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Original Article

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DEVELOPMENT AND *IN VITRO* EVALUATION OF PROTEIN LOADED PLGA MICROSPHERES

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ABSTRACT

Protein drug delivery has emerged to be an important area of research in the field of novel drug delivery technology. The objective of the study was to prepare poly (D, L-lactide-co-glycolide) (PLGA) microspheres containing bovine serum albumin (BSA) as a model drug and to evaluate the various physicochemical characteristics of the formulations, namely microspheres' morphology, particle size, zeta potential, BSA encapsulation efficiency and *in-vitro* BSA release profile. BSA-loaded microspheres were prepared by double emulsion solvent evaporation method with different BSA: PLGA ratios and at different speeds of homogenization keeping the amount of BSA constant in all the formulations. Accelerated stability testing was performed with the optimized formulations for a period of eight weeks. The mean particle size and encapsulation efficiency of the microspheres were found to decrease as the speed of homogenization increased. And the same were found to increase simultaneously with increase in the amount of polymer. The zeta potential values confirmed stability of the formulations in colloidal condition. The *in vitro* release study showed a slow and steady release pattern of BSA. Thus, a sustained release formulation of protein loaded PLGA microspheres was developed.

Keywords: Bovine serum albumin, poly (D, L-lactide-co-glycolide), microspheres, novel drug delivery.

INTRODUCTION

Protein drug delivery has occupied an eminent area in the field of pharmaceutical research. With the advent of r- DNA technology, various protein and peptide drugs are now commercially available (1, 2). However, administration of such drugs poses a lot of problem. Oral administration of proteins is not feasible owing to inherent instability in the GIT and low permeability across biological membranes due to high molecular weight and polar surface characteristics, which imply that proteins required for systemic treatment should

be administered via parenteral route (3 - 6). However, frequent administration of proteins through parenteral route to keep protein drug at minimum effective concentration is cumbersome, expensive and non patient compliant. Therefore, development of sustained release parenteral dosage forms is necessary to circumvent these problems (7, 8).

Biodegradable polymeric matrices like microspheres and nanospheres have emerged to be promising for delivery of protein over a prolonged period of time. In this regard poly (D, L-lactide-co- glycolide) is widely used because it is fully biodegradable and biocompatible (9-12). Drug release from such dosage forms is an important criteria and it depends largely on the physical properties of the polymer such as molecular weight, hydrophillicity and ratio of lactide to glycolide (2, 13, 14).

In the present investigation, the objective was to prepare poly (D,L- lactide- co - glycolide) microspheres with bovine serum albumin as a model protein at three different speeds of homogenization and at three different ratios of protein and polymer and to characterize the microspheres for surface morphology, particle size, zeta potential, encapsulation efficiency , *in vitro* release study and stability study. The effect of two process variables namely protein: polymer ratio and speed of homogenization on encapsulation efficiency have been evaluated using a 3^2 factorial design (15).

Experimental

Materials

Bovine serum albumin (BSA) and poly (D, L- lactide- co- glycolide) (85:15) were purchased from Sigma Aldrich Chemicals Ltd., India. Polyvinyl alcohol was obtained from S.D. Fine Chemicals Ltd., India. Dichloromethane (DCM) was purchased from E. Merck Ltd., India. All other reagents used were of analytical grade.

Methods

Preparation of microspheres

Specified amount of PLGA was dissolved in dicloromethane and 2.5% w/v polyvinyl alcohol solution containing 5 mg BSA was homogenized for 4 min at specified speed. The above mixture was added drop wise to 75 ml of 1.5% w/v polyvinyl alcohol in 400 ml of glass tube and homogenized for 6 minutes at the same speed to produce w/o/w emulsion. The emulsion was then stirred on a magnetic stirrer for 3 hours to evaporate dichloromethane. The microspheres were then washed for 3 times using double distilled water by centrifugation at specified rpm for 40 minutes. Samples were frozen for 2 to 3 hours at -20^0C and freeze dried for 48 hours in 10 ml freeze drying vial (initial self temperature was -15^0C and it was reduced to -40^0C and kept for 48 hours). Self

temperature was then elevated to 15^{0}C. Samples were then removed and lyophilised microspheres were obtained.

Batches of microspheres were depending on BSA: PLGA ratio and speed of homogenization. Formulation codes are given in Table 1.

Table 1. Composition of BSA loaded microspheres

Formulation code	Speed of homogenization (rpm)	BSA: PLGA
S1	6000	1:50
S2	6000	1:60
S3	6000	1:70
S4	8000	1:50
S5	8000	1:60
S6	8000	1:70
S7	10,000	1:50
S8	10,000	1:60
S9	10,000	1:70

BSA-excipient interaction study

Pure bovine serum albumin (BSA), mixture of BSA and excipients and excipients alone, were mixed with infra-red grade potassium bromide and compressed into a pellet by applying 5.5 metric ton of pressure. They were scanned over a wave number range of 4000 to 400 cm^{-1} in an FT-IR instrument (Magna IR series II, Nicolet, USA).

Study of particle morphology

The external morphology of the microspheres was analysed by Scanning Electron Microscopy (SEM). The microspheres were placed on the metal stubs, coated with gold and examined under a scanning electron microscope (JEOL, JSM 5200, Tokyo Japan).

Particle size distribution analysis and zeta potential study

Particle size distribution and zeta potential were studied by Zetasizer nano ZS using DTS software (Malvern Instrument Limited, Malvern, UK). The microspheres were reconstituted (5 mg/ ml) by phosphate buffer saline solution, 0.1 M, pH 7.2 and then introduced into the instrument.

Determination of protein encapsulation efficiency

About 5 mg of the protein loaded microspheres were taken in an eppendorff and 1 ml of 5% SDS- NaOH solution was added to it and incubated at 37 ± 0.5 ^{0}C on a shaker till a clear solution was obtained. The supernatant was obtained by centrifugation and the amount of protein loaded was assayed using Bio-Rad Protein Assay Kit.

In-vitro release study

In vitro release study was conducted in saline phosphate buffer solution, pH 7.2, 0.1 M. Several accurately weighed 5 mg BSA loaded micropheres were dispersed in 1ml of 0.1 M phosphate buffer saline solution (pH 7.2) in eppendorffs. They were incubated at 37± 0.5^0 C on a shaker for a period up to 50 days. At predetermined intervals, the supernatants were

collected by centrifugation and the amount of BSA released was determined by Bio-Rad Protein Assay Kit.

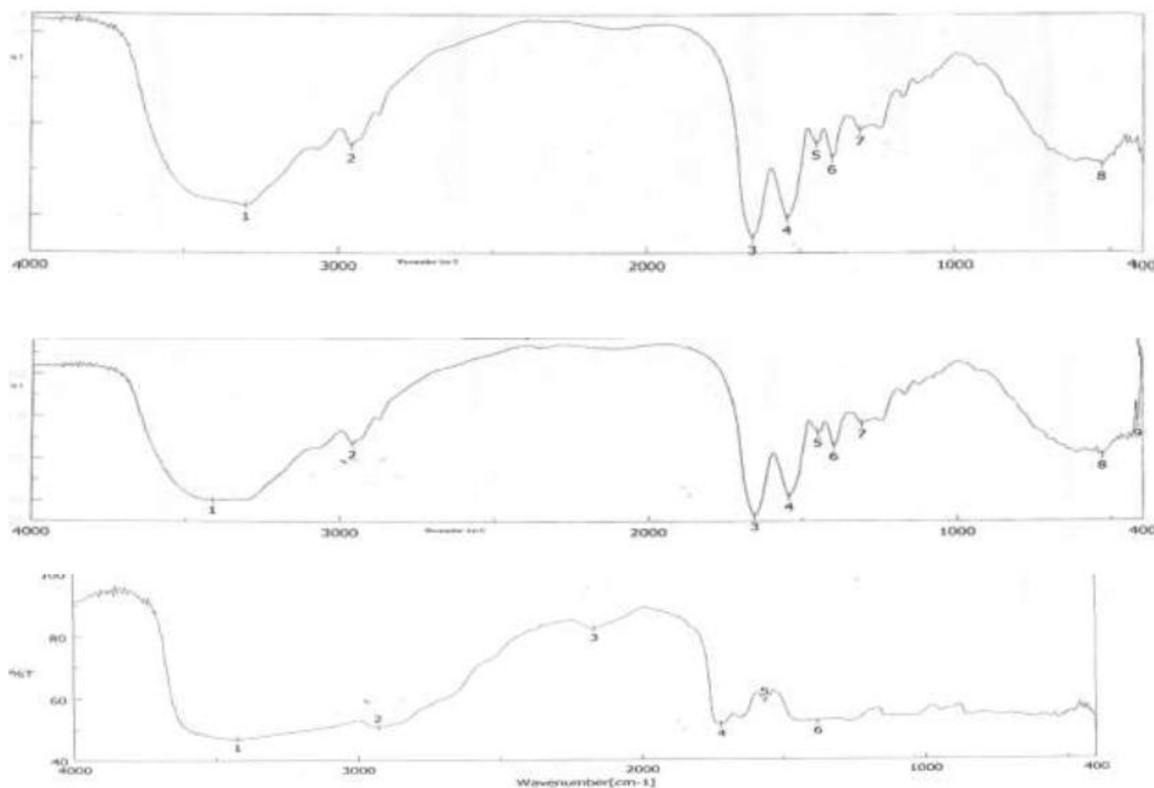
Stability study

Stability study was performed by incubating lyophillised microspheres at four different temperatures of 4^0C, 25^0C, 45^0C and 65^0C, respectively for 12 weeks. They were then analysed by FT-IR spectroscopy. Spectroscopic data of the stored samples were compared with those from the freshly prepared samples.

Results and discussion

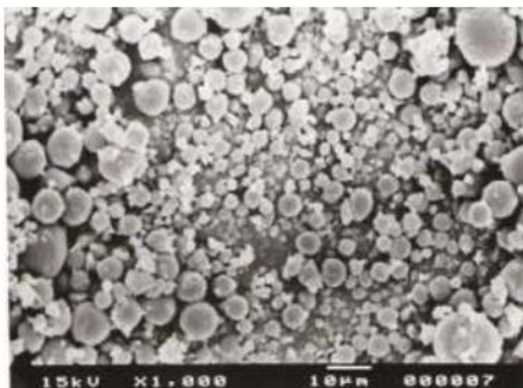
Drug-excipient interaction is one of the most important characteristics that regulate the availability of drug from a formulation, its release pattern and its stability in the formulation. The FT-IR spectrum can accurately determine drug-excipient interactions at various functional groups between the drug and excipient molecules. FT-IR spectra of BSA, a mixture of BSA and excipients and excipients only, are displayed in Figures 1a, b and c, respectively. The drug-excipient interaction study revealed that there was no significant interaction between BSA and the other excipients used in the formulation. Hence, the polymer could be used to formulate the microspheres.

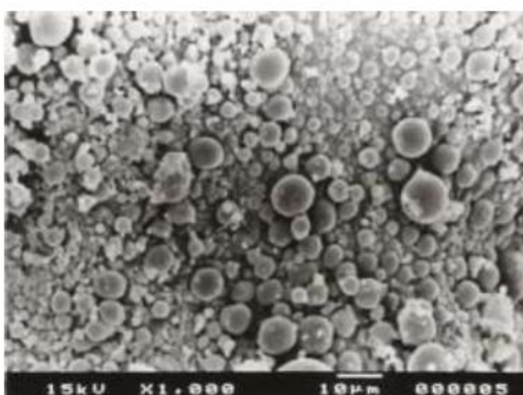
Figure 1. FT-IR spectra of (a) bovine serum albumin, (b) bovine serum albumin and excipients (c) excipients only

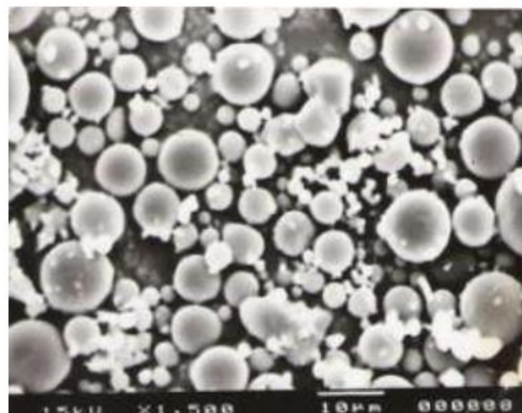


SEM is helpful to examine surface characteristics of microspheres. The photographs of formulations S7, S8 and S9 are shown in figures 2(a), (b) and (c). They reveal that surface of the microspheres was smooth and spherical and a major portion of the microspheres were less than 10 µm. It is observed that increase in speed of homogenization led to decrease in particle size.

Figure 2. SEM photographs of formulations (a) S7, (b) S8 and (c) S9.







Present study was conducted with PLGA (85:15) than the more commonly used PLGA (50:50) variety. Lactic acid is more hydrophobic than glycolic acid and hence lactide-rich PLGA (85:15) is less hydrophilic, absorbs less water, and subsequently degrade more slowly than PLGA (50:50) (17). PLGA polymer containing lactic and glycolic acids in 85:15 ratio is hydrolyzed at a much slower rate than the PLGA (50:50) containing equal proportion of lactide and glycolide. Higher molecular weight of PLGA (85:15) than PLGA (50:50) due to the presence of higher lactide chain lengths enhances the hydrophobicity and decreases the degradation of the polymers and rate of protein release. In the present study, the microspheres with larger particle sizes and with increased polydispersity indices showed slower release of BSA and it might be due to longer diffusion pathways of BSA in larger particles. However, more content of PLGA (85:15) showed the slowest BSA release in S9 among

the formulations studied and it might be due to the enhancement of hydrophobicity. Among the prepared formulations, S8 was found to exhibit a sustained release pattern of drug delivery.

Data of particle size analyses are presented in Table 2 showing the values of particle size and polydispersity indices. Particle size was influenced by two parameters: protein: polymer ratio and speed of homogenization. The average particle size of the microspheres decreased with increase in speed of homogenization and with decrease in protein: polymer ratio. The particle size distribution curve of the formulation, S8 is displayed in Figure 3, which shows unimodal size distribution. Narrow size distribution may be because of the lower viscosity of the external phase, which offered less resistance to the spheres formed. It is also observed that almost all the formulations have polydispersity indices less than 0.20. Formulation S8 was found to have a polydispersity index of 0.004 ± 0.001, hence this formulation was found to have the narrowest particle size distribution among the prepared formulations. There was a clear distribution of both the small and large particles and they were not conglomerated.

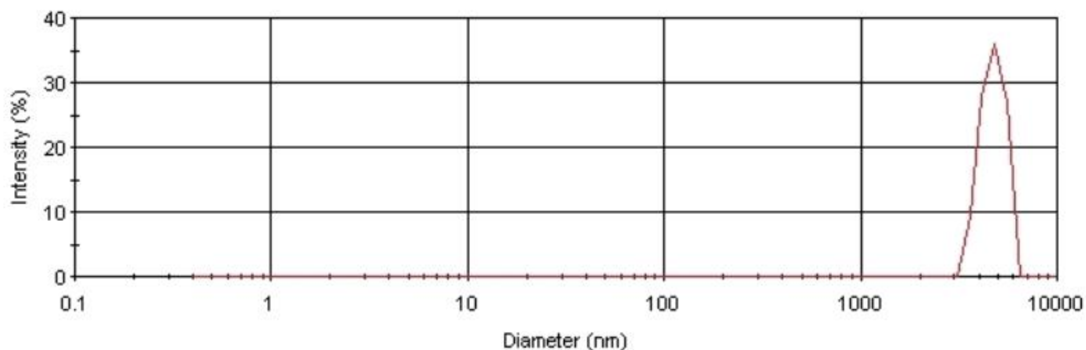
Table 2. Values of particle size, polydispersity indices and zeta potential of the miceospheres. Data represent mean ± SD

Formulation code	Particle size ± SD (µm)	Polydispersity index ± SD	Zeta potential ± SD (mV)
S1	7.95 ± 0.95	0.286 ± 0.01	- 4.67 ± 0.32
S2	9.18 ± 0.88	0.050 ± 0.007	- 4.98 ± 0.45
S3	12.65 ± 1.05	0.013 ± 0.009	-5.12 ± 0.69
S4	5.12 ± 0.77	0.04 ± 0.005	-3.85 ± 0.88
S5	6.78 ± 0.86	0.089 ± 0.008	- 4.72 ± 0.35
S6	8.26 ± 0.95	0.125 ± 0.009	-5.54 ± 0.47
S7	2.70 ± 0.65	0.191 ± 0.01	- 4.30 ± 0.65
S8	3.46 ± 0.88	0.004 ± 0.001	-5.16 ± 0.95
S9	4.42 ± 0.93	0.035 ± 0.005	-5.77 ± 0.29

Table 3. Values of encapsulation efficiency of microspheres. Data represent mean ± SD

Formulation code	Encapsulation Efficiency ± SD (%)
S1	79.62 ± 0.54
S2	88.72 ± 0.52
S3	96.91 ± 0.85
S4	62.35 ± 1.28
S5	79.22 ± 0.96
S6	85.65 ± 1.67
S7	48.06 ± 0.87
S8	59.65 ± 1.01
S9	77.88 ± 0.95

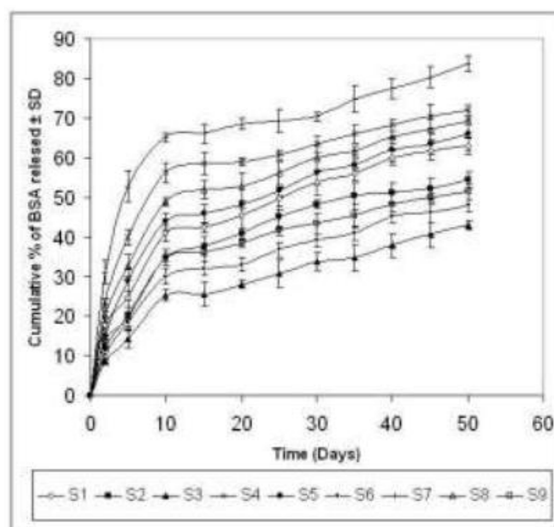
Figure 3. Particle size distribution plot of S8



Zeta potential is an important physicochemical parameter of the microspheres that influences the physical stability of the microspheres. Table 2 depicts the values of zeta potential of the respective formulations and it is observed that the values ranged from -3.85 to -5.77 mV. Particles with zeta potentials more positive than +30 mV and more negative than −30 mV are normally considered stable for colloidal dispersion [18]. From the data it is evident that all the formulations are unstable in the colloidal state. This suggests that the particles should not be stored in a liquid suspension form and rather they should be stored in a lyophilized state. Immediately before the administration they should be reconstituted.

Encapsulation efficiencies of the formulations are reported in Table 3. It was also dependent on two variables: BSA: PLGA ratio and speed of homogenization. It was observed that with increase in speed of homogenization, the loading capacity of the formulations decreased. This may be because increased homogenizer speed produced smaller particles. But with increase in amount of polymer, the capacity increased since more amount of polymer can entrap more amount of protein as expected.

The *in vitro* BSA release study was conducted in saline phosphate buffer solution, 0.1 M, pH 7.2. Figure 4 depicts the plot of the release study. From the figure it is evident that with an increase in speed of homogenization, there was an increase in cumulative amount of BSA released. But as amount of polymer was increased, keeping amount of BSA and speed of homogenization constant, there was a decrease in cumulative amount of BSA released. For all formulations, initial burst effect is observed which may be due to release of loosely bound or surface associated protein. BSA: PLGA ratio and speed of homogenization had a significant influence on protein release; higher ratios retarded protein release whereas increased homogenizer speed enhanced amount of drug release. Figure 4. Plot showing the *in vitro* release of cumulative percentage of BSA. Datashows mean ± SD, n=6.



FT-IR spectra of microspheres stored at 4^0C, 25^0C, 45^0C and 65^0C, respectively for 12 weeks are displayed in figures 5 (a – d). From the spectra it is evident that there was no significant change in the spectra of the formulations stored at different temperatures and hence it can be inferred that BSA loaded PLGA microspheres were stable up to 65^0C.

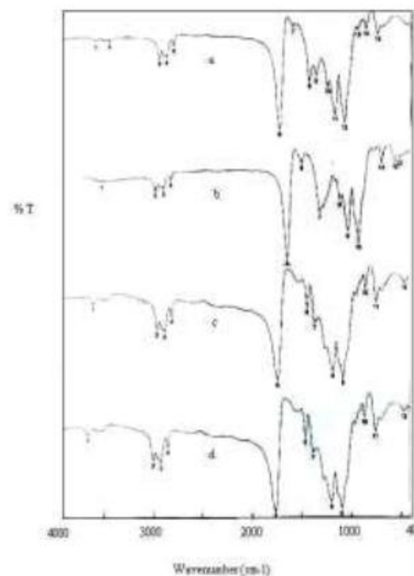


Figure 5 (a). FTIR of microspheres stored at 4^0C (b) FTIR of microspheres stored at 25^0C (c) FTIR of microspheres stored at 45^0C (d) FTIR of microspheres stored at 65^0C.

Conclusion

The above study shows that the microspheres prepared with the polymer PLGA (85:15) with a protein: polymer ratio of 1:70 at a speed of 10,000 rpm, were not only stable at different temperatures ranging from 4^0 – 65 ^{0}C but they also exhibited desirable physicochemical characteristics. Hence, it may be considered as the best among the prepared formulations, for further investigation. It is thus evident from the above results that PLGA microspheres prepared with BSA as a model drug could effectively provide sustained release effect.

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