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Research Article

EFFECT OF PROCESSING VARIABLES IN FORMULATION AND DEVELOPMENT OF BIODEGRADABLE MICROPARTICLES

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ABSTRACT

The aim of the present work was to investigate the preparation of microspheres as potential drug carriers for proteins, intended for controlled release formulation. The hydrophilic Bovine Serum Albumin (BSA) was chosen as a model protein to be encapsulated within two different grades of poly (D, L-lactide-co-glycolide) (50:50) in three different concentrations of 5, 10, 15 % W/V for each. Microspheres were prepared using a w/o/w double emulsion solvent evaporation method. Different parameters influencing the molecular weight, particle size, entrapment efficiency and *in vitro* release profiles were investigated. The microspheres prepared with different concentration and hydrophilicity of poly(D,L-lactide-co-glycolide) polymers were non porous, smooth surfaced and spherical in structure under scanning electron microscope with a mean particle size ranging from 34.58 to 97.56 µm. The protein loading efficiency determined by Hartree lowrey's protein detection method was found varied from 40.25 to 71.54 % of the theoretical amount incorporated. The *in vitro* release profile of bovine serum albumin from microspheres presented two phases, initial burst release phase due to the protein adsorbed on the microsphere surface, followed by slower and continuous release phase corresponding to the protein entrapped in polymer matrix. The release rate was fairly constant after an initial burst release. Consequently, these microspheres can be proposed as controlled release protein delivery system.

Keywords: Bovine serum albumin, Lowrey's protein detection method, Microspheres, Poly (D,L-lactide-co-glycolide), w/o/w double emulsion.

INTRODUCTION

Proteins/peptides are bioactive compounds that are poorly stable in biological fluids and unable to pass across phospholipids membrane. To be active and used in therapeutics and immunology, it is necessary to administer large doses of these compounds and to repeat frequently drug administration. The development of polymeric biodegradable delivery systems has allowed to protect these molecules against degradation and to allow their delivery by mucosal sites or in a prolonged fashion. These molecules can be entrapped within implantable or particulate systems made of hydrophobic or hydrophilic polymers. Applications using polymers such as Poly (methilidene malonate), poly (lactide-co-glycolide) and pectins will be described after an overview of the major issues concerning pharmacokinetics and biopharmaceutics of these molecules[1].

In order to avoid the inconvenient surgical insertion of large implants, injectable biodegradable and biocompatible polymeric particles (microspheres, microcapsules, nanocapsules, nanospheres) could be employed for controlled-release dosage forms[2]. Microparticles of size less than 250 micron, ideally less than 125 micron is suitable for this purpose[3]. Biodegradable polymers are natural or synthetic in origin and are degraded in vivo, either enzymatically or non-enzymatically or both to produce biocompatible, toxicologically safe by-products which are further eliminated by the normal metabolic pathways[4]. Drugs formulated in polymeric devices are released either by difusion through the polymer barrier, or by erosion of the polymer material, or by a combination of both difusion and erosion mechanisms[5]. The polymers selected for the parenteral administration must meet several requirements like biocompatibility, drug compatibility, suitable biodegradation kinetics and mechanical properties, and ease of processing[5-6].

A wide variety of natural and synthetic biodegradablepolymers have been investigated for drug targeting or prolonged drug release. However, only a few of them are actually biocompatible. Natural biodegradable polymers like bovine serum albumin (BSA), human serum albumin (HSA), collagen, gelatin, and hemoglobin have been studied for drug delivery[2]. The use of these natural polymers is limited due to their higher costs and questionable purity[2].Since the last two decades, synthetic biodegradable polymers have been increasingly used to deliver drugs, since they are free from most of the problems associated with the natural polymers. Poly (amides), poly (amino acids), poly(alkyl-a-cyano acrylates), poly(esters), poly(orthoesters), poly(urethanes), and poly (acrylamides)have been used to prepare various drug loaded devices. Amongst them, the thermoplastic aliphaticpoly(esters) like PLA, PGA, and especially PLGA have generated tremendous interest due to their excellent biocompatibilityand biodegradability[2-8].The discovery and the synthetic work on low molecularweight oligomeric forms of lactide and/or glycolide polymers was first carried out several decades ago[4-6].The methods to synthesize high molecular weights of these polymers were first reported by Lowery[4]. During the late 1960s and early 1970s a number of groups had published pioneering work on the utility of these polymers to make sutures/fibers. These fibers had several advantages like good mechanical properties, low immunogenicity and toxicity, excellent biocompatibility, and predictable biodegradation kinetics[3,4-6]. The wide acceptance of the lactide/glycolide polymers as suture materials, made them an attractive candidate for biomedical applications like ligament reconstruction, tracheal replacement, surgical dressings, vascular grafts, nerve, dental, and fracture repairs[4-6].

MATERIALS AND METHODS

Bovine serum albumin (BSA, MW 66 430 Da) and sodium azide were purchased from Sigma (St. Louis, USA). PLGA (50:50) polymers, PURASORB polymers PDLG 5002 and PDLG 5002A were gifted exclusively by PURAC BIOMATERIALS, The Netherland. Polyvinyl alcohol (PVA) (MW 70,000 Da), mannitol, Potassium dihydrogen phosphate, di sodium hydrogen phosphate, sodium dodecyl sulphate (SDS), sodium hydroxide, Tween 80 and Dichloromethane (DCM), isopropyl alcohol (IPA), tetrahydrofuran (THF), Acetone and Ethyl acetate were purchased from S. D. fine Chemicals, Mumbai. All the solvents used were of analytical grade and the materials were used as received.

PLGA purification method

PLGA 50:50 copolymer was purified prior to use. The polymer was dissolved in tetrahydrofuran (THF) (5 g/dL) and the solution was slowly added to isopropyl alcohol (IPA) with continuous stirring. A volume ratio of 100mL of polymer solution to 500mL of IPA was not exceeded. The fibrous precipitate was then vacuum dried at room temperature for 48 h to remove solvent prior to use. The molecular weight distribution of the material before and after purification was measured using Gel permeation chromatography (GPC). No significant differences were found before or after purification[9].

Microspheres preparation of BSA loaded PLGA

BSA loaded PLGA microspheres were prepared by a double emulsion solvent evaporation technique as previously described by Rajeev A. Jain[10].and conveniently modified. Briefly, PLGA was dissolved in 2.25 ml dichloromethane and emulsified with 10 mg BSA in 0.22 ml aqueous solution(PBS) using a homogenizer (REMI, INDIA) at a speed of 5000 rpm for 1 min to form a primary emulsion (w/o). The polymer concentrations in organic phase were 5, 10, and 15 % w/v. This primary emulsion was rapidly transferred into 25 ml of aqueous solution containing 0.5 % w/v of PVA as an emulsifier and homogenized for 0.5 min at the half speed of the primary emulsion to produce a double (w/o/w) emulsion in the ratio of 1:10:100 by volumes. The resultant double emulsion was magnetically stirred for 4 h at ambient room temperature at 250 rpm to evaporate dichloromethane. The hardened microspheres were isolated by centrifugation at 7000 rpm for 3 min (REMI, INDIA) and washed thrice using distilled water. Mannitol (1% w/v) was added before lyophilization (Cyberlab, USA) to prevent aggregation of microspheres. The microspheres were stored at 4ºC until the time of evaluation.

Effect of formulation and processing variables

The effect of the following formulation and process variables on the characterization of microspheres was investigated. The effect of different speeds of magnetic stirrer (50, 150, 250 rpm) and homogenizer (1500, 3000, 6000 rpm) were studied. The effect of time of homogenization (0.5, 1, 2 min) were studied. The effect of PVA concentrations (0.1, 0.5, 1 % w/v) to achieve desired particle size in the range of 20.85 – 95.85 µm. The effect of organic solvents with different water solubility such as acetone, ethyl acetate and dichloromethane on the particle size and entrapment efficiency of microspheres was studied. The PLGA polymer microspheres were prepared with the speed of 6000 rpm using Remi homogenizer. The effect of molecular weight and hydrophilicity of PLGA (50:50) biodegradable polymers on the particle size, morphology, entrapment efficiency and *in vitro* release profiles of microspheres was investigated. PLGA (50:50) PURASORB polymers PDLG 5002 for this study.

Characterization of microspheres

The particle size of microspheres was estimated using Malvern Mastersizer (Mastersizer S, Malvern Instruments Limited, U.K.). The freeze dried microspheres were dispersed by bath sonication in saline medium (0.9% NaCl) containing a surfactant (0.1% Tween 80) to prevent aggregation before samples examination. Samples were analyzed in triplicate. The particle size was expressed as the mean volume diameter in µm.

Morphological study

The morphological examination of microspheres containing BSA was performed using SEM (SEM, Leica Cambridge S360, UK). The formulations which were used for the study of effect of different molecular weight and hydrophilicity of PLGA polymers were subjected to SEM analysis. For the shape and surface analysis, the freeze dried microspheres were mounted onto aluminum stub using double-sided adhesive tape and then sputter coated with a thin layer of gold under argon atmosphere (Emitech K750, Kent, UK) before examination. The coated specimen was then examined under the microscope at an acceleration voltage of 20 kV and photographed.

Entrapment efficiency

The BSA content of microspheres was analyzed using hydrolysis technique as previously described by Igartua et al[11]. Briefly, 15 mg of lyophilized microspheres were digested with 5 ml of 0.1 M NaOH containing 5% w/v SDS and stirred for 15 h at ambient temperature until a clear solution was obtained. Sodium hydroxide catalyzes the hydrolysis of the polymer and SDS ensures the complete solubilization of the protein during the polymer hydrolysis. The resulting clear solution was then neutralized to pH 7 by addition of 1 M HCl and centrifuged at 1726×g for 15 min. The samples were analyzed in triplicate for each batch of microspheres using Hartree-Lowry and Modified Lowry Protein Assays[12] by UV-Visible Spectrophotometer (Hitachi 2000, Japan) at 650 nm. The encapsulation efficiency was expressed as the ratio of actual to theoretical BSA content. The entrapment efficiency was calculated using following equation, Entrapment efficiency (%) = (Weight of BSA in microspheres/Weight of BSA fed initially)×100.

***In vitro* release studies**

In vitro release studies were carried out by suspending 100 mg of microspheres in 60 ml of phosphate buffered saline (PBS, pH 7.4) containing 0.02% sodium azide as bacteriostatic agent and 0.01% Tween 80 to prevent the microspheres from aggregation in the dissolution medium in stoppered flasks. The flasks were placed in a reciprocal shaking water bath maintained at 37±0.5º at a speed of 60 cycles/min. At predetermined time intervals of 2, 12, 24, 72, 120 and 168 h, samples were collected and centrifuged at for 15 min. The supernatant was assayed for the protein release using Hartree-Lowry and Modified Lowry Protein Assays[12] by UV-Visible Spectrophotometer (Hitachi 2000, Japan) at 650 nm. The collected amount of supernatant was replaced with fresh PBS to maintain sink conditions. The percentage of protein release at different intervals was calculated by using a freshly prepared calibration curve using the standard samples which were run along with test samples. Release experiments were done independently in triplicate for each batch of microspheres.

Statistical Analysis

The results are presented as mean±standard deviation. The particle size and entrapment efficiency of BSA loaded microspheres were treated statistically using one-way analysis of variance (ANOVA). When there was a statistically significant difference, a post-hoc Tukey-HSD (Honestly Significant Difference) test was performed. A statistically significant difference was considered at $p<0.05$.

RESULTS AND DISCUSSION

Microencapsulation by the solvent evaporation method is, in principal, quite simple and involves two major steps, the formation of stable droplets of the drug-containing polymer solution and the subsequent removal of solvent from the droplets. In practice, however, the reproducible manufacturing of microspheres with the desired properties (good encapsulation efficiency, suitable release profile and particle distribution, acceptable solvent residuals), can be difficult, due to the large number of factors influencing the outcome, such as solvent composition, total volume and phase volume ratio of the phases, polymer concentration, type of stabilizer, stirring speed, stirring time etc. The effect of each of these parameters has to be determined empirically, predictions and scale up remain a problem. Therefore, more information is needed in order to identify the relevant parameters and save development resources[13] .

The selection of a particular encapsulation method is usually determined by solubility of the protein and the coating polymer; it also has utmost importance for the efficient entrapment of active substance. In this study, double emulsion solvent evaporation technique was adopted for the efficient incorporation of BSA in the biodegradable polymeric microspheres due to the solubility of protein in aqueous phase and the organic phase or oil phase acts as a barrier between the two aqueous compartments, preventing the diffusion of the active material toward the external aqueous phase[14]. It is also known to be superior to other encapsulation methods in terms of stability of proteins due to minimize exposure to organic solvent during preparation of microspheres. Encapsulation by the solvent evaporation technique involves two major steps, emulsification of an organic solvent containing dissolved polymer and dissolved/dispersed protein in an excess amount of aqueous continuous phase to form a stable droplets and the subsequent removal of organic solvent from the droplets of the second emulsion. Particle size is one of the most important characteristics of the microspheres. In the preliminary study of this work, effect of different speeds of magnetic stirrer and homogenizer were studied to achieve smaller size of microspheres. Preparation condition of standard formation is given in Table 1. The particle size of microspheres decreased from 95.85 to 22.04 µm with the increase of stirring speed of magnetic stirrer from 50 to 250 rpm and homogenizer speed from 1500 to 6000 rpm, respectively. The results are shown in Table 2.

Table 1: Preparation Conditions of Standerd Formulaation

Preparation parameter	W:O:W-multiple emulsion method
Internal aqueous phase (0.5 ml)	Phosphate Buffer Saline(PBS), pH 7.4
Internal organic phase	Methylene Chloride
Volume of internal organic phase	5 ml
External phase	Water (50 ml)
Stabilizer (0.5 % w/v)	Poly Vinyl Alcohol

Note: Phase volume ratio was maintain 1:10:100 for W: O: W.

Table 2: Optimization Of Stirring Speed (Homogenizer And Magnetic Stirrer) and Poly vinyl alcohol (PVA) concentration for microspheres

Formulation	BSA (mg)	PDLG 5002 (mg)	PDLG 5002 A (mg)	Homogenizer Stirring speed (rpm)	Magnetic Stirring speed (rpm)	PVA % (W/V)	Particle size (µm) (Mean±SD, n=3)
A1	20	500	-	1500	50	0.25	95.85±0.34
				3000	100	0.5	64.23±0.97
				6000	250	1	22.04±0.49
A2	20	-	500	1500	50	0.25	85.47±0.29
				3000	100	0.5	52.53±0.31
				6000	250	1	20.85±0.34

Size of microspheres was determined by the stirring speed. Stirring speed was parameter of primary importance in the emulsification step because it provides the energy to disperse the oil phase in aqueous phase. Our experimental results demonstrated that mean particle size of microspheres was inversely proportional to stirring speed; consequently increase in stirring speed decreased the size of microspheres because the second emulsion was broken up into smaller droplets at a higher input power in accordance with the study of Yang *et al*[15]. Thus, the stirring speed was optimized in order to obtain a desired size of microspheres in the range of 20.85 to 52.53 µm because the size of microspheres plays a crucial role in determination of the uptake of the encapsulated protein by immune system, *in vivo* distribution of the particles after administration and syringeability through hypodermic needles[16]. There was a statistically significant difference ($p<0.05$) in the size of microspheres prepared with different speeds of magnetic stirrer and homogenizer. A homogenizer stirring speed at 6000 rpm was chosen for further study as given in Table 3.

Table 3: Effect of stirring speed on particle size of BSA loaded PLGA microspheres

Formulation	BSA (mg)	PDLG 5002	PDLG 5002 A	Stirring speed (rpm)	Particle size (µm) (Mean±SD, n=3)
F1	20	250	-	6000	16.85±0.34
F2	20	500	-	6000	22.04±0.49
F3	20	750	-	6000	82.04±0.49
F4	20	-	250	6000	15.47±0.29
F5	20	-	500	6000	20.85±0.34
F6	20	-	750	6000	87.04±0.49

The selection of suitable organic solvent is critical in developing a successful formulation of biodegradable microspheres containing protein. The parameters, solvent's miscibility/solubility in water and its ability to dissolve the polymer should be considered while choosing organic solvent. In this process, acetone, ethyl acetate and dichloromethane organic solvents with percentage of solubility of 100, 8.7 and 1.6% w/w in water respectively were used.

The results of particle size and entrapment efficiency of microspheres prepared with different organic solvents are shown in Table 4. The size of microspheres was highly influenced by the water solubility of organic solvent. The mean particle size of microspheres with acetone, ethyl acetate and dichloromethane were 91.53, 54.26 and 22.04µm, respectively. The larger size of microspheres were formed with the higher water solubility of organic solvent because of the irregular polymer agglomeration upon emulsification due to rapid solvent exchange into aqueous phase[17]. The use of dichloromethane instead of acetone and ethyl acetate resulted in particles which were smaller in size. This was probably because dichloromethane is a better solvent for PLGA than acetone and ethyl acetate, leading to the formation of denser microspheres. There was a statistically significant difference ($p<0.05$) in the size of microspheres prepared with different organic solvents.

Table 4: Effect of organic solvent on particle size and encapsulation efficiency of BSA loaded PLGA microspheres

Formulation	Organic solvent	Particle size (µm) (Mean±SD, n=3)	Encapsulation efficiency (%) (Mean±SD, n=3)
F7	Dichloromethane	22.04±0.09	66.25±1.27
F8	Ethyl acetate	54.26 ±0.06	38.15±4.17
F9	Acetone	91.53±0.38	41.57±4.87

The successful entrapment of protein was dependent to a large extent on the type of organic solvent used. The entrapment efficiency of microspheres prepared with acetone, ethyl acetate and dichloromethane were 41.57, 38.15 and 66.25%, respectively. Bodmeier and McGinity found from the experiments that the rate of polymer precipitation from the organic solvent phase was strongly affected by the rate of diffusion of the organic solvent into the aqueous phase[17]. Organic solvents of low water solubility resulted in slow polymer precipitation which facilitated complete partitioning of the drug into the aqueous phase, resulting in empty

microspheres[18]. On the other hand, highly water-miscible solvents did not form droplets but large irregular polymer agglomerates upon emulsification due to rapid solvent exchange it leads to non uniform and poor encapsulation efficiency. The relatively high water miscible organic solvents acetone and ethyl acetate did not encapsulate the protein efficiently and produced larger particle size of microspheres compared to dichloromethane which is relatively a low water miscible solvent. The only organic solvent which could successfully encapsulate higher amount of protein (66.25%) with smaller size of microspheres under the selected experimental conditions was dichloromethane. This might be due to the optimum solubility of dichloromethane in water. There was statistically significant difference ($p < 0.05$) among the formulations prepared with acetone and dichloromethane as well as ethyl acetate and dichloromethane. These results indicated that dichloromethane is a good solvent for the formation of microspheres with higher entrapment efficiency due to its desirable physical properties such as extremely low solubility in water, ability to dissolve large amounts of polymer and required the lowest heat of evaporation. The organic solvent, dichloromethane was selected for further study.

The influence of concentration, molecular weight and hydrophilicity of PLGA polymers on particle size, morphology, entrapment efficiency and *in vitro* release profiles of microspheres were studied. PLGA (50:50) polymers, PDLG 5002 and PDLG 5002A were used for this study. The results of particle size and entrapment efficiency of the protein loaded microspheres are shown in Table 5.

Table 5: Effect of molecular weight and concentration of PLGA polymers on particle size and encapsulation efficiency of microspheres

Formulation	Concentration (%w/v)	Particle Size (µm) (Mean±SD, n=3)	Encapsulation efficiency (%) (Mean±SD, n=3)
PDLG 5002	5	34.58±0.65	40.25±0.34
(M. W. 0.19 kd)	10	67.29±0.22	47.58±0.26
	15	84.32±0.39	65.23±0.25
PDLG 5002A	5	52.35±0.56	42.58±0.25
(M. W. 0.44 kd)	10	81.54±0.08	46.35±0.46
	15	97.56±0.25	71.54±0.06

The mean particle size of PLGA microspheres, PDLG 5002 and PDLG 5002A were 34.58 – 84.32µm and 52.35 – 97.56 µm respectively.

The particle size of the microspheres was mainly affected by the concentration and molecular weight of the polymers. At a constant solvent volume, the viscosity of the polymeric solution was proportional to concentration and molecular weight of the polymer. Low viscosity polymer solution could be dispersed in the external aqueous phase to a greater extent than high viscosity polymer solution and hence reduced the microspheres to smaller sizes.

The morphological examination of the BSA loaded microspheres was performed by the SEM. The BSA loaded PLGA microspheres were spherical in shape and had a smooth surface without pores or cavities which could affect the release of encapsulated protein. The same appearance was observed for all formulations, independent of the type of polymer. The SEM photographs are shown in Fig. 1.

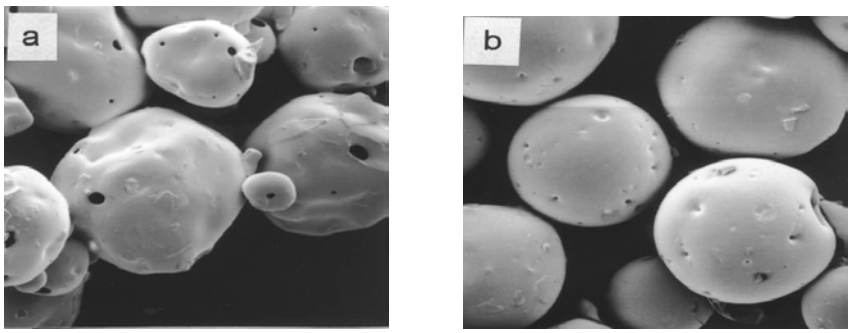

Fig. 1: Scanning electron micrographs of Bsa containing plga microspheres formulated by double emulsion technique: (A) PDLG 5002 (B) PDLG 5002 A

The encapsulation efficiency of protein loaded PLGA (50:50) polymer of three different concentration 5, 10 and 15 % were 40, 47 and 65 %, respectively. The encapsulation efficiency of protein was highly influenced by the concentration (% w/v), molecular weight and hydrophilicity of PLGA polymers. BSA encapsulation efficiency was higher for 15% w/v concentration compared to 5 and 10% w/v concentration. This might be attributed to the higher viscosity of polymeric solution as a result of high concentration of the polymer which could prevent the transfer of protein from internal aqueous phase to external aqueous phase and/or pronounced molecular weight dependent attraction forces between protein and polymer also caused the entrapment of more protein. The higher encapsulation efficiency was observed for the hydrophilic polymer PLGA 50:50A compared to PLGA 50:50. This might be due to the strong affinity between positively charged amino acid groups in protein and negatively charged free carboxyl end groups in the polymer chain. There was a statistically significant difference ($p<0.05$) in the entrapment efficiency of protein among the PLGA polymers.

The *in vitro* release profiles of microspheres are intended to assist in predicting the ultimate behavior of a given microsphere formulation. The release of BSA from the microspheres showed a biphasic profile. The microspheres showed an initial rapid release of a certain amount of protein which was deposited on the surface of the microspheres, followed by a slow and continuous release which corresponds to release of protein entrapped in the microspheres.

The polymer with terminal carboxylic groups, PLGA 50:50A showed low initial burst release compared to PLGA 50:50. This could be due to the hydrophilicity of polymer which causes favourable interaction between free carboxylic end groups in the polymer chains and protein. The cumulative percentage release of protein from PDLG 5002 and PDLG 5002A in one week is given in Fig. 2 and 3. It was clearly observed that higher the mol. weight and concentration of polymer, slower would be the release.

This might be attributed to low molecular weight of PLGA polymer degraded into small fragments which are soluble in aqueous release medium, thus leading to faster erosion of the microspheres. During erosion, the polymer matrix becomes more and more hydrophilic, allowing more water to penetrate, thereby enhancing polymer degradation and thus, protein release[19].

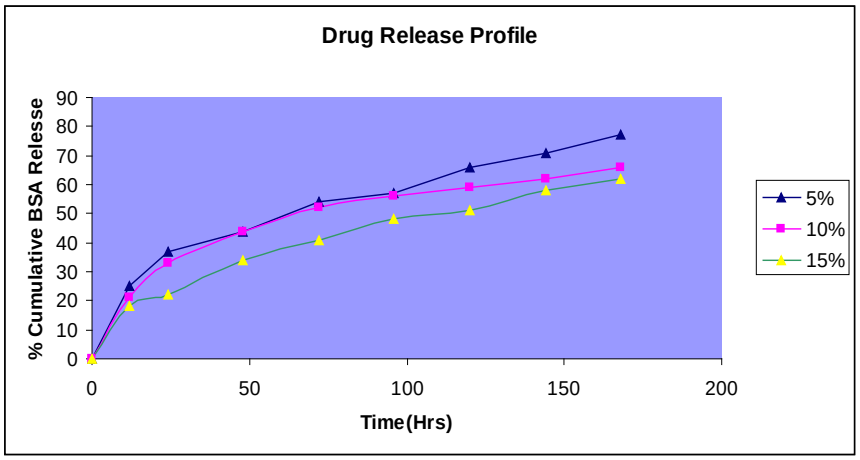

Fig. 2: The Cumulative percentage release of BSA from PLGA 5002 in one week

Note: 5, 10 and 15%w/v showing three different concentration of PDLG 5002.

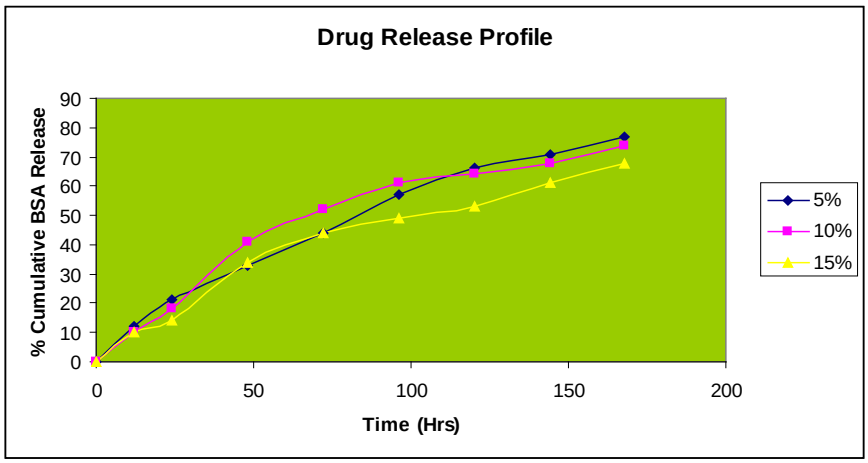

Fig. 3: The cumulative percentage release Of BSA from PDLG 5002A in one week

Note: 5, 10 and 15%w/v showing three different concentration of PDLG 5002 A

Legends

µg	Micro Gram
µm	Micro Meter
kDa	Kilo Dalton
MW	Molecular Weight
PHEMA	Poly(2-Hydroxyethyl Methacrylate)
PAA	Poly(Acrylic Acid)
PA	Polyacrylamide
PVA	Poly Vinyl Alcohol
PMAA	Poly (Methacrylic Acid)
DCM	Dichloro methane
IPA	Iso propyl alchohol
PEG	Poly Ethylene Glycol
PLA	Poly(DL -Lactide)
PLG	Poly(DL -Lactide-*Co*-Glycolide)
PLC	Poly(DL-Lactide-*Co*-Caprolactone)
BSA	Bovine Serum Albumin

CONCLUSION

Protein loaded PLGA microspheres were prepared successfully using the double emulsion solvent evaporation technique. The size of the microspheres was influenced by the stirring speed, organic solvent and molecular weight of polymers. The microspheres were spherical in shape with a smooth and non porous surface. The water miscibility of organic solvents plays a role in successful entrapment of proteins. The *in vitro* protein release study from PLGA microspheres proved that the present microspheres had the properties of an ideal sustained release formulation. Furthermore, the present microspheres are attractive for parenteral application because of their smaller size and biodegradability.

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