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USP apparatus 4 method for *in vitro* release testing of protein loaded microspheres

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ABSTRACT

The objective was to develop an *in vitro* release method for protein loaded poly(lactide-co-glycolide) (PLGA) microspheres. A modified USP apparatus 4 and sample and separate methods were compared using a microsphere formulation encapsulating a model protein, bovine serum albumin (BSA). Microsphere characteristics such as porosity, drug loading, particle size and burst release were significantly affected by the formulation parameters (i.e., phase ratio, internal aqueous phase composition and theoretical drug loading). Incomplete release of BSA was observed using the sample and separate method and this was attributed to microsphere loss during sampling. This problem was overcome using the modified USP apparatus 4 method. However, an unusual decrease in cumulative percent release was observed which was considered to be due to BSA adsorption onto the hydrophobic surfaces of the modified USP apparatus 4. Addition of SDS to the release media prevented BSA adsorption and a zero order release profile was observed. The presence of SDS did not change the microsphere degradation kinetics. The results indicate the importance of understanding protein adsorption and aggregation behavior during *in vitro* release testing. The USP apparatus 4 method appears to be useful for investigation of *in vitro* release of protein loaded microspheres.

1. Introduction

Recent advancements in the field of biotechnology have resulted in the advent of various recombinant peptide and protein therapeutics. These therapeutics are gaining popularity due to their safety and efficacy in the treatment of diseases that were incurable in the past (Shofner and Peppas, 2009). However, there are challenges associated with protein and peptide delivery that include: (a) instability in the gastrointestinal tract; (b) short half life; (c) immunogenicity and (d) loss of activity due to physical or chemical degradation (Jorgensen et al., 2006, 2009). Therefore, most of these products require repeated parenteral administration. At present, solution or lyophilized powder for reconstitution are the most popular formulations used to deliver protein and peptide therapeutics *via* the intravenous, subcutaneous or intramuscular routes (Jorgensen et al., 2009; Rawat and Burgess, 2009).

Efforts are being made to improve patient compliance by reducing the frequency of injections using controlled and targeted drug delivery systems such as microspheres, liposomes, implants, *etc.* (Brown, 2005; Jorgensen et al., 2006; Wright et al., 2001; Ye et al., 2000). These drug delivery systems have reduced side effects due to the steady release of low amounts of therapeutics over periods of days to months. Accordingly, these systems are being considered as potential alternatives to solution or lyophilized formulations of proteins and peptides. Poly(lactide-co-glycolide) (PLGA) microspheres are one of the most promising controlled drug delivery systems for peptides and proteins. Lupron Depot® (leurpolide) was the first injectable microsphere formulation approved by the US FDA for the treatment of prostate cancer (Chen, 2005; Rhee et al., 2010). Other peptide/protein microsphere formulations approved by the US FDA include: Sandostatin® LAR (octreotide acetate), Nutropin® Depot (Somatropin) and Trelstar™ Depot (Triptorelin) for the treatment of acromegaly, growth hormone deficiency and prostate cancer, respectively (Chen, 2005; D'Souza and DeLuca, 2006).

It is essential to develop appropriate performance tests for microsphere formulations to ensure their safety and efficacy. *In vitro* release is an important performance test that is used to: monitor batch-to-batch variability, evaluate any change in the manufacturing process, optimize formulations, as well as develop *in vitro–in vivo* correlations (IVIVCs) (Shah et al., 2002). At present, there are no standard *in vitro* release testing methods for microspheres. The methods that are being used for *in vitro* release testing of microspheres encapsulating peptide and protein therapeutics include sample and separate, dialysis membrane and continuous flow-through methods.

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Conventionally, sample and separate methods have been used where microspheres are dispersed in small volumes of release media in vials, tubes or bottles and sampling is performed by separating the microspheres from the release media using centrifugation or filtration (D'Souza and DeLuca, 2006). However, there are limitations of this method such as violation of sink conditions, aggregation of microspheres due to inadequate agitation, loss of microspheres during sampling, disruption of the formulation due to centrifugation force, and the use of vials/tubes/bottles of different dimensions that make inter-laboratory comparisons impossible (Berkland et al., 2007; Clark et al., 2005; D'Souza and DeLuca, 2006; Hernádez et al., 1998; Voisine et al., 2008; Zolnik et al., 2005). All these factors can lead to variable and inaccurate results.

The membrane diffusion method has also been used for *in vitro* release testing of microspheres (D'Souza et al., 2005; D'Souza and DeLuca, 2005). In this method a dialysis membrane is used to separate the formulation and the release media. This method suffers the disadvantage that the formulations are placed in a small volume of release media within the dialysis sac and hence sink conditions may be violated (Clark et al., 2005). In addition, inadequate agitation inside the dialysis sac can cause microsphere aggregation.

Continuous flow-through methods involve the release media flowing through a cell containing the formulation. Continuous flow-through systems using HPLC columns as the sample compartment have been used by some groups to mimic *in vivo* conditions (Aubert-Pouëssel et al., 2002; Cleland et al., 1997). Continuous flow-through methods vary among laboratories and may result in variable release profiles. Zolnik et al. (2005) have modified USP apparatus 4 for *in vitro* release testing of microspheres. In this modification of USP apparatus 4, microspheres were mixed with glass beads to minimize microsphere aggregation and the system was operated in a closed loop configuration. Studies indicated the USP apparatus 4 (flow through cell) method to be better than conventional sample and separate methods for *in vitro* release testing of microspheres (Voisine et al., 2008; Zolnik et al., 2005). The advantages of the continuous flow-through method using USP apparatus 4 include: (a) separation of the formulation from the release media in the flow-through cell which makes sampling and media replacement easy; (b) minimum aggregation of the microspheres when mixed with glass beads in the flow through cell; (c) the flexibility of using small media volumes (to allow detection of drug in case of microspheres with low drug loading) or large media volumes (to maintain sink conditions); and (d) better reproducibility as it is based on a compendial apparatus with well defined geometry and hydrodynamics.

USP apparatus 4 has been shown to be useful for PLGA microspheres encapsulating small molecular weight drugs (Voisine et al., 2008; Zolnik et al., 2005). However, it is necessary to evaluate the feasibility of using this method for protein/peptide loaded microspheres.

In this study, the feasibility of using USP apparatus 4 for *in vitro* release testing of PLGA microspheres encapsulating a model protein, bovine serum albumin (BSA) was evaluated. The USP apparatus 4 method (continuous flow-through) was compared with the conventional sample and separate method. BSA loaded PLGA microsphere formulations were developed by varying some of the critical formulation parameters and the most appropriate formulation was selected for *in vitro* release testing using USP apparatus 4 and sample and separate methods.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA) polymer, PLGA Resomer® RG503H 50:50 (MW: 25 kD) was a gift from Boehringer–Ingelheim. Bovine serum albumin, poly(vinyl alcohol) (PVA) (MW: 30–70 kD), sodium dodecyl sulfate (SDS, ACS grade), sodium chloride (NaCl, ACS grade), sodium azide (N_3Na) and dimethyl sulfoxide (DMSO, ACS grade) were purchased from Sigma–Aldrich (St. Louis, MO). Bicinchoninic acid assay (BCA) reagents, sodium hydroxide (NaOH, ACS grade), sodium monohydrogen phosphate (NaH_2PO_4, ACS grade) and tetrahydrofuran (THF) (HPLC grade) were obtained from Fisher Scientific (Pittsburg, PA). Disodium hydrogen phosphate (Na_2HPO_4, ACS grade) was purchased from VWR International. Nanopure™ quality water (Barnstead, Dubuque, IA) was used for all studies.

2.2. Methods

2.2.1. Preparation of microspheres

BSA containing PLGA microspheres were prepared using the $W_1/O/W_2$ emulsion solvent extraction/evaporation technique (Okada, 1997). Two grams of PLGA were dissolved in 8 ml of methylene chloride. BSA was dissolved in a small amount of water/phosphate buffered saline pH 7.4 (refer to Table 1 for phase ratio: W_1:O and theoretical drug loading) and the solution thus obtained was added to the methylene chloride solution of PLGA. The mixture was homogenized for 40 s at 10,000 rpm using a homogenizer (PowerGen 700 D, Fisher Scientific) to obtain a primary emulsion (W_1/O). This primary emulsion was then added slowly to 40 ml of 1% (w/v) aqueous poly(vinyl alcohol) (PVA) solution and homogenized at 10,000 rpm for 30 s to obtain a secondary emulsion ($W_1/O/W_2$). The resulting double emulsion was then added to 500 ml of 0.1% (w/v) aqueous PVA solution and stirred at 600 rpm under vacuum for 5–6 h at 25 °C for extraction/evaporation of methylene chloride. The resulting microspheres were filtered (Durapore® membrane filter, 0.45 μm, Fisher Scientific), washed three times with de-ionized water and vacuum dried for 24 h.

Six different BSA containing microsphere formulations were prepared by varying the processing parameters such as phase ratio, theoretical drug loading and using de-ionized water or phosphate buffered saline pH 7.4 (PBS) as the internal aqueous phase (as indicated in Table 1). All the microsphere formulations were prepared in triplicate (3 batches).

2.2.2. Characterization of microspheres

2.2.2.1. Method of analysis. Quantitative analysis of BSA was performed using a bicinchoninic acid (BCA) assay (Wiechelman et al.,

Table 1
BSA loaded PLGA microsphere formulations.

	Theoretical drug loading (% (w/w))	Internal aqueous phase (W_1)	Phase ratio (W_1:O)	Experimental drug loading (% (w/w))	Encapsulation efficiency (%)	Burst release (24 h) (%)	Particle size (μm)
A	1.00	Water*	1:16	0.89 ± 0.04	88.76 ± 4.04	9.98 ± 1.43	10.50 ± 0.39
B	1.00	Buffer**	1:16	0.91 ± 0.02	88.17 ± 1.77	42.10 ± 1.23	16.63 ± 0.57
C	2.50	Water*	1:8	2.09 ± 0.20	83.48 ± 8.08	41.04 ± 2.01	13.86 ± 0.60
D	2.50	Buffer**	1:8	1.87 ± 0.09	74.79 ± 3.71	66.44 ± 2.63	24.68 ± 0.46
E	5.00	Water*	1:8	4.28 ± 0.26	85.57 ± 5.22	53.31 ± 1.74	12.39 ± 0.38
F	5.00	Water*	1:16	4.18 ± 0.28	83.65 ± 5.62	24.55 ± 4.16	9.30 ± 1.03

% (w/w) – percent weight by weight; W_1:O – ratio of internal aqueous phase to organic phase; * de-ionized water; ** PBS pH 7.4; polymer concentration: 25% (w/v).

1988). A 0.1 ml aliquot sample was mixed with two ml of BCA reagent (50:1 ratio of BCA reagent A and B) in 5 ml polystyrene tubes. The tubes were incubated at 60 °C for 30 min. After incubation the tubes were cooled and the absorbance was determined at 562 nm using a UV-visible spectrophotometer (Cary 50, Varian).

2.2.2.2. Drug loading and encapsulation efficiency. The amount of BSA encapsulated within the microspheres was determined by dissolving 10 mg of microspheres in 1 ml of DMSO. The volume was made up to 10 ml with a solution of 0.05 N NaOH and 0.5% (w/v) SDS. The microspheres were dissolved for 1 h by occasional shaking and the BSA content was analyzed using a BCA assay as described in Section 2.2.2.1 (Sah, 1997). All chemicals were used in concentrations compatible with the BCA assay.

Drug loading was determined as: percent drug loading = (weight of drug entrapped/weight of microspheres used) × 100. The encapsulation efficiency was determined as: (experimental drug loading/theoretical drug loading) × 100. All measurements were conducted in triplicate and the results were reported as the mean ± SD.

2.2.2.3. Particle size analysis. An AccuSizer 780A autodiluter particle sizing system was used to determine the mean particle diameter. Approximately 50 mg of microspheres were dispersed in 2 ml of 0.1% (w/v) PVA solution. 200 μl of the dispersion was used for particle size analysis. All measurements were conducted in triplicate and the results were reported as the mean ± SD.

2.2.2.4. Microsphere morphology. Microsphere morphology was characterized using scanning electron microscopy (SEM). Samples were mounted on carbon taped aluminum stubs and gold coated in a sputter coater for 1 min at 6 mA. The samples were analyzed using a DSM982 Gemini SEM (Carl Zeiss, Inc.) at an accelerating voltage of 2.0 kV.

2.2.2.5. Molecular weight determination. The molecular weight of the microspheres was determined by gel permeation chromatography (GPC; Waters) with an evaporative light scattering detector (ELSD). The mobile phase was THF with a flow rate of 3 ml/min at 40 °C. Ten mg of microspheres were dissolved in 10 ml tetrahydrofuran (THF) and filtered through 0.45 μm filters for GPC analysis. The data collection and analysis was performed using Waters Millenium software. Polystyrene standards (2000, 900, 824, 400, 200, 110, 43, 18.80, 17.60, 6.93, 2.61, 0.98 kD) were used for calibration and weight average molecular weights (Mw) were calculated. All measurements were conducted in triplicate and the results were reported as the mean ± SD.

2.2.2.6. In vitro release testing using USP apparatus 4. A modified USP apparatus 4 (Sotax CE7 smart with CY 7 piston pump, Sotax, Horsham, PA) was used for *in vitro* release testing (Zolnik et al., 2005). Flow through cells (12 mm diameter) packed with glass beads (1 mm diameter) were used in a closed system. The system was temperature controlled at 37 ± 0.1 °C. Approximately 40 mg of microspheres were dispersed in the flow through cells (fitted with regenerated cellulose filters 0.45 μm) and 40 ml of 0.05 M phosphate buffered saline pH 7.4 (PBS) (with and without 0.01% SDS) was circulated at a flow rate of 8 ml/min. One ml samples were withdrawn and replaced with fresh media at suitable time intervals. The samples were analyzed using BCA assay as described in Section 2.2.2.1. The *in vitro* release was determined in triplicate and the results were reported as the mean ± SD.

2.2.2.7. In vitro release testing using the sample and separate method. 20 mg of microspheres were dispersed in 2 ml of 0.05 M PBS pH 7.4 (with 0.01% (w/v) SDS) in 5 ml polystyrene tubes (BD Falcon). The tubes were placed in a C76 water bath shaker (New Brunswick Scientific) maintained at 37 °C and 100 rpm. The tubes were centrifuged (4000 rpm for 15 min) at each sampling time point. The entire supernatant was collected and filtered through 0.45 μm syringe filters (Whatman) and analyzed for BSA content using a BCA assay as described in Section 2.2.2.1. Fresh medium (2 ml) was the placed in the tubes. The tubes were vortexed to disperse the microspheres and returned to the shaker water bath. The *in vitro* release was determined in triplicate and the results were reported as the mean ± SD.

3. Results and discussion

3.1. Microsphere formulations

$W_1/O/W_2$ emulsion/solvent evaporation is a commonly used method for the preparation of protein/peptide loaded microspheres. Various formulation and processing parameters have been reported to affect the characteristics of microspheres prepared using this method (such as burst release and drug loading) (Mao et al., 2008; Yang et al., 2000; Yeo and Park, 2004). In the present study, BSA loaded PLGA microsphere formulations were prepared using the $W_1/O/W_2$ emulsion/solvent evaporation method and some of the critical formulation parameters such as theoretical drug loading, internal aqueous phase and phase ratio were varied to select an optimum formulation for *in vitro* release studies. These formulations are referred to as A, B, C, D, E and F (Table 1). Formulations A, B, C and D were prepared to evaluate the effect of using de-ionized water or PBS pH 7.4 as the internal aqueous phase (W_1). These formulations were prepared with constant theoretical drug loading and phase ratio (1% (w/w) theoretical drug loading and 1:16 phase ratio for Formulations A and B; 2.5% (w/w) theoretical drug loading and 1:8 phase ratio for Formulations C and D). The effect of increasing the theoretical drug loading (1–5% (w/w)) was evaluated with de-ionized water as the internal aqueous phase and phase ratios of 1:16 (Formulations A and F). Formulations E and F were prepared with de-ionized water as the internal aqueous phase (W_1) and 5% (w/w) theoretical drug loading. The effect of changing the phase ratio from 1:8 to 1:16 (W_1:O) was evaluated.

3.2. Morphology

Fig. 1 shows SEM micrographs of BSA loaded PLGA microspheres (Formulations A–F) prepared as per the parameters shown in Table 1.

3.2.1. Effect of internal aqueous phase composition (i.e., de-ionized water or PBS pH 7.4)

The microspheres prepared with water as the internal aqueous phase (W_1) had smooth surfaces with a few pores (Fig. 1A, C, E and F) whereas the microspheres prepared with phosphate buffer pH 7.4 as the internal aqueous phase were highly porous (Fig. 1B and D-(a)). Buffers (*e.g.*, phosphate, citrate buffers) have been used as the internal aqueous phase (W_1) to dissolve proteins/peptides for microspheres preparation (Aubert-Pouëssel et al., 2002; Cleland et al., 1997; Couvreur et al., 1997; Crotts and Park, 1997; Crotts et al., 1997). However, the effect of using buffers as the internal aqueous phase on the microspheres morphology has not been reported in any of these studies.

In the present work, the porous microsphere surfaces observed in Formulations B and D (PBS pH 7.4 as internal aqueous phase) may be due to the formation of an osmotic pressure gradient (as a result of the presence of buffer salts) between the internal and external aqueous phases across the polymer phase boundary. The polymer phase has been reported to act as a semi-permeable membrane in the semi-solid state (Yeo and Park, 2004). This osmotic pressure

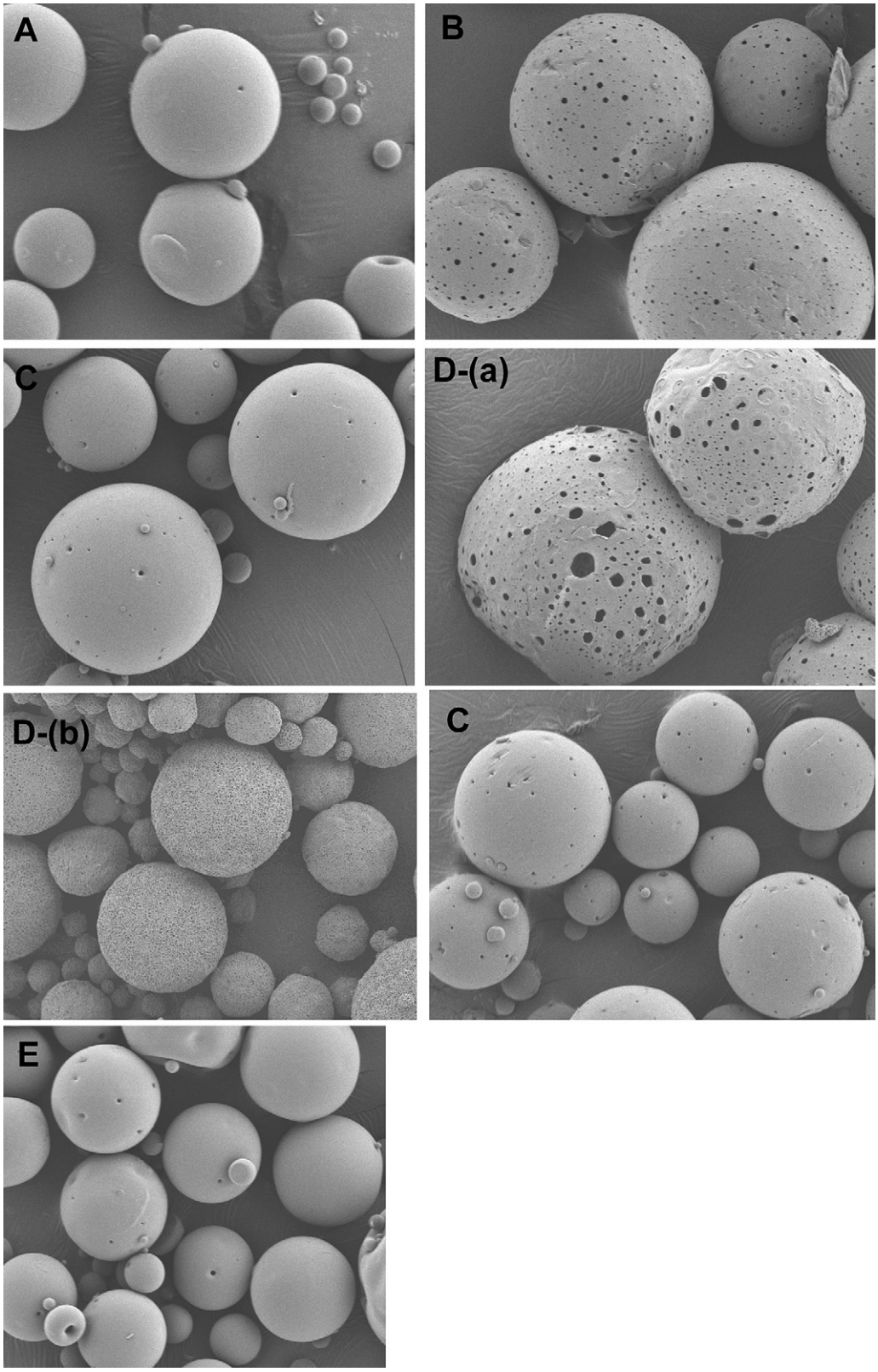

Fig. 1. SEM micrographs of BSA loaded PLGA microspheres. A: Formulation A; B: Formulation B; C: Formulation C; D-(a): Formulation D; D-(b): Formulation D with PBS as internal (W_1) and external (W_2) aqueous phases; E: Formulation E; and F: Formulation F.

gradient might have caused mixing of the internal and external aqueous phases during microencapsulation processing resulting in the porous structure. The pores on the microsphere surfaces were significantly reduced when PBS pH 7.4 was used as both internal (W_1) and external (W_2) aqueous phases as shown in Fig. 1D-(a) and D-(b) due to the reduction in the osmotic pressure gradient. Uchida et al. (1997) observed that the addition of sodium chloride to the external aqueous phase (W_2) resulted in a decrease in the

encapsulation efficiency of insulin due to osmotic pressure driven mixing of the two aqueous phases.

3.2.2. Effect of phase ratio (W_1:O)

In formulations where pH 7.4 PBS was used as the internal aqueous phase, the microspheres prepared with a phase ratio 1:8 (W_1:O) (Formulation D) showed larger pores when compared to the microspheres prepared with a phase ratio of 1:16 (W_1:O) (Formulation B). This may be attributed to the higher volume fraction of the internal aqueous phase (Formulation D) resulting in an increased number and size of aqueous droplets within each polymer droplet. This effect was also observed in microspheres prepared with de-ionized water as the internal aqueous phase (Formulations E and F). A similar observation on the effect of phase ratio on microsphere porosity has been reported by Yang et al. (2000).

3.3. Drug loading and encapsulation efficiency

As shown in Table 1, the experimental drug loading of the microspheres increased significantly with an increase in the amount of BSA (theoretical drug loading) at constant polymer concentration (25% (w/v)). There was an increase in drug loading irrespective of the change in phase ratio and the internal aqueous phase composition (Formulations A–F) ($p < 0.05$).

The encapsulation efficiency of the microspheres was not significantly affected by change in the theoretical drug loading, phase ratio or internal aqueous phase composition. Couvreur et al. (1997) used pH 8 buffer as the internal aqueous phase (W_1) to improve the encapsulation efficiency of a cholecystokinin analog by increasing its solubility in the internal aqueous phase (p*I* of cholecystokinin is 4.8). At pH values above and below their isoelectric points (p*I*), proteins/peptides have net negative and positive charge, respectively. This results in repulsion between molecules preventing protein aggregation and hence there is an increase in solubility. However in the present work, no significant increase in the encapsulation efficiency of BSA was observed when PBS pH 7.4 was used as the internal aqueous phase (Formulations B and D) instead of de-ionized water (Formulations A and C).

3.4. Particle size analysis

3.4.1. Effect of phase ratio (W_1:O)

There was a significant increase in the mean particle size of the microspheres with change in the phase ratio from 1:16 (W_1:O) (approximately 10 μm for Formulations A and F; water as the internal aqueous phase) to 1:8 (approximately 13 μm for Formulations C and E; water as internal aqueous phase) ($p < 0.05$). This is considered to be due to the relative increase in the volume fraction of the internal aqueous phase at a phase ratio of 1:8. Hence larger polymer phase droplets are formed to accommodate the internal aqueous phase droplets. A significant increase in particle size with change in the phase ratio was also observed for microspheres prepared with PBS (pH 7.4) as the internal aqueous phase (Formulations B and D) as shown in Table 1 ($p < 0.05$).

3.4.2. Effect of internal aqueous phase composition (i.e., de-ionized water or PBS pH 7.4)

The particle size of the microspheres prepared with PBS pH 7.4 as the internal aqueous phase (Formulations B and D) was larger than the corresponding microspheres prepared with de-ionized water as the internal aqueous phase (Formulations A and C). The mixing of internal and external aqueous phases due to the osmotic pressure gradient across the polymer phase can cause faster extraction of the organic solvent methylene chloride leading to faster solidification (Formulations B and D) (Yeo and Park, 2004). Faster solidification rate has been reported to result in larger size microspheres (Yeo and Park, 2004).

3.5. Burst release

High burst release is one of the challenges with protein/peptide loaded PLGA microspheres (Yeo and Park, 2004). Proteins are hydrophilic and have a tendency to adsorb at interfaces during microsphere preparation and hence concentrate on the microsphere surfaces contributing to the high initial burst release.

3.5.1. Effect of theoretical drug loading

In the present work, the burst release significantly increased with increase in theoretical drug loading (microspheres with the same phase ratio and internal aqueous phase composition) ($p < 0.05$). The burst release increased from approximately 10% to 25% when the theoretical drug loading was increased from 1% (w/w) (Formulation A) to 5% (w/w) (Formulation F) (Table 1). This affect has been attributed to: (1) the formation of interconnected aqueous channels of hydrophilic protein within the microspheres and (2) surface precipitation of BSA at high drug loading. These interconnected aqueous channels facilitate drug diffusion (Okada, 1997; Yeo and Park, 2004). Okada (1997) used hydrophilic excipients in the formulation of leuprolide loaded PLGA microspheres to form aqueous channels and enhance increase drug diffusion during the lag phase where drug release from microspheres is usually low.

3.5.2. Effect of phase ratio (W_1:O)

Formulations E and F were prepared with the same theoretical drug loading (5% (w/w)) but different phase ratios (1:8 and 1:16, respectively). As shown in Table 1, there was a significant increase in burst release (from approximately 25% to 53%) when the phase ratio was changed from 1:16 (Formulation F) to 1:8 (Formulation E) ($p < 0.05$). The higher internal aqueous phase volume (W_1:O; 1:8) is considered to facilitate mixing of the two aqueous phases resulting in porous microsphere surfaces as explained in Section 3.2 (morphology). The high burst release is associated with the larger number of pores on the surface of Formulation E when compared to Formulation F (Fig. 1E and F).

3.5.3. Effect of internal aqueous phase composition (i.e., de-ionized water or PBS pH 7.4)

Formulations A and B were prepared with the same theoretical drug loading (1% (w/w)) and phase ratio (1:16). However, higher burst release was observed in microspheres prepared with PBS (pH 7.4) as the internal aqueous phase (Formulations B) when compared to the microspheres prepared with de-ionized water (Formulations A) (Table 1). This was due to the highly porous surface of Formulation B as shown in Fig. 1B which is expected to result in rapid diffusion of surface and pore associated BSA.

3.6. In vitro release

Formulation F was selected for *in vitro* release testing due to its high drug loading and relatively lower burst release (approximately 4% (w/w) experimental drug loading and 25% burst release). *In vitro* release was determined using the USP apparatus 4 method and 0.05 M phosphate buffer saline (pH 7.4) as the release medium. An initial burst release of approximately 27% was observed. BSA release increased to 30% on day 5 and then an unexpected decrease in the cumulative percent release was observed (Fig. 2, PBS pH 7.4). There was almost no release detected up to 55 days as shown in Fig. 2. Slow and incomplete release of protein from microspheres has been observed for proteins such as BSA, lysozyme, thyroglobulin from PLGA microspheres and this was attributed to protein

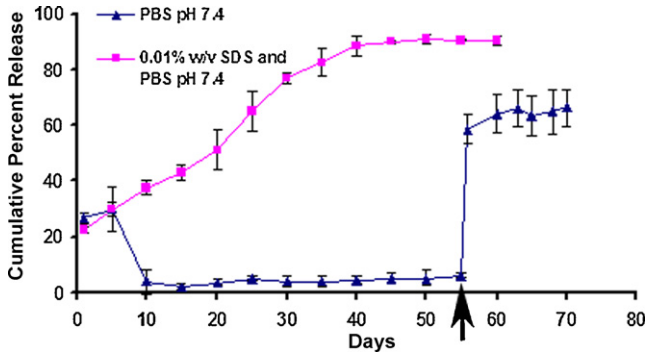

Fig. 2. *In vitro* release profile of BSA loaded PLGA microspheres (Formulation F) using USP apparatus 4 (PBS pH 7.4 with and without 0.01% (w/v) SDS). The arrow indicates the addition of 0.01% (w/v) SDS on day 55 in the study performed without 0.01% (w/v) SDS in the release medium (PBS pH 7.4).

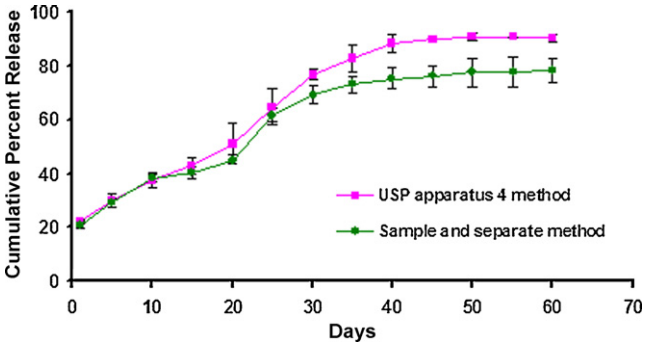

Fig. 3. *In vitro* release profile of BSA loaded PLGA microspheres (Formulation F) using USP apparatus 4 and sample and separate methods with 0.01% (w/v) SDS in PBS pH 7.4 as the release medium.

denaturation, aggregation and adsorption on the degrading polymer surface (Crotts and Park, 1997; Crotts et al., 1997; Park et al., 1998). In particular, Crotts et al. (1997) emphasized that incomplete release of BSA from PLGA microspheres was predominantly due to BSA adsorption on the degrading polymer surface rather than a result of aggregation.

However, none of these studies have reported a decrease in the percent cumulative release as observed in the present study with USP apparatus 4. Decrease in the cumulative percent release from the microspheres after the initial burst release may be due to the large amount of hydrophobic surfaces in the modified USP apparatus 4 (such as glass beads, flow-through cell surface, filter, tubings as well as the polymer). BSA can adsorb onto these surfaces by hydrophobic interactions and hence, may not be detected in the release medium.

Addition of sodium dodecyl sulfate (SDS) (below its critical micelle concentration (CMC) in 0.05 M PBS pH 7.4 (Yamamoto et al., 2004)) to the release medium on day 55 resulted in 70% BSA recovery (Fig. 2, PBS pH 7.4). This can be attributed to the preferential adsorption of SDS onto the hydrophobic interfaces, thus replacing adsorbed BSA (Bilati et al., 2005; Crotts and Park, 1997; Crotts et al., 1997). Preferential adsorption of surfactants on hydrophobic interfaces is well known and hence, surfactants are often used to stabilize proteins in solution formulations (Nakanishi et al., 2001; Wang et al., 2008).

All subsequent *in vitro* release tests of BSA loaded PLGA microspheres (Formulation F) were performed using USP apparatus 4 with 0.01% (w/v) SDS in PBS pH 7.4 as the release medium. As shown in Fig. 2 (0.01% SDS in PBS pH 7.4), a burst release of approximately 22% followed by a zero order release for approximately 40 days was observed. These results indicate that the presence of SDS in the release medium, at concentrations below its CMC, helped to prevent adsorption of released BSA by competing for adsorption onto the hydrophobic surfaces. Duncan et al. (2005) used SDS above CMC (0.1% (w/v)) and reported an increase in the cumulative percent release from microspheres which was considered to be due to solubilization of denatured protein.

It was important to investigate the effect of SDS on the PLGA degradation rate, since change in the degradation rate can alter the cumulative percent release. It was observed that the addition of 0.01% (w/v) SDS in the release medium did not affect the degradation kinetics of the PLGA microspheres. The degradation rate constants determined for microspheres incubated in PBS pH 7.4 with and without SDS were not significantly different ($0.0581 \pm 0.007\ day^{-1}$ and $0.0477 \pm 0.005\ day^{-1}$, respectively). Therefore, it appears to be beneficial to add a surfactant into the release medium for accurate estimation of the *in vitro* release of protein therapeutics.

The USP apparatus 4 method was compared with the conventional sample and separate method with 0.01% (w/v) SDS in PBS pH 7.4 as the release medium. As shown in Fig. 3, the release profiles up to day 25 were similar using the USP apparatus 4 and sample and separate methods. However, the release profiles started deviating around day 25. The percent cumulative release for USP apparatus 4 was approximately 12% higher than the sample and separate method after day 40. In the sample and separate method, the microspheres are directly dispersed in the release medium and sampling involves replenishing the entire release medium at every time point. This process can result in loss of microspheres and hence, lower cumulative percent release as compared to the USP apparatus 4 method where microspheres are separated from the release medium in the flow-through cells. Similar results have been reported for dexamethasone loaded PLGA microspheres by Zolnik et al. (2005) where the cumulative percent release using the USP apparatus 4 method was approximately 16% higher than the sample and separate method after 30 days. Cleland et al. (1997) used high speed centrifugation sample and separate and a flow-through method (microspheres dispersed in HPLC column) for *in vitro* release testing of growth hormone loaded PLGA microspheres. The authors reported that the release profile using the flow-through method was similar to the *in vivo* release from the microspheres. However, the sample and separate method resulted in faster release of growth hormone. This was attributed to the use centrifugal force for microsphere separation during sampling which can result in microsphere disruption.

It is evident that the flow-through method is better than the conventional sample and separate method. The flow through method based on USP apparatus 4, with the modified flow-through cell configuration, also has the advantage of utilizing a compendial apparatus. The prerequisites for a quality control test such as reproducibility, ease of operation and automation for GMP laboratories can be easily fulfilled using the USP apparatus 4 method.

4. Conclusions

The modified USP apparatus 4 method was shown to be useful for investigation of *in vitro* release of protein loaded microspheres. The work provided useful information on the effect of various formulation parameters on BSA loaded PLGA microsphere characteristics. Buffers are used to increase solubility of certain peptides and proteins in the internal aqueous phase during microsphere preparation (using emulsion/solvent evaporation method). According to the current study, the presence of buffer salts in the internal aqueous phase resulted in a highly porous microsphere structure and associated high burst release. Therefore, an understanding of the effects of various formulation parameters is critical in microsphere formulation design as such parameters are likely to affect

product performance. Adsorption of the released BSA on to the hydrophobic surfaces of the modified USP apparatus 4 system decreased the cumulative percent release. Therefore, the protein adsorption and aggregation behavior should be considered during *in vitro* release testing. The present work shows that incorporation of surfactants such as SDS in the release media would be useful for accurate estimation of cumulative percent release of proteins from the microspheres. The modified USP apparatus 4 method was better when compared to the sample and separate method for *in vitro* release testing of protein (BSA) loaded PLGA microspheres. The problem of microspheres loss encountered in sample and separate method was avoided using USP apparatus 4 method. Hence, the modified USP apparatus 4 method led to an accurate determination of cumulative percent release of microsphere. The method also offers the advantage of a compendial method that can minimize variation in results and hence, provides a useful tool for routine quality control of protein/peptide loaded microspheres.

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