) because there were observed particles with sizes > 200 microns (data not shown). Thus, the rate of acetone diffusion is controlled primarily by the concentration of MgCl$_2$ 6H$_2$O On the other hand, the size of the droplets was dependent mainly on the amount of PVA, mechanical energy used to form the emulsion, and the volume of EAP. PVA is a popular stabilizer for the production of polymeric MP (Vandervoort and Ludwig, 2002), whereby its hydrocarbon regions adsorb strongly on the surface of the MP via hydrophobic bonding with Eudragit® S100. AC diffusion from nascent particles has been shown to precipitate PVA and in turn, acts as an effective stabilizing agent around the droplet and restricting aggregation (Murakami et al., 1997). At a concentration of 1% (w/w), particles < 1 micron were obtained (data not
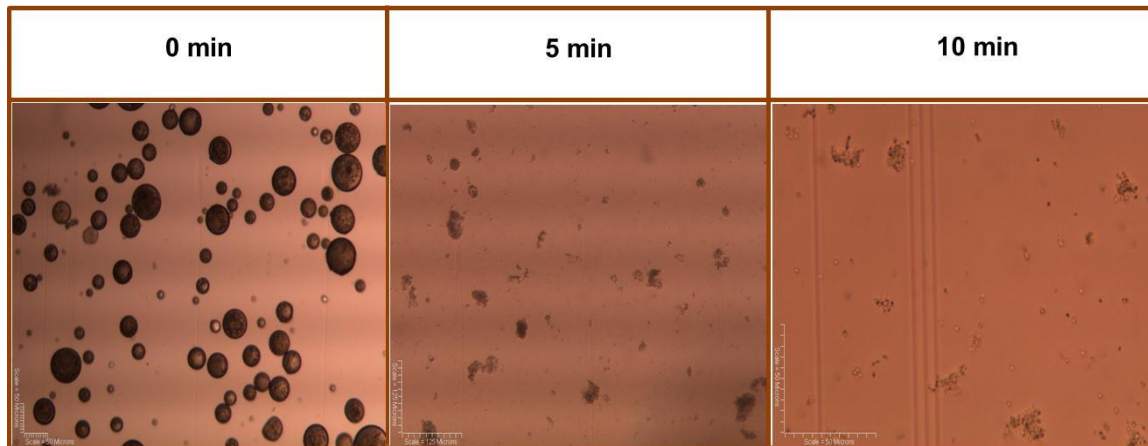
Figure 7. Optical microscopy of ovoalbumin-loaded MP in pH 10.3, at 0.0, 5.0 and 10.0 minutes of exposition.
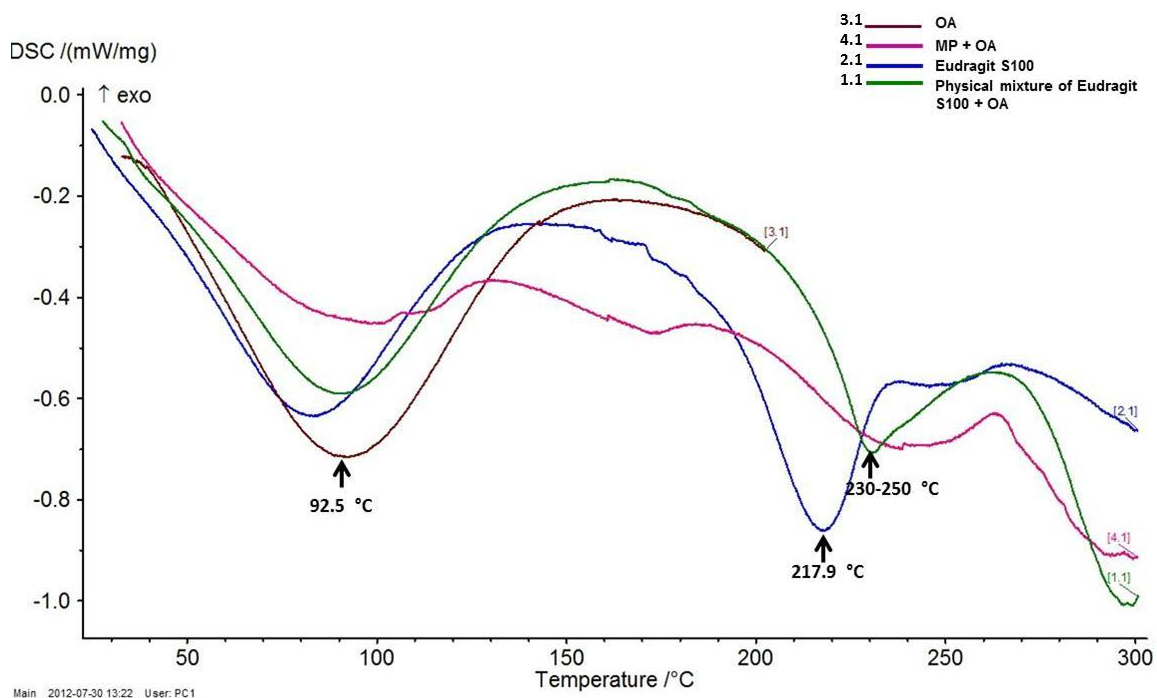

Figure 8. Differential scanning calorimetry for OA-loaded Eudragit® S100 microparticles, OA pure, Eudragit® S100 pure and physical mixture of OA and Eudragit® S100. MP were prepared using 13 mL of 1 % w/w PVA to 75 % w/w $MgCl_2$ $6H_2O$ in EPA, with 300 rpm of mechanical stirring speed and an organic volume ratio 3: 0.5: 1.5 AC: ISP: CH.

shown), and a polymer aggregates possibly because the interfacial tension resulted in low globules formation, because the PVA concentration was insufficient to stabilize the globules. The formulation of PVA 3% (w/w) presented a size around 16.2 ± 6.1 microns with no aggregates (with a PI of 0.38), allowing globules stability. This result is based on the fact that the acetone fraction was able to diffuse to EAP which is instrumental in forming a shell

around the quasi-MP during the former part of the particle assembly.

In Table 3 and Figure 3, it can be observed that as the AC ratio increases in the organic phase, the particle size decreased probably due to the solvent mixture having a low interface tension with the aqueous phase, allowing more stable globules. As Eudragit® S100 is solubilized by the solvent mixture, the ratio of the three solvents was

critical. Therefore, removing a relatively small volume of AC precipitates the polymer (Eudragit® S100) and a coacervate phase may not actually be formed. This fast precipitation impacts the morphology and size of the capsules produced (Atkin et al., 2004; Murakami et al., 1997). It could be mentioned that the organic phase composition influenced the encapsulation efficiency, but this effect was minimal on the PI. Despite low percentage encapsulation efficiency, formulation with the best PI (OP composed by AC: ISP: CH (3.0:0.5:1.5, v/v/v)) from Table 3 was chosen to evaluate the influence of EAP volume on particle size and encapsulation efficiency.

With the decrease on volume of EAP, the EE and particle size also increased. Less effective stirring (300 rpm) and accumulation of internal phase globules generated an emulsion with larger globules and increased the particle size (Zolfaghanan and Mohammadpour, 2009). Furthermore, it was observed that the effect on particle size by EAP volume would not yield to obtain particles > 20 microns (Table 4). However, a formulation was obtained with a PI and acceptable encapsulation efficiency percent at a volume of 13 mL of EAP (0.35 and 57.5 ± 8.9, respectively). Due system behaved as an emulsion, in order to increase particle size it was decided to decrease the concentration of PVA.

Table 5 shows that preparation at a high speed (500 rpm) had submicron-sized particles and polymer aggregates (data not shown), possibly because tensoactive concentration was not enough to prevent coalescence among yielded globules, giving origin to a polymer film. On the otherhand, low speed stirring (100 rpm) yielded higher polymer aggregates and less particle production because the energy provided during emulsification was not enough to promote the formation of a disperse system (Makame et al., 2005). This can be sustained by the polydispersity values, which showed t- value of ≥ 0.4, indicating heterogeneity in particle size or the presence of polymer aggregates. The PI and encapsulation efficiency were acceptable for the formulation produced at a speed of 300 rpm. A lack of diffusion control gives rise to a bimodal distribution with particle sizes around 15 µm (Boury et al, 1995; Shakesheff et al., 1997).

Manu Sharma et al. (2011) encapsulated papain in Eudragit® S100 (and other polymers) microparticles using a $W_1/O/W_2$ emulsion solvent evaporation and observed an encapsulation efficiency of 82.35%. In the present experiment, the best encapsulation efficiency of OA was 56.5%. The higher drug loading observed in the study of Manu Sharma et al. (2011) appears to be due to the use of a higher polymer: protein ratio compared with the present study (100: 3.5 against 100:10, respectively).

In vitro **release profile and mechanism of drug release**

The release may be due to dissolution and diffusion,

because the polymer (Eudragit® S100) is pH-dependent (dissolves at pH > 7.0). It could be demonstrated that the alkaline medium promotes the almost immediate release of OA. Furthermore the isoelectric point for OA (4.7) contributes too, because as the polymer, OA exists in anionic form at the alkaline pH (West et al., 1974; Palmieri et al., 2000; Bykov et al., 2000). To investigate the kinetics and mechanism of drug release, the release data were fitted to zero order, first order, Higuchi and Korsmeyer-Peppas models. The 'n' value that can be obtained from the slope of a plot of log $Mt/M\infty$ versus log time (Korsmeyer-Peppas model) is indicative of drug release mechanism. If n is 0.45 or less, the release mechanism follows Fickian diffusion, higher values (0.43 < n < 0.85) for mass transfer follow a non-Fickian model (anomalous transport), where release is controlled by a combination of diffusion and polymer relaxation. When n reaches a value of 0.85 or above, the mechanism of drug release is regarded as case-II transport or super case-II transport which means the drug release rate does not change over time and involves polymer relaxation and chain disentanglement (Cox et al., 1999; Harland et al., 1988).

The values of n were > 0.43 and < 0.85, indicating non Fickian (anomalous) transport for the investigated formulation in these pH. Thus, it was proposed that these formulations delivered their active ingredient by coupled diffusion and relaxation. Whereas the kinetic release of OA from MP at alkaline pH was not fitted to mathematical models used and fast release was supported by optical microscopy showing the dissolution of the particles at less than 10 min of exposure (Figure 7).

DSC analysis

OA crystals still exist in physical mixture (Bharate et al., 2010; Pignatello et al., 2001). This type of interaction was previously observed in the physical mixture of piroxicam with Eudragit® S100 and diflunisal with Eudragit RL 100 (Pignatello et al., 2001; Maghsoodi et al., 2010). However, for microparticles, the intensity of melting peak of OA decreases considerably. The reduction of the protein endothermic peak in the microparticles suggested that OA might be imbedded into Eudragit® S100 and existed in an amorphous state in the microparticles, indicating a thermodynamic compatibility between OA and Eudragit® S100 (Hu et al., 2011). In this way, the intermolecular interaction between protein molecules is favored.

Conclusion

Ovoalbumin was successfully encapsulated in enteric microparticles by controllable combination of diffusion and emulsification steps as part of the salting out procedure, optimizing the various formulation parameters

in order to attain maximum encapsulation efficiency and a spherical shape, with an almost monodispersed particle size distribution and an optimum *in vitro* release profile. The release profiles for the formulations allowed pH-dependent release of ovoalbumin in alkaline pH.

Eudragit® S100 particles disintegrated and showed complete release of ovoalbumin in the pH environment of the ileum intestine within few minutes. Thus, Eudragit® S100 particles have great potential as oral carriers for delivery of proteins to the small and large intestines to facilitate their digestion.

ABBREVIATIONS

MP: Polymeric microparticles, **PI:** polydispersity index, **PVA:** poly(vinyl-alcohol), **OA:** ovoalbumin, **IAP:** inner aqueous phase, **AC:** acetone, **OP:** organic phase, **CH:** chloroform, **EPA:** external aqueous phase, **ISP:** isopropanol, **SEM:** scanning electronic microscopy, **EE:** entrapment efficiency.

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