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Article

Preparation and *in vitro* release profiling of PLGA microspheres containing BSA as a model protein

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Conventional drug formulations are incapable of adequate delivery of proteins and peptides for therapeutic purposes. As these molecules have very short biological half-life, multiple dosing is required to achieve the desirable therapeutic effects. Microspheres are able to encapsulate proteins and peptide in the polymeric matrix while protecting them from enzymatic degradation. In this study Bovine Serum Albumin (BSA) matrix type microspheres were fabricated using Polylactide-co-glycolide (PLGA) by double emulsion solvent evaporation method. The effects of variables such as homogenizer speed, molecular weight of polymer and the effect of pH of the water phases, were investigated against factors such as drug loading, encapsulation efficiency, morphology, size, drug distribution and release profile of the microspheres. Results, suggested that an increase in homogenization speed leads to a decrease in microsphere size. The increase in homogenization speed also caused a significant effect on the release profile only when higher molecular weight of polymer had been used.. The pH change of the internal aqueous phase led to modification of surface morphology of spheres to a porous structure that significantly increased the total amount of released protein. Integrity of protein structure was intact as shown by SDS-PAGE. According to the results, it can be concluded that we achieved a reproducible method regarding controlled protein delivery for different sizes of particles.

Keywords: Microsphere. Polylactide-co-glycolide. Efficiency. Release profile. Bovine serum albumin.

INTRODUCTION

There are many limitations in conventional delivery of proteins and peptides for therapeutic purposes. Enzymatic degradation of proteins and absorption limitations in gastrointestinal system prevents oral use of proteins by conventional systems (Sinha, Trehan, 2003). As some of these molecules have very short biological half-life, multiple and frequent dosage is required to achieve the desirable therapeutic effects which in turn leads to an increase in the drug associated toxicity.

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Microspheres as free flowing powders (<200 μm) are capable of releasing the active drug continuously over extended period of time (Alagusundaram *et al*., 2009; Sinha, Trehan, 2003). Also, microspheres have the ability to encapsulate many drugs such as small molecules, nucleic acids and proteins (Kim, Pack, 2006). Due to these properties, microspheres can be used to overcome several problems associated with conventional drug formulation by reducing the number of doses required. Hence, reducing the adverse effect of drugs and improving the patient compliance.

It has been reported that microspheres are able to successfully deliver insulin because of their ability for prolonged sustained release (Björk, Edman, 1988). Moreover, microspheres have been used as a targeted

drug release system for delivering anti-cancer drugs to tumour site (Jameela *et al*., 1996; Wang *et al*., 1996; Salem *et al*., 2005). They also have been used in medical and/or biomedical imaging to deliver the contrasting agents (Shell, See, 1993; Klibanov, 1999; Sundaram, Wen 2014).

Several factors can influence the microsphere size, drug release rate and entrapment efficiency for proteins in the polymeric matrix (Yeo, Park, 2004; Degim, Çelebi, 2007). Firstly, type and molecular weight of polymer used in the formulation can determine the biodegradation and release of drug from the microsphere (Crotts, Park, 1998; Tracy *et al*., 1999; Kim, Pack, 2006). The method of microsphere preparation is another important factor influencing the drug release pattern.

In this study, a PLGA matrix type microsphere containing BSA as a model for protein drugs has been prepared using w/o/w double emulsion solvent evaporation method (Ogawa *et al*., 1988; Alonso *et al*., 1994; Boury *et al*., 1997).

PLGA is a synthetic polymer approved by FDA with proven biodegradability and biocompatibility that has been studied extensively for decades (Alonso *et al*., 1994; Kim, Pack, 2006; Makadia, Siegel, 2011).

For optimal formation of an emulsion, the use of emulsifiers such as poly vinyl alcohol (PVA) is required. Emulsifiers and polymer surfactants act by reducing the surface tension which result in formation of the stable emulsion (Sahoo *et al*., 2002). PVA is one of the most widely used emulsifiers because of its biocompatibility, limited toxicity without any adverse effect on protein loading or the size of microspheres.

Selection of an organic solvent is another important factor for a successful preparation of biodegradable protein microspheres. It has been shown that the extent of protein encapsulation in microspheres is dependent on the organic solvent used (Ravi *et al*., 2008). Dichloromethane has been widely used in preparation of microsphere with great success. Therefore it was used in our study as the organic solvent to dissolve the polymer and form the oil phase.

BSA has also been used as a model protein in this study, which can be later utilized for encapsulation of other proteins. Previously, it has been shown that encapsulation of BSA is independent of PLGA molecular weight (Blanco, Alonso, 1998). In this study we aimed to prepare microspheres on the basis of previous studies with emphasizes on high efficiency and loading capacity. The aimed microspheres must be capable of releasing most of the encapsulated drug in an intact form with a controlled manner and without significant burst release. Another goal was to prepare reproducible microspheres in which the protein is uniformly distributed. BSA is used as a model drug to apply and extend its results for all protein based medicines. The loading determination is a very important criterion for efficiency of microsphere preparation practically. In a large number of articles, the encapsulation efficiency is calculated but reported as loading by mistake.

Effect of variables such as homogenizer speed used for the second emulsification was also investigated in our study. Effects of molecular weight of polymer and changes of pH (that seems to be reported for the first time) on the morphology of microsphere and protein release from the polymeric matrix were also studied.

MATERIAL AND METHODS

PLGA (50:50, RG 502, Mw 14.1 and RG 503, MW 19.8 KDa) was obtained from Sigma Aldrich, Germany. BSA (MW = 66000) and PVA (MW = 13000-23000, 87-89% hydrolysed) were purchased from Sigma Aldrich Chemical Co. (Germany). Dichloromethane and all other solvents were analytical grade and obtained from Merck Company. Deionised water was used throughout the study.

Microsphere preparation

PLGA microspheres containing BSA were prepared by double emulsion method designed on the basis of our previous knowledge. Briefly, 60 mg of BSA was dissolved in 1 mL of deionised water as the internal aqueous phase (W1) that was emulsified into a solution of PLGA (0.3 g) in 5 mL of dichloromethane (oil phase) using homogenization at 12000 rpm for 2 min to form w/o emulsion. This primary emulsion was then added to the external aqueous phase consisted of 50 mL of 2% PVA aqueous solution (W2) under homogenization at 9000 or 19000 rpm for 5 min to form w/o/w double emulsion. The resultant emulsion was then transferred to a rotary evaporator (90 min, 50 rpm at RT, 400 mm Hg vacuum) for its dichloromethane to be evaporated.

Obtained spheres were isolated by centrifugation for three times at 1500 rpm for 10 min. Each time pellet was washed with deionised water. The microspheres were then dried in a desiccator at 4° C and stored for further experiments.

Three independent variables were considered in formulations at two levels and accordingly 6 different batches were designed. Independent variables consisted of polymer molecular weight (14.1 and 19.8 KDa), speed of homogenization in second emulsion preparation (9000 and 19000 rpm), and pH of aqueous phases (internal aqueous phase or both of internal and continuous aqueous phases). Effects of these formulation factors on the size, shape, drug distribution, loading efficiency and release of the microspheres were determined. Each formulation was made in triplicate and data were expressed as mean ± STD. Result of statistical analysis (Anova single factor or t-test) with $p<0.05$ was considered significant.

Total of 6 batches of microspheres (S1-S6) have been prepared in triplicate where, first 2 batches have been prepared using the lower molecular weight PLGA but at different homogenization speed, 3rd batch was prepared using pH alteration in the internal aqueous phase (W1), 4th batch was prepared using pH alterations in both the internal and continuous aqueous phase (W1 and W2), 5th and 6th batches were both prepared by the PLGA polymer with higher molecular weight using different homogenization speed.

Formulations related to pH alterations were made by lower molecular weight of polymer and lower speed of homogenisation (9000 rpm) in the second emulsification step.

In the 3rd set of formulations, pH of internal aqueous solution (containing BSA) was raised from 6 to 8.5 with addition of 0.5 mL 1 N NaOH solution in order to increase the solubility of BSA in internal aqueous phase (Machado *et al*., 2007). Theoretically, the higher the solubility of drug in internal aqueous phase of double emulsion the higher the level of drug encapsulation.

On the other hand the lower the solubility of drug in the external aqueous phase of double emulsion, the less the escape of drug from entrapment and therefore the higher the encapsulation efficiency. To verify this, in addition to adjustment of pH of internal aqueous phase to 8.5, pH of external aqueous phase was also decreased from 8 to 4.5 using diluted HCl, in the 4th set of experiments.

Characterisation of microspheres

Yield of preparation, efficiency of encapsulation, drug loading, Size distribution, particle morphology, drug distribution, drug integrity and drug release were evaluated *in vitro*.

Yield of microsphere preparation in percentage, was calculated by dividing the mass of dry sphere powder by the total mass of polymer and protein initially used and multiplying this figure by 100.

For the encapsulation efficiency, 10 mg of dried microspheres was dissolved in 2 mL of 1 N NaOH (Jeffery, Davis, O'Hagan, 1993) and pH was adjusted to 7.4 by diluted HCl. UV absorption was measured at 278.5 nm (the peak wavelength) using the UV/Vis spectrophotometer (Cecil-7350, England) (Ravindran *et al*., 2010; Shiri *et al*., 2018). Based on the calibration curve of BSA ($R^2 = 0.9854$), corresponding amount of protein was calculated. Three samples for each measurement with repeated readings for each sample were employed. Total amount of protein found in dry spheres was divided by the initial amount of protein used multiplied by 100 to calculate the efficiency of encapsulation.

Total amount of encapsulated drug found in 100 mg of dry microsphere powder was also calculated as drug loading. In many articles the value reported as "loading" actually means "efficiency of encapsulation". Here we reported both of these values.

The particle size of microspheres was determined using Mastersizer 3000 laser particle size analyser (Malvern, UK) equipment. Particle size was reported as volume mean diameter.

To study the surface morphology of microspheres, they were imaged by the scanning electron microscope (SEM) (Leo-906, Germany). Confocal laser scanning microscope (CLSM) was also employed to study drug distribution in microspheres (Nikon Ins., Japan) using BSA fluorescence properties (Yang, Chung, Ng, 2001).

In vitro release studies

Amount of BSA released was determined using UV spectrophotometer. 20 mg of microspheres was dispersed in 10 mL of freshly made Phosphate Buffered Saline (PBS, pH 7.4) and placed in shaker incubator (Heidolph, Unimax 1010 + Incubator, Germany) at 37 ºC and at 100 rpm. 1 mL of sample was collected at predetermined intervals and 1 mL of PBS was added to solution after each sampling. Concentration of BSA was obtained using UV absorption of supernatant. Percentage of cumulative concentration of BSA was determined and graphed.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the primary structure of BSA protein (Determan *et al*., 2004) released from all batches of sphere preparation.

RESULTS AND DISCUSSION

Yield, encapsulation efficiency and drug loading

Results of the preparation yield, encapsulation efficiency, size, drug loading and release data in different formulations are reflected in Table I. Changes of pH in both aqueous phases, unlike our expectation, resulted in the lowest encapsulation efficiency and drug loading. Other formulations had similar results. In fact all batches (except for S4) had relatively high amount of drug loading.

The encapsulation efficiency has not changed ignificantly neither by increasing the homogenization speed nor by an increase in the molecular weight of PLGA.

TABLE I - Characteristics of microspheres in different formulations. Mean ± STD (n≥3)

Series	Polymer MW	Homogenizer Speed (2nd emulsion)	pH change	Yield %	Efficiency %	Loading Capacity%	Size µ	Release 1st hour	Cumulative release 120th hour
S1	14100	9000	N*	65.9 ± 2.1	51 ± 10	12.5 ± 2.5	13.6 ± 5	4.8 ± 1	47.7 ± 9
S2	14100	19000	N	76.6 ± 6.7	58 ± 9	12.6 ± 2.5	6.6 ± 0.9	6.2 ± 1	52.7 ± 1.3
S3	14100	9000	Y** -1 phase	75 ± 0.7	45 ± 4	9.9 ± 0.5	20 ± 1	5 ± 0.3	60.2 ± 1.3
S4	14100	9000	Y -2 phases	50 ± 0.1	25 ± 4	8.5 ± 1	-	1.2 ± 0.3	20.9 ± 1.3
S5	19800	9000	N	67 ± 2.7	44 ± 5	10.9 ± 1	10 ± 0.8	4.9 ± 1.8	51.9 ± 4.6
S6	19800	19000	N	71.8 ± 1.7	47 ± 7	10.9 ± 2	5.63 ± 1	7.2 ± 2	78.8 ± 19

*No (Not applied)
**Yes (Applied).

Particle size

Result of particle size measurement (as the volume mean diameter) in different batches is shown in Figure 1. It is concluded that an increase in homogenization speed during second emulsification, reduced the particle size that is in agreement with the other studies (Yang, Chung, Ng, 2001). Statistically there was a significant difference between particle sizes of S1 and S2 formulations ($p<0.05$) as well as S5 and S6 ($p<0.001$) where the homogenization speed was increased. Any change in the pH of internal aqueous phase (S3) was resulted in the formation of larger microspheres with an insignificant difference compared to S1.

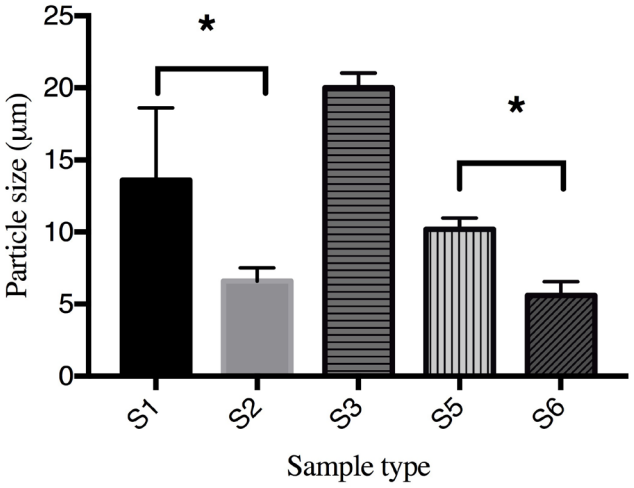

FIGURE 1 - Mean volumetric particle size measured by Mastersizer. Mean ± STD, n≥3.
* Represents a statistical significance after t-test. P< 0.05 for S1 & S2, P< 0.001 for S5 & S6.

Morphology and drug distribution study

Representative SEM of microspheres surface morphology has been shown in Figure 2. SEM images of our BSA loaded microspheres revealed that most of our microspheres were spherical in shape with a smooth surface. There was no apparent morphological differences in microsphere prepared by different variables, except for the S3 microspheres (image C in Figure 2) that exhibited porosity on their surfaces caused by change in pH of the internal aqueous phase, which could affect the release of drug. Moreover, the S4 preparation resulted in low amount of microsphere due to formation of an unstable emulsion (image not shown).

Confocal laser scanning microscope equipped with different filters was used to observe the fluorescence light emitted by encapsulated BSA. It revealed the matrix type structure of spheres marked by thorough distribution of BSA as seen in Figure 3.

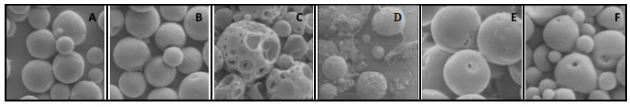

FIGURE 2 - Scanning electron micrographs of microspheres surface morphology. Where A=S1, B=S2, C=S3, D=S5, E, F=S6 at magnification of X5000.

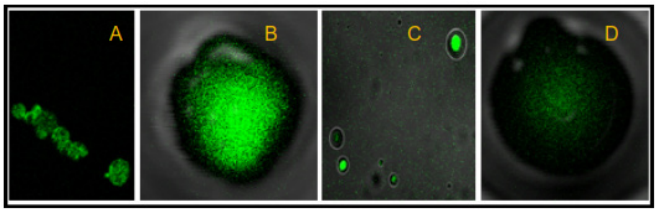

FIGURE 3 - CLSM images of microspheres representing BSA matrix type distribution inside the particles in different batches. A=S1, B=S2, C=S3, D=S5.

In vitro release

Percentage of cumulative release of BSA is shown in Figure 4. With lower molecular weight of PLGA there was no significant difference ($p<0.05$) between the release rate of S1 and S2 microspheres. However there was a significant increase in the release rate of S5 compared to S6 batch ($p<0.05$) because of higher speed of homogenization in the second emulsification step.

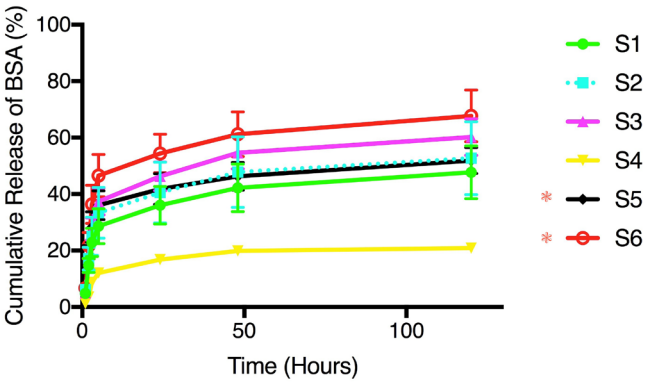

FIGURE 4 - Percentage of cumulative concentration of BSA released over time. Each point represents the average of at least 3 readings for each replicate. * represents a statistically significant difference where p value is <0.005.

It appears that in formulations with higher molecular weight of PLGA, the initial release of BSA is higher. There was a significant rise in release rate of S6 in comparison to S2 ($p<0.05$), where higher molecular weight of PLGA was used. Although S5 had a higher release rate compared to S1 batch, this difference was not statistically significant, however.

Change of pH in the internal aqueous phase (S3) to an alkaline pH, increased the release rate of BSA significantly compared to S1 batch ($p<0.01$). This effect might be related to high porosity of the surface

of microspheres that facilitated drug release. However, change of pH of both internal and continuous phase resulted in a catastrophic decrease ($p<0.001$) in BSA release rate (compared to S1 batch), which, could be explained by the low encapsulation efficiency and low amount of loaded drug inside microspheres. Percentage of cumulative release after 120 hours for S1 formulation was almost 48% versus 21% in S4 batch.

The highest overall release of BSA was about 79% obtained in S6 microspheres. This batch was made using PLGA with higher MW and contained lower particle size as a result of higher homogenization speed used.

SDS-PAGE

To investigate the integrity of loaded protein the SDS-PAGE was employed (Figure 5). Results of SDS-PAGE of the BSA released from spheres after 120 hours showed that there was no change in structure and molecular weight of BSA in any formulations imposed by encapsulation stages and the release period.

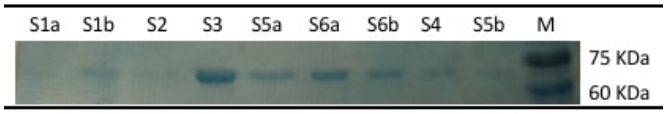

FIGURE 5 - SDS-PAGE of BSA released from microspheres after 5 days in different batches (S1-S6).

CONCLUSION

Process design (based on our knowledge) was accomplished to produce microspheres containing high amount of encapsulated protein that could release its functional drug in a continuous pattern. Variables such as ratio of second aqueous phase to oil phase, ratio of polymer to drug, amount of inner water phase and concentration of PVA, were of the parameters precisely selected for the preparation procedure. For example, we used a small volume of water (1 mL) as inner aqueous phase to improve encapsulation as reported by Parikh too (Parikh *et al*., 2003).

The aim of this study was to determine the effects of polymer molecular weight, pH and homogenization speed, as the investigational variables on different characteristics of microspheres such as size, surface, efficiency of encapsulation, drug loading and release properties of microspheres in order to make reproducible and efficient protein loaded particles.

There was no statistically significant difference between the yield or encapsulation efficiency among produced different batches. This is in agreement with other studies emphasizing that process parameters like the ratio of second aqueous phase to oil phase and concentrations of PLGA and drug could play a more important role on the encapsulation efficiency (Feczkó *et al*., 2011).

S3 batches were also resulted in high yield, but this might be due to the higher size of microspheres produced. Efficiency and drug loading were the same as batches without the pH change. Variation in the pH of both internal and continuous phases produced the lowest yield and encapsulation efficiency unexpectedly. Theoretically by shifting the pH of internal aqueous phase to a pH in which solubility of drug increases, drug entrapment is expected to increase. Entrapment would be expected to increase, if pH of external aqueous phase is also changed to a pH in which the drug shows less solubility. Preventing the escape of the drug could lead to higher amount of drug entrapment inside spheres (Giri *et al*., 2013). However in practice lower drug loading and encapsulation were observed. According to the literature within the pH ranges of our work, BSA is stable and negatively charged (isoelectric pH of BSA = 4.5-4.9) (Kondo, Oku, Higashitani, 1991). Therefore, formation of an unstable emulsion in the S4 batches might explain the low amount of encapsulation. Although decrease in the loading and efficiency in S3 batches was insignificant, but further investigation is needed to clarify the possible causes.

The SEM images revealed the smooth and non-porous surface of S1, S2, S5 and S6 batches. The change in homogenization speed and also molecular weight of PLGA polymer did not affect the surface morphology while change in pH resulted in porous microspheres.

Perhaps the most important effect of pH was the morphologic change of sphere from a smooth surface to a porous surface in S3. This might be responsible for the increase in released drug after 120 hrs in comparison with S1 batches. Another effect of pH change in S3 series, is the production of larger particles (20 ± 1 μ) compared to S1 (13.6 ± 5 μ) that might contribute to the increase in cumulative amount of released BSA.

Within the range of 14.1 to 19.8 KDa any increase in the molecular weight showed a slight increase on the release profile that was insignificant when lower speed of homogenisation was used. This is in agreement with a

number of studies. As reported that in the low molecular weight polymers (below 20-23 KDa) a relatively slow and constant release profile was observed after initial burst stage (Freiberg, Zhu, 2004). We also noted that, regardless of the molecular weight of PLGA polymer, the particle size reduced significantly with an increase in homogenization speed. It was observed that the higher homogenization speed resulted in an increase in the release rate of protein from microspheres. One can conclude that larger surface area of smaller particles may lead to location of higher amount of drug near the surface which could be released rapidly after contact with dissolution medium. With higher molecular weight of polymer, the amount of cumulative release after 120 h was significantly increased by an increase in homogenization speed.

Changes of pH of internal and external phases (S4) resulted in a catastrophic decrease in BSA release rate, which could be explained by the low yield and encapsulation efficiency of this type of microsphere as a result of the unstable emulsion that was formed.

One of the major disadvantages of microspheres is the release of high proportion of the encapsulated drug during the first stage of release. This phenomenon is known as "burst effect" (Yang, Washington, 2006; Fu *et al*., 2003). In fact immediately after exposure to dissolution medium, large amount of drug located on the surface of particles is released. The initial high release rate could either lead to toxic concentration level of drug or its shorter half-life (Huang, Brazel, 2001). In our study, percentage of cumulative release of BSA during the 1st hour ranged from about 1.2% to 7.4%w/v in different series. Percentages of cumulative release after the first three hours were from 23% for 1st batch to 28% for the 5th batch utmost. Surface adsorption and porosity of spheres are of the main reasons of burst release. Displacement of drug during isolation, drying and storage of spheres are other reasons for burst release (Kishida *et al*., 1998). In a number of studies initial burst release of BSA from PLGA microspheres were up to 60% (Wang *et al*., 1991). Uniform drug distribution inside the matrix systems and increase in molecular weight of PLGA may prevent the burst phenomenon (Cohen *et al*., 1991; Huang, Brazel, 2001).

Cumulative amount of drug, which was released from our particles during the period of 5 days, was quite satisfactory. The released protein was intact as shown by SDS-PAGE. CLSM images showed thorough distribution of drug in microspheres confirming matrix type structure of carrier particles.

In conclusion, relatively high encapsulation efficiency, high drug loading, retention of BSA stability and suitable release profile with low amount of burst release, are of major achievements of our study to be considered in a protein delivery system, however it needs further evaluations *in vivo*.

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