Research in Pharmaceutical Sciences, December 2014; 9(6): 407-420
Received: Sep 2013
Accepted: Nov 2013

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

The effects of technical and compositional variables on the size and release profile of bovine serum albumin from PLGA based particulate systems

B. Taghipour[1], M. Yakhchali[2], I. Haririan[3], A.M. Tamaddon[4] and S. Mohammadi Samani[1,*]

[1]*Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, I.R. Iran.*
[2]*Deptartment of Pharmaceutics, School of Pharmacy, Tehran University of Medical Sciences, Tehran, I.R. Iran.*
[3]*Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences and Medical Biomaterials Research Centre (MBRC), Tehran, I.R. Iran.*
[4]*Center for Nanotechnology in Drug Delivery, Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, I.R. Iran.*

Abstract

Double emulsion solvent evaporation technique is one of the most attractive methods used to prepare micro and nanoparticles in pharmaceutical areas of interest, but because of the effects of many formulation factors on the size and release behavior of the fabricated particles, optimization of the formulation factors is needed. In this study various parameters including technical and compositional variables were considered to achieve an optimized formulation with desire characteristics especially size and the release profiles, using high shear homogenizer. In this regard, bovine serum albumin (BSA) was used as the model protein and double emulsion was formed with the addition of Tween 80 and Span 80 as surfactants for inner aqueous phase and oil phase, respectively. Hydroxypropyl beta cyclodextrin was used as protein stabilizer. After optimization steps, composite nanoparticles (core-shell) were made based on optimized formulation by hyaluronic acid as shell and poly lactic-co-glycolic acid (PLGA) as core material. Formulation of the BSA loaded PLGA nanoparticles using core shell strategy improved the release pattern of the BSA and diminished burst release. The final composite nanoparticles had the particle size of about 160 nm and 70 % of the loaded BSA was released during 14 days and the release data was better fitted to zero order release kinetics.

Keywords: Protein; Drug delivery; Biodegradable; PLGA; Nanocomposite

INTRODUCTION

Protein-based therapeutics have changed the face of the modern medicine and they continue to provide new and effective therapies for numerous diseases ranging from cancers to infertility (1), but short biological half life of the proteins is a serious limitation which should be tackled. During the past decades a significant interests have been focused on the extended release formulations of therapeutic proteins (2-3). In addition to higher safety and efficacy, such formulations enhance the compliance of the patients due to reduction in the frequency of administration (3-4). Various approaches have been examined to establish effective formulations containing proteins to extend frequency of peptide and protein-administration, including protein engineering, PEGylation, conjugation of fatty acids and so on; but in many of these strategies chemical modification of the therapeutic peptides and proteins is needed and the efficacy and therapeutic use of the final modified molecule may be compromised. Other approaches such as microencapsulation using biodegradable polymers are very attractive, because they do not require chemical modification (3,5-6).

The hydrophobic nature of the interface in double emulsification solvent evaporation technique has been identified as a major cause of protein denaturation and aggregation (7). Protein aggregation results in incomplete

*Corresponding author: S. Mohammadi Samani
Tel. 0098 711 2424127, Fax. 0098 711 2424126
Email: smsamani@sums.ac.ir

release of the protein from the formulation and not only protein aggregate are therapeutically inactive, but also might be toxic and immunogenic (8). Using the stabilizing agents such as surfactants, sugar, polyols and basic salts in the inner aqueous phase is a rational approach to minimize protein exposure to the organic solvents (7,9-10). Another approach is the protection of protein conformation in a cavity created by cyclodextrin derivatives (10-11). The stabilizing effect of cyclodextrin is the result of increased hydrophilicity of protein by shielding of hydrophobic parts of protein by cyclodextrin molecule (12).

Microencapsulation of proteins into poly lactic-co-glycolic acid (PLGA) micro or nanoparticles is a multifactorial method that requires optimization of many factors which could affect the characteristics of the final product. For instance, polymer/protein ratio, type of PLGA, the aqueous/oil phase ratio and type and concentration of the surfactants are some compositional factors which strongly affect the size and structure of the particles (13).

Although PLGA is a suitable polymer for microencapsulation of peptides and proteins, there are some limitations. Generally, protein loaded PLGA microparticles show a burst phase in the release study which is then followed by a non-complete release phase (8,14). It seems that the protein aggregates located in the surface of the particles are responsible for such a behavior (8). In addition, hydrolysis of PLGA leads to the formation of acidic by-products which could result in the denaturation of peptides and proteins in the acidic microenvironment (15-16). As an effective approach, composite particles, namely, core-shell structure plays a key role in the prevention of these obstacles (17). Through this method, the entering rate of the medium into the particles is controlled and the acidic microenvironment is not a determinant factor in bulk hydrolysis of the polymer and consequently the burst release is suppressed.

Therefore, the purpose of this study was to evaluate the most influencing factors on the characteristics of protein loaded PLGA micro and nanoparticles prepared by double emulsion solvent evaporation method. To avoid limitations associated with PLGA, the composite strategy was applied to produce core-shell structure, using PLGA and hyaluronic acid (HA). Bovine serum albumin (BSA) in this study was used as a model protein, due to its availability in high purity and low cost and the effects of several variables on the release profile of protein from manufactured particles and the size of particles were assessed. In addition, the effects of additives such as cyclodextrin were investigated to optimize a successful sustained release formulation for therapeutic proteins.
As mentioned previously, burst release of protein from the PLGA loaded particles is another limitation of PLGA-based drug delivery systems (18). Core-shell strategy was also used to increase encapsulation efficiency and to retard and stabilize the loaded drug in encapsulated particles (18).

MATERIALS AND METHODS

Materials

Bovine serum albumin, PLGA (50:50 and 65:35 of lactide: glycolide), Span 40, hyaluronic acid were purchased from Sigma-Aldrich (USA). Tween 20, Tween 80, Span 80 and dichloromethane were purchased from Merck (Germany). Polyvinyl alcohol (PVA) in different molecular weights (27000, 72000) was from Fluka. All other reagents used in this study were of analytical grade.

Preparation of particles

Micro and nanoparticles were prepared using double emulsion solvent evaporation method reported in previous studies (14, 19-26) with some modifications. Briefly, the inner aqueous phase (W_1) was prepared by dispersing predetermined amounts of the model protein BSA in phosphate buffer saline (PBS) and this aqueous phase was then added to the organic phase (O) containing PLGA in dichloromethane.

Hydroxylpropyl beta cyclodextrin was introduced into W_1 phase as protein stabilizer. Mixing of two phases led to the formation of initial emulsion and then, the mixture immediately was poured into outer aqueous phase (W_2) consisting of water and PVA as

stabilizer. Homogenization of this mixture led to the formation of final double emulsion. To eliminate the organic solvent, double emulsion was stirred (IKA RCT basic mixer, Germany) for 2 h at 800 rpm which resulted in the solidification of particles after evaporation of dichloromethane. Micro and nanoparticles were washed by double distilled water and were separated by ultracentrifuge (sigma 3-30K, Germany) at 60,000 g for 15 min. Finally, trehalose as lyoprotectant was added and after freezing, the particles were lyophilized for 48 h.

Optimization of technical variables

In the current study, two types of variables including technical and compositional variables were studied. In the technical variables, the effects of preliminary emulsion addition method into the W_2 phase, homogenization technique and homogenization time were studied.

Two different methods were used to add preliminary emulsion to W_2 aqueous phase including continuous and dropwise addition. In doing so, preliminary w/o emulsion was added to the second aqueous phase in 2 different rates, using a glass syringe. Depending on the applied pressure to the piston of the syringe, two different rates were considered. The mixture was homogenized simultaneously during the addition.

High shear homogenizer (IKA T25 digital, ULTRA- TURRAX, Germany) and probe sonicator (Hielscher, UP400S, Germany) were used to homogenize the primary and secondary emulsion. Mixing rate was set to 17500 rpm for high shear homogenizer. During the use of probe sonicator, the magnitude of sonication was adjusted to 80% and 0.5 for amplitude and cycle, respectively.

Homogenization duration is one of the crucial parameters which could play a key role in the characteristics of the final particles, especially on the size and encapsulation efficiency (23). The effect of homogenization time was studied in 3 levels for both techniques already described. In the probe sonication technique, settings were 1 and 2, 1 and 3, 1 and 6 min for two steps of emulsification and for high shear homogenizer the durations were adjusted to 1 and 2, 2 and 3, 4 and 6 min.

Optimization of compositional variables

Several compositional parameters including protein/PLGA weight ratios, polymer concentration, lactide/glycolide ratios, phase ratios and surfactant concentrations in each phase were evaluated.

Protein concentration

Theoretically, higher protein concentration can lead to lower encapsulation efficiency. For a certain volume of inner aqueous phase, a range of 2.5–10 % protein (w/v for W_1) was studied.

Polymer concentration

The concentration of polymer can affect the characteristics of particles (13). According to previous studies, a range of 3-8 % w/v PLGA (in oil phase) was considered and respective effects on particle properties were evaluated.

Lactide:glycolide ratio of poly lactic-co-glycolic acid

Based on the previous works, two different types of PLGA which induce a reliable release profile contained 50:50 and 65:35 lactide-to-glycolide ratios (14,21,27). Therefore, the same types of PLGA were used to produce micro and nanoparticles.

Phase ratios

Different phase ratios are among the other factors affecting the particles characteristics. In this regard, according to previous study two different ratios were considered in the preparation of double emulsion including 1:10:35 and 1:10:80 ratios for W_1, O and W_2, phases, respectively (13).

Inner aqueous phase for surfactants selection

Many surfactants have been examined to stabilize the initial W_1/O emulsion and also to protect the protein from deformation and denaturation at interface. To protect the protein in aqueous phase, water soluble surfactant with higher HLB should be considered. In this study, five surfactants with different concentrations from 1-10% w/v were studied individually, including PVA,

Tween 20, Tween 80, Pluronic F68 and Pluronic F127.

Oil phase surfactant selection

To stabilize the system and prevent the merging of droplets, it is essential to utilize appropriate surfactants in the oil phase. Span 40 and Span 80 were used separately with concentrations ranging 0.2-7% in the oil phase.

Polyvinyl alcohol concentration and molecular weight

Stabilizing effect of PVA is strongly affected by its concentration in outer aqueous phase (23). Therefore, three concentrations of PVA including 1, 2.5 and 5 % were assessed. Also, to clarify the effect of the PVA molecular weight on stabilization of the double emulsion, two different molecular weights of PVA were examined (27000 and 72000 Dalton). Table 1 and 2 demonstrates the different formulation factors based on technical and compositional variables.

Design of experiment

To evaluate the influence of different variables on the particle size, a statistical study was performed using Minitab Ver. 16 software. Although there were many variables (introduced in the next paragraphs), the analysis was done considering five independent variables including homogenization time, polymer concentration, W_1 surfactant concentration, O surfactant concentration and PVA concentration and the particle size was taken as the dependent variable.

The studied ranges of independent variables were obtained from preliminary experiments. Having considered these five independent variables, a 5-factorial 3-level Box-Behnken experimental design was chosen.

Table 1. Different setting of technical variables.

Code of Formulation	Technical variables		
	Mode of addition	Homogenization technique	Mixing time (min)
F1	Continuous	Sonicator	1-2
F2	Continuous	High shear	4-6
F3	Continuous	Sonicator	1-3
F4	Continuous	Sonicator	1-6
F5	Continuous	High shear	4-6
F6	Dropwise	High shear	4-6
F7	Dropwise	High shear	4-6
F8	Dropwise	High shear	4-6
F9	Dropwise	High shear	4-6
F10	Dropwise	High shear	4-6
F11	Dropwise	High shear	4-6
F12	Dropwise	High shear	4-6
F13	Dropwise	High shear	4-6
F14	Dropwise	High shear	1-2
F15	Dropwise	High shear	2-3
F16	Dropwise	High shear	1-2
F17	Dropwise	High shear	1-2
F18	Dropwise	High shear	1-2
F21	Dropwise	High shear	1-2
F22	Dropwise	High shear	1-2
F23	Dropwise	High shear	1-2
F24	Dropwise	High shear	1-2
F25	Dropwise	High shear	1-2
F26	Dropwise	High shear	1-2
F27	Dropwise	High shear	1-2
F28	Dropwise	High shear	1-2
F29	Dropwise	High shear	1-2
F30	Dropwise	High shear	1-2

Table 2. Different setting of compositional variables.

Code of formulation	Compositional variables									
	Protein (%)	Polymer (%)	Phase ratio	L/G ratio	W_1 surfactant	O surfactant	W_1 surfactants (%)	O surfactant (%)	PVA (%)	PVA MW (Da)
F1	2.5	8	1:10:80	50/50	-	-	-	-	1	72000
F2	2.5	8	1:10:80	50/50	-	-	-	-	1	72000
F3	2.5	8	1:10:80	50/50	PVA	-	1	-	1	72000
F4	2.5	8	1:10:80	65/35	-	-	-	-	1	72000
F5	2.5	4	1:10:80	65/35	-	-	-	-	1	72000
F6	2.5	4	1:10:80	65/35	-	Span 40	-	0.2	1	72000
F7	2.5	4	1:10:80	65/35	Tween 20	-	2	-	1	72000
F8	2.5	4	1:10:80	65/35	Tween 20	Span 40	2	0.2	1	72000
F9	2.5	3	1:10:80	65/35	-	-	-	-	1	72000
F10	2.5	3	1:10:80	65/35	Tween 20	Span 40	2	0.2	1	72000
F11	10	3	1:10:80	65/35	Tween 20	Span 40	2	0.2	1	72000
F12	2.5	4	1:10:80	65/35	Tween 20	Span 40	2	0.2	2.5	72000
F13	2.5	4	1:10:80	65/35	Tween 20	Span 40	2	0.2	5	72000
F14	2.5	4	1:10:80	65/35	Tween 20	Span 40	2	0.2	1	72000
F15	2.5	4	1:10:80	65/35	Tween 20	Span 40	2	0.2	1	72000
F16	2.5	4	1:10:80	65/35	Tween 20	Span 40	2	0.2	2.5	72000
F17	2.5	4	1:10:80	65/35	Tween 20	Span 40	2	0.2	1	27000
F18	2.5	4	1:10:80	65/35	Tween 20	Span 40	2	0.2	2.5	27000
F21	2.5	4	1:10:80	65/35	Tween 20	Span 40	10	1	1	72000
F22	2.5	4	1:10:80	65/35	Pluronic F68	Span 40	10	1	1	72000
F23	2.5	4	1:10:80	65/35	Pluronic F127	Span 40	10	1	1	72000
F24	2.5	4	1:10:80	65/35	Tween 80	Span 80	7	7	1	72000
F25	3.7	4	1:10:35	65/35	Tween 80	Span 80	-	-	1	72000
F26	3.7	4	1:10:35	65/35	Tween 80	Span 80	7	7	1	72000
F27	3.7	4	1:10:35	65/35	Tween 80	Span 80	3.5	3.5	2.5	72000
F28	3.7	4	1:10:35	65/35	Tween 80	Span 80	7	7	2.5	72000
F29	3.7	4	1:10:35	65/35	Tween 80	Span 80	7	7	1	72000
F30	3.7	4	1:10:35	65/35	Tween 80	Span 80	2	2	1	72000

O; Oil, W_1; Inner aqueous phase.

It is noticeable that although 46 experiments were suggested by the statistics software, during the study some of the obtained results indicated that it was not necessary to perform some of the suggested experiments and instead, other settings were studied as described in the following sections.

Core-shell structure

Hyaluronic acid (HA) was selected as the second polymer to fabricate core-shell structure. Two different approaches were considered, at first HA was used as core forming polymer and PLGA as shell former and in the second approach, PLGA was selected as core former polymer and HA as shell forming polymer.

As for the first approach, a certain amount of HA was added to inner aqueous phase (W_1) of the double emulsion during preparation of the optimized formulation. In the second approach, the optimized formulation was used as the core and the HA was used to coat the previously fabricated PLGA nanoparticles. To do so, a certain amount of lyophilized particles were suspended in a viscous solution of HA (with or without Pluronic F127 0.02 %w/v) and after mixing, the formulation was lyophilized.

Characterization of the particles

Particle size and zeta potential determination

Particle size analysis was carried out using a Malvern zetasizer (ZS90, United Kingdom). Five ml of sample (after ultracentrifugation and washing) was used to determine the particle size. The effects of lyophilization and lyoprotectant on the size and the zeta potential were also assessed.

Encapsulation efficiency

The amount of encapsulated BSA was determined in triplicate by dissolving 10 mg of lyophilized particles in 3 ml of 0.1 N NaOH

solution containing 0.5% sodium dodecyl sulfate. Samples were incubated at 37 °C for 48 h (7). Then, the samples were centrifuged at 60,000 g and 4 °C for 10 min and finally the concentration of BSA in the supernatant was determined by Bradford protein assay method (28).

Release study

The release profile of BSA from PLGA micro and nanoparticles, was studied in triplicate by adding 2 ml of PBS containing 0.01% sodium azide to 5 mg of lyophilized particles. The samples were placed in bath shaker at 37 °C and were gently shaken. Sampling was considered at 24 h intervals and in each sampling time (every 24 h) a triplicate set of samples was assigned; therefore, buffer replacement was not required. The release study continued for 7-14 days, and finally the samples were ultracentrifuged at 60,000 g for 10 min and the supernatants were analyzed by Bradford protein assay method at 595 nm.

In order to characterize the release kinetics, the *in vitro* release data were fitted to the following models (29); Zero order kinetic (1), First order kinetic (2),Higuchi equation (3), and Korsmeyer-Peppas equation was used to elucidate the release mechanism (4).

$$Q = kt \quad (1)$$

$$\ln(100 - Q) = \ln 100 - kt \quad (2)$$

$$Q = kt^{\frac{1}{2}} \quad (3)$$

$$Q = kt^{n} \quad (4)$$

where, Q is the percent of released drug at time *t*, and *k* is the release constant. In equation (4) *n* is release exponent and indicates the type of release mechanism. When *n* approaches to 0.5 the release mechanism is Fickian and if *n* approaches to 1 the release mechanism approaches to zero order and when 0.5<n<1, non Fickean transport could be expected (30).

RESULTS

The effects of several formulation factors including the phase ratios, type of surfactants, mixing rates, mixing techniques, protein /polymer ratios and type of PLGA were considered in this study. Because of the importance of the particle size and zeta potential on the fate of particles, at first the optimization was performed based on the size and zeta potential and then, the optimized formulations from this step were used to optimize other characteristics related to the particles.

Optimization of technical variables

The results showed that continuous addition of W_1 into oil phase, produced larger particles with a wide range of distributions and with sizes bigger than 2 μm. On the other hand, dropwise addition of inner aqueous phase to the organic phase resulted in considerable reduction in the particle size. In the dropwise addition method, the particles had a Z-average of about 1700 nm.

Essentially, during the first and second emulsification processes by probe sonicator, it was observed that the mixing did not performed efficiently. In spite of several settings for power and operation cycle of probe sonication technique, no significant differences were observed by these variables. In different experiences, the power was adjusted at 50-75% of maximum power and also the operation cycle (the duration of on/off process) was changed within 0.5-0.7 sec. However, the manufactured particles were too large to be determined by zeta sizer.

Because of different natures of homogenization techniques (probe sonicator and high shear homogenizer), two series of settings were applied. For probe sonicator, the primary mixing time was set on 1 min and duration of mixing in second step was 2, 3 and 6 min; however, this process could not reduce the size of produced particles. Nevertheless, at the high shear homogenization technique considerable differences among different settings have been reported. Previous studies confirmed that decreasing the duration of homogenization to lower than 3 min, produced small and more stable particles (31). Similarly, the results of the present study confirmed the same outcomes.

As mentioned, a Box-Behnken 5-factorial 3-level design was chosen for statistical study here. Effects of different variables were

evaluated using Minitab Ver.16 software. Fig. 1 shows the effect of homogenization time and polymer concentration on the particle size. The numbers 1, 2 and 3 in the homogenization axis shows the duration of 1-2, 2-3 and 4-6 min for primary and secondary homogenization time. It was observed that increasing the homogenization time strongly affected the particle size and led to the production of larger particles.

Optimization of compositional variables

Protein concentration

The effects of protein content on the size and zeta potential of the particles were evaluated. In this study, different protein concentrations were considered including 2.5 to 10% (w/v) protein to W_1. The results confirmed that increasing the protein concentration up to 4 times, did not change the particle size significantly.

Polymer concentration

The results revealed that at higher concentrations of polymer (8%), the size of particle increases significantly (Fig. 1). Theoretically, increasing the polymer concentration leads to a profound increase in viscosity in continuous phase (oil phase) of preliminary emulsion and consequently with the same power, the process of breaking down of droplets is not carried out satisfactorily in comparison to the formulation possess lower polymer concentration.

Lactide-to-glycolide ratio of poly lactic-co-glycolic acid

According to the results, change in the lactide:glycolide ratio from 50:50 to 65:35 did not affect the average size of the particles significantly. Due to the effect of lactide/glycolide ratios on particle hydrolysis and then on the release profiles, it seems that formulator can manipulate the release rate without fear of significant change in particle size and size distribution.

Phase ratios

Comparison of the results of different ratios, showed that although there was not a significant difference between particle size of formulations produced by 1:10:35 ratio of $W_1/O/W_2$ (W_1,O and W_2 containing PBS buffer solution/BSA/PVA, dichloromethane-span 80-PLGA and water-PVA respectively) and 1:10:80, ratio of $W_1/O/W_2$ the lower ratio (1:10:35), produced more homogenous dispersion than the 1:10:80 ratio. In fact, the particle size distribution in 1:10:35 ratio was more uniform. Yeo and coworkers have reviewed some factors affecting the characteristics of particles produced by double emulsion method. It has been reported that the particle size increased with the increase of continuous phase volume (13). In the formulation with higher phase ratio (1:10:80) after 24 day refrigeration at 2-8 ^{o}C, the larger particles precipitated; however, this phenomenon was not observed in the lower ratios.

Type and concentration of W_1 phase surfactant

The main evaluations in this study were focused on the application of Tweens as W_1 surfactant. There are several studies indicating the successful application of Tweens as surfactant in the double emulsion technique. However, other surfactants such as Pluronic or PVA were also assessed. Results showed that Tweens, especially Tween 80, can produce smaller particles by stabilizing the system more efficiently and, therefore, there was a significant difference between Tween 80 and other surfactants. In addition, different concentrations of Tween 80 showed various effects. It appears that the higher the concentration of Tween 80 (7% w/v in W_1), the greater the reduction of particle size of the final particles. Fig. 2 shows the effect of surfactant concentrations of inner aqueous (W_1) and organic (O) phases on the particle size.

Type and concentration of oil phase surfactant

Although Span 40 was used in some formulations, acceptable results were obtained during the addition of the span 80 (19). Span 80 was introduced into these formulations in two different concentrations of 3.5 and 7% w/v of the oil phase. Results showed that Span 80 can reduce the particle size efficiently when the Tween 80 was applied in the inner aqueous phase (Fig. 2).

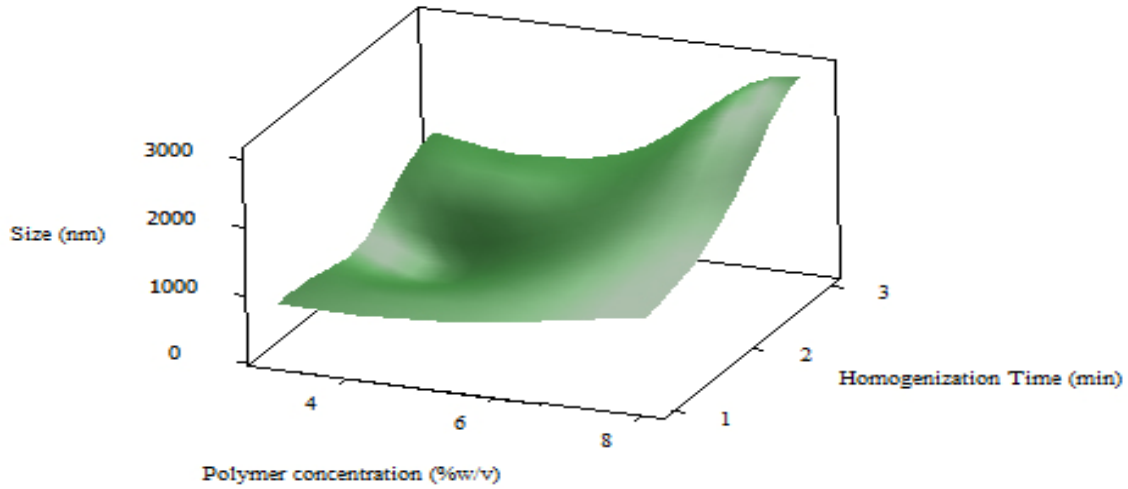

Fig. 1. The effect of polymer concentration and homogenization time on the particle size.

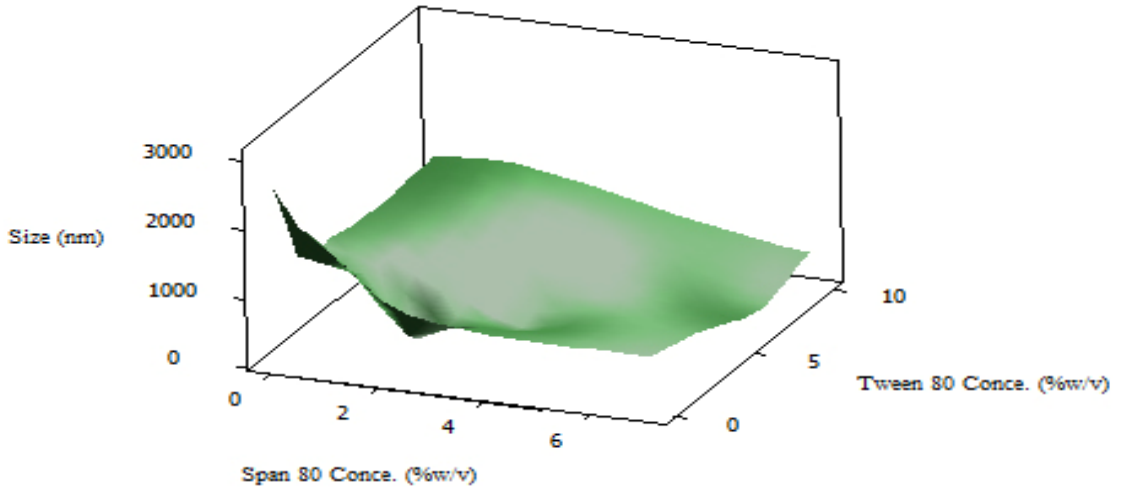

Fig. 2. The effect of surfactants concentration on the particle size.

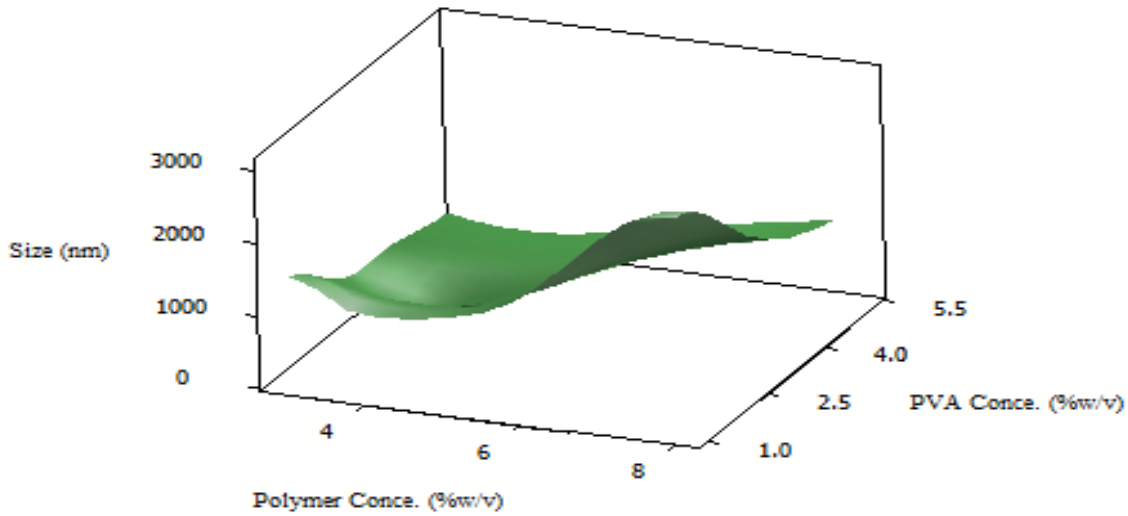

Fig. 3. The effect of polymer concentration and polyvinyl alcohol concentration on the particle size.

Polyvinyl alcohol concentration and molecular weight

There are several reports challenging the optimum concentration of PVA in the outer aqueous phase as stabilizer (23). In the present study, three different concentrations of PVA (1, 2.5 and 5% w/v in W_2) were evaluated. Fig. 3 shows the effect of PVA concentration on the particle size. The results showed that increasing the PVA concentration led to the smaller particles. But this behavior was not linear and further increase of PVA diminished this phenomenon. This effect was parallel to the viscosity increments. It seems that higher viscosity of W_2 phase limits the dispersing of the inner phase and prevents the breaking down during the second emulsification step.

The results showed that the formulations prepared by PVA with higher molecular weight (72000) produced smaller particles, in comparison to the formulations containing low molecular weight PVA (27000). It seems that higher molecular weight PVA is more efficient than low molecular weight PVA in prevention of the particles association prior to evaporation of organic solvent. This effect can be attributed to higher viscosity of high molecular weight PVA solution at the same concentration.

Optimized formulations

In order to obtain the submicron sizes and the desired characteristics, the formulation variables were set as follows: High shear homogenizer was used at the rate of 17500 rpm and duration of 1 and 2 min for primary and secondary emulsification steps, respectively. In addition, dropwise addition of the primary emulsion into outer aqueous phase also reduced the particle size more efficiently. Although in this study the different concentrations of BSA did not affect the particle size, this parameter was set on 3.7% (w/v) as in a similar study by Feczko' and coworkers.(25). Polymer concentration was adjusted at 4% (w/v) and manufacturing of particles was performed by the most common L/G ratio of PLGA, namely, as 50/50. The phase ratios were set to 1:10:35 according to the results of the present study and the previously published reports (25). The PVA concentration was fixed on 1% (w/v) and high molecular weight PVA (72000) was used. All of these parameters were applied to produce a pre-optimized formulation coded, F25.

To produce more stable emulsions and also to achieve smaller particles, the optimized surfactants were used in inner aqueous phase and oil phase. For W_1, Tween 80 and for organic phase (O), Span 80 were used with the same concentration (7% w/v). All other formulation conditions were the same as F25. The new formulation was considered to be F26.

Given the proper particle size and size distribution resulted from formulation F26, some modifications were performed to improve the protein stability. As mentioned earlier, hydroxypropyl beta cyclodextrin is a suitable excipient which protects protein from aggregation (11-12). In order to evaluate its effect, a subtype of F26 formulation was generated and coded as F29.

Particles characterization

Particle size

Depending on the settings of multiple variables chosen during optimization processes, the particle size was set between 160 – 2000 nm. As mentioned above, surfactants play a key role in reducing the particle size. The only difference between formulations F25 and F26 was in surfactant type, but their sizes were completely different. The particle size of F25 was large to the extent that could not be determined by zeta sizer (approximately larger than 2000 nm). Certainly, surfactants stabilize the double emulsion and prevent merging of droplets which lead to the smaller droplets and smaller particles (32). This effect resulted in the production of nanoparticles with the size of about 200 nm in F26.

Further optimization during this study led to the application of HPβCD as an aggregation inhibitor. This formulation (F29) showed a smaller particle size about 160 nm. Arun and coworkers also have reported that co-encapsulation of HPβCD with PLGA reduced the particle size of the microspheres (12).As described, the optimized formulation (F29) was used for the production of composite nanoparticles which were coded as F30-F32

(depending on application order of PLGA and HA as core or shell former). The F30 formulation consisted of HA as the core which was coated by PLGA as shell. F30 was produced based on F29 with the exception of adding HA into W_1. This formulation had a particle size of 138 ± 7 nm. On the other hand, using the PLGA nanoparticles (F29) as core and HA as the shell, led to the formation of F31 and F32. The differences of these formulations were in the presence of Pluronic F127 (as porogen) in HA solution in F32. The F31 and F32 had the particle size of 157 and 167 nm, respectively. Fig. 4 displays the SEM images of F30, F32.

Zeta potential

During the optimization steps, the zeta potential was also monitored. Theoretically, highly positive or highly negative values of zeta potential indicate the higher stability, according to the DLVO theory (33), but from biological standpoint, particles having high positive or negative zeta potential are prone to fast clearance from the body.

Here, the results showed that the increase in polymer concentration or changing the mixing mechanism did not change the zeta potential value (~ +17mv); however, changing the type and concentration of surfactants strongly affected the zeta potential. When Pluronic F68 or Pluronic F127 was used, a considerable change in the zeta potential (-30 to -32 mv) was reported, as in previous research (33), however, in this study, replacing the Pluronic with Tween 80 (as W_1 surfactant), had no significant effect on the zeta potential.

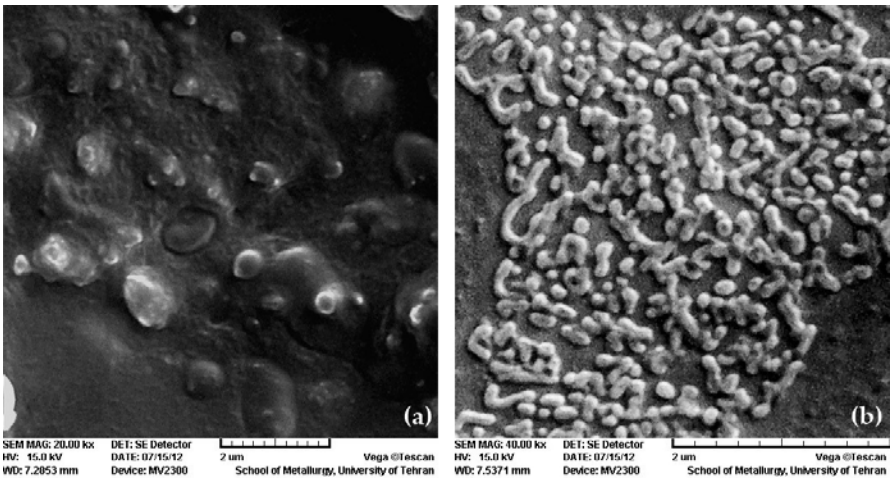

Fig. 4. SEM images of nanoparticles; (a) F30 and (b) F32.

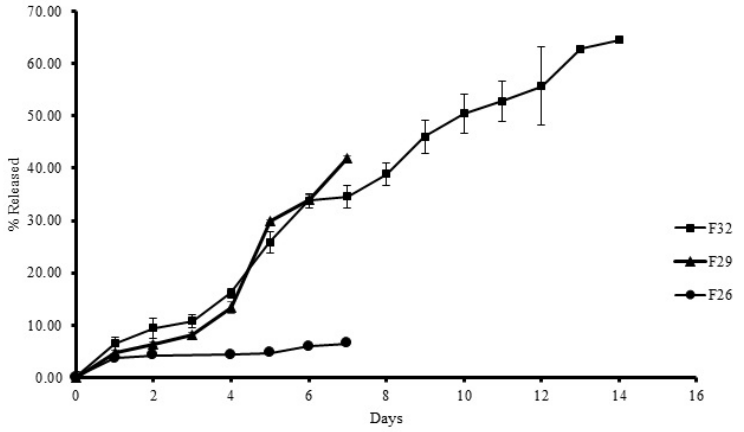

Fig. 5. The release profile of bovine serum albumin from different formulations. (a) F26, (b) F29, (c) F32

Table 3. Particle size and zeta potential of some of selected formulations.

Code of formulation	Particle size (nm)	Zeta potential (mv)
F26	223.3	-8.31
F27	209.1	-11.7
F28	129.5	-15.2
F29	155.1	-30.5
F30	138.1	-32.2
F31	156.9	-46.6
F32	167.4	-58.3

Table 4. The release kinetics of optimized formulation (F32).

Formulation	Zero order		First order		Higuchi		Korsmeyer-Peppas	
	K (%.day^{-1})	R^2	K (day^{-1})	R^2	K (%.cm^{2}day$^{-1/2}$)	R^2	n	R^2
F32	4.781	0.988	0.17	0.894	19.51	0.923	0.9676	0.968

Comparison of F29 and F32 formulations indicates that addition of shell layer (HA) on the PLGA nanoparticles, shifted the zeta potential from -30 mv (F29) towards -58 mv (F32) which was more desired according to DLVO theory, and consequently could suppress the merging of the particles. Table 3 shows the particle size and zeta potential of different formulations.

Encapsulation efficiency

According to the results of loading studies, the encapsulation efficiency (EE) was calculated using equation (5) (34) .

$$EE = \frac{\text{actual loaded drug}}{\text{theoritical added drug}} \times 100 \quad (5)$$

As mentioned in the method section, a proper dilution was prepared and was assayed by Bradford method. The EE values for F26 and F29 were 89% and 76%, respectively.

Release and kinetic study

The release profile of BSA from selected formulations including F26, F29 and F32 was assessed. According to the results presented in Fig. 5, the initial burst and subsequent non-complete release profile which led to the plateau (in formulation F26), strongly indicated that the absence of a suitable stabilizer in formulation led to the aggregation of BSA.

The BSA release from aggregates located in the surface of nanoparticles, has led to the burst effect. Aggregation of protein also resulted in insufficient release after initial release (8).As indicated previously, addition of stabilizers (HPβCD) can enhance the release profile of BSA from nanospheres. As shown in Fig. 5, the release profile of BSA was significantly enhanced in F29, compared to F26. It is evident that the presence of HPβCD stabilized the BSA molecules and prevented extensive aggregation, as the limiting factor in continuous and uniform release profile in formulation F29.

In part (c) of Fig. 5, also, the effect of composite (core-shell) structure on the release profile was presented. Formulation F32 shows more linear release profile than F29 and as it is clear from this figure, release rate is slower than PLGA base nanoparticles. Kinetic analysis of the release profiles also showed that formulation F32 better fitted to zero order kinetics with coefficient of correlation about 0.994 and during the 14 days release study, 70% of protein released uniformly without significant burst effect.

Fitting the release data on four kinetic models (zero, first, Higuchi and Korsmeyer-Peppas) showed that the zero order kinetic had the highest value of R^2 in comparison to the others. On the other hand, the "n" constant of Korsmeyer-Peppas which was near 1, shows that a case 2 transport (zero order) exists within the release profile of F32 (Table 4).

DISCUSSION

Based on the results of this study, mixing techniques can affect the mean size of the particles. Dropwise addition of the first aqueous phase to the oil phase produces

smaller particles than continuous addition technique. It seems that dropwise addition of the W_1 phase provide sufficient time for effective breakdown of the inner phase within the continuous phase. The same results also were reported by Adams and coworkers, when they used a jet device to induce higher flow rates than dropwise addition procedures (35). On the other hand, probe sonication of the mixture was not sufficiently effective in reducing the average particle size although different magnitude and duration of probe sonication was examined. Burapapadh and colleagues studied the effect of homogenization technique on the droplet size of the pectin-based emulsion.

They reported that applying the probe sonicator led to the generation of droplet with the size of approximately 2 micron, but in the present study, no supporting data were found to show the effect of sonication on particle size and size distribution (36).

Mixing of phases using high shear homogenizer resulted in more uniform and smaller particles which were due to more efficient breaking down of the droplets. Under the same conditions, the high shear technique produced particles with narrow particle size distribution. The results showed that duration of homogenization also are important and prolong homogenization time using high shear homogenizer produced larger particles. In this regard it seems that an appropriate homogenization time is needed and prolonging the time increases the chance of the inner phase droplet collision and increase of the average particle size could be possible.

Protein content of the aqueous phase at different concentration from 2.5 -10 % were examined and at these concentration ranges its effect on average size was less important than the other formulation parameters. This finding indicates that drug content of this type of formulations have minimum effect on formulation characteristics. Increase in protein concentration mainly affects the encapsulation efficiency and variation in drug content has minimal impact on size of the particles.

Polymer concentration had profound effect on average particle size. This effect can be attributed to the effect of polymer concentration on the viscosity of the polymeric solution and need more energy to disperse the system. This trend was also reported by Yeo and coworkers during concentration modification from 4 % to 8 %, the particle size increased linearly with increase in polymer concentration (11). However, differences between particle sizes of formulations containing 3% and 4% polymer (PLGA) were not very significant. The results also showed that change in lactide/glycolide ratios did not significantly affect the average particle size of the different formulations having various ratios of lactide/glycolide.

Although phase ratios didn't change the average particle size significantly but according to the results, particle size distribution is a function of the phase ratios, the phases ratio can affect the efficacy of the mixing and homogenization pattern of the emulsion and an optimum ratios would be necessary.

Different surfactants were examined to stabilize the emulsified systems. Evaluation of the particle size in the presence of the different surfactant revealed that Tweens (especially Tween 80) in aqueous phase and span 80 in organic phase are better than the others (ploxamers or span 40) and surfactant concentrations greatly affect the particle size. The effect of surfactant on stability of dispersed phase and also surfactant effect on viscosity of the emulsified system can affect the average size and particle size distribution of the particles.

PVA also was used as stabilizer in outer aqueous phase and its concentration and also molecular weight of PVA plays an important role on average particle diameter. Increase in PVA molecular weight has increasing effect on viscosity of the outer aqueous phase and then can prevent coalescence of the emulsified droplet during the mixing and possible collision between them.

In order to clarify the role of stabilizers, the F29 formulation was designed in the presence of HPβCD as stabilizer. According to earlier reports (26), hydroxypropyl beta cyclodextrin (8% w/v) when added to inner aqueous phase (W_1), was able to stabilize the protein containing delivery systems and can prevent

extensive aggregation. The same results were also obtained during this study (11,37). HPβCD also has significant effect on the release pattern of proteins from the PLGA loaded particles. This effect is directly from the deaggregation effect of HPβCD which facilitate the freely diffusion and movement of the individual protein molecules.The release studies revealed that composite formation (core-shell coating) are able to limit the release rate of the BSA from the nanoparticles as model protein and also diminished the burst effect in PLGA based formulations.

CONCLUSION

In this study, several factors were investigated for their corresponding effects on the characteristics of micro and nanoparticles. According to the results, although compositional variables can affect properties of fabricated particles, the effects of technical variables are more significant. For example, changing the method of W_1 phase addition to organic phase in primary emulsion formation, or the selection of homogenization technique, could reduce the particle size significantly. On the other hand, among the compositional variables, the type of surfactant for both aqueous and oil phases were among the most important variables. Finally, application of a suitable stabilizer such as HPβCD could enhance the release profile by reducing the aggregation. Therefore, applying the optimized conditions for technical and compositional variables, leads to the fabrication of particles with desired characteristics such as particle size, zeta potential and release profile. Using core-shell strategy also led to the formation of composite nanoparticles with excellent release characteristic which met the requirements of a zero order kinetics.

ACKNOWLEDGMENTS

The authors acknowledge the financial support of the Shiraz University of Medical Sciences (grant No. 90-5700). This project was a part of Ph.D thesis and authors greatly appreciate the support of the vice chancellor for research of the Shiraz University of Medical Sciences. Our thanks go to Hassan Khajehei for copy editing of the manuscript.

REFERENCES

1. Rezaei M, Zarkesh-Esfahani SH, Gharagozloo M. The effect of different media composition and temperatures on the production of recombinant human growth hormone by CHO cells. ResPharm Sci. 2012;8:211-217.
2. Hahn SK, Kim SJ, Kim MJ, Kim DH. Characterization and In vivo study of sustained-release formulation of human growth hormone using sodium hyaluronate. Pharm Res. 2004;21:1374-1381.
3. Govardhan C, Khalaf N, Jung CW, Simeone B, Higbie A, Qu S, *et al*. Novel Long-Acting Crystal Formulation of Human Growth Hormone. Pharm Res. 2005;22:1461-1470.
4. Kim SJ, Hahn SK, Kim MJ, Kim DH, Lee YP. Development of a novel sustained release formulation of recombinant human growth hormone using sodium hyaluronate microparticles. J Control Release. 2005;104:323-335.
5. Nandakumar V, Geetha V, Chittaranjan S, Doble M. High glycolic poly (DL lactic co glycolic acid) nanoparticles for controlled release of meropenem. Biomed Pharmacother. 2013;67:431-436.
6. Zhou J, Wang X, Hua K, Zhang W, Ji J, Yang X. Enhanced mechanical properties and degradability of poly (butylene succinate) and poly (lactic acid) blends. Iran Polym J. 2013;22:267–275.
7. Wei G, Lu LF, Lu WY. Stabilization of recombinant human growth hormone against emulsification-induced aggregation by Pluronic surfactants during microencapsulation. Int J Pharm. 2007;338:125–132.
8. Weert Mvd, Hennink WE, Jiskoot W. Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm Res. 2000;17:1159-1167.
9. Crommelin DJA. Formulation of Biotech Products, Including Biopharmaceutical Considerations. In: Crommelin DJA, Sindelar RD, editors. Pharmaceutical Biotechnology : An Introduction for Pharmacists and Pharmaceutical Scientists. London: Taylor & Francis Routledge; 2002. p. 73.
10. Brange J. Physical Stability of Proteins. In: S. Frokjaer, L. Hovgaard, editors. Pharmaceutical Formulation Development of Peptides and Proteins. London: Taylor & Francis Inc; 2000. p. 89-112.
11. Yeo Y, Baek N, Park K. Microencapsulation methods for delivery of protein drugs. Biotechnol Bioprocess Eng. 2001;6:213-30.
12. Arun R, Ashok K. Cyclodextrins as drug carrier molecule: A Review. Sci Pharm. 2008;76:567–598.
13. Yeo Y, Park K. Control of encapsulation efficiency and initial burst in polymeric microparticle systems. Arch Pharm Res. 2004;27:1-12.
14. Kim HK, Park TG. Comparative study on sustained release of human growth hormone from semi-

crystalline poly(L-lactic acid) and amorphous poly(D,L-lactic-co-glycolic acid) microspheres: morphological effect on protein release. J Control Release. 2004;98:115–125.
15. Stevanovic M, Uskokovic D. Poly(lactide-co-glycolide)-based Micro and Nanoparticles for the Controlled Drug Delivery of Vitamins. Curr Nanoscience. 2009;5:1-15.
16. Vrignaud S, Benoit J-P, Saulnier P. Strategies for the nanoencapsulation of hydrophilic molecules in polymer-based nanoparticles. Biomaterials. 2011;32:8593-8604.
17. Chitkara D, Kumar N. BSA-PLGA-based core-shell nanoparticles as carrier system for water-soluble drugs. Pharm Res. 2013; 30:2396-2409.
18. Ma M, Tan L, Dai Y, Zhou J. An investigation of flavor encapsulation comprising of regenerated cellulose as core and carboxymethyl cellulose as wall. Iran Polym J. 2013;22:689–695.
19. Kwak HH, Shim WS, Choi MK, Son MK, Kim YJ, Yang HC, *et al*. Development of a sustained-release recombinant human growth hormone formulation. J Control Release. 2009;137:160-165.
20. Takada S, Yamagata Y, Misaki M, Taira K, Kurokawa T. Sustained release of human growth hormone from microcapsules prepared by a solvent evaporation technique. J Control Release. 2003;88:229-242.
21. Kim HK, Park TG. Microencapsulation of dissociable human growth hormone aggregates within poly(D,L-lactic-co-glycolic acid) microparticles for sustained release. Int J Pharm. 2001;229:107–116.
22. Mukherjee B, Santra K, Pattnaik G, Ghosh S. Preparation, characterization and in-vitro evaluation of sustained release protein-loaded nanoparticles based on biodegradable polymers. Int J Nanomedicine. 2008;4:487–496.
23. Feczk OT, Toth J, Gyenis Ja. Comparison of the preparation of PLGA–BSA nano- and microparticles by PVA, poloxamer and PVP. Colloids and Surfaces A: Physicochem Eng Aspects. 2008;319:188–195.
24. Ravi S, Peh KK, Darwis Y, Murthy BK, Singh TRR, Mallikarjun C. Development and characterization of polymeric microspheres for controlled release protein loaded drug delivery system. Indian J Pharm Sci. 2008;70:303-9.
25. Feczkó T, Tóth J, Dósa G, Gyenis J. Optimization of protein encapsulation in PLGA nanoparticles. chemical engineering and processing. 2011;50:757–765.
26. Feczkó T, Tóth J, Dósa G, Gyenis J. Influence of process conditions on the mean size of PLGA nanoparticles. Chemical Engineering and Processing: Process Intensification. 2011;50:846-853.
27. Makadia HK, Siegel SJ. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. Polymers (Basel). 2011;3:1377–1397.
28. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-254.
29. Samani SM, Montaseri H, Kazemi A. The effect of polymer blends on release profiles of diclofenac sodium from matrices. Eur J Pharm Biopharm. 2003;55:351-355.
30. Ritger PL, Peppas NA. A simple equation for description of solute release II. Fickian and anomalous release from swellable devices. J Control Release. 1987;5:37-42.
31. Lamprecht A, Ubrich N, Pe´rez MH, Lehr C-M, Hoffman M, Maincent P. Influences of process parameters on nanoparticle preparation performed by a double emulsion pressure homogenization technique. Int J Pharm. 2000;196:177–182.
32. Pachuau L, Mazumder B. A study on the effects of different surfactants on Ethylcellulose microspheres. Int J Pharm Tech Research. 2009;1:966-971.
33. Vandervoort J, Ludwig A. Biocompatible stabilizers in the preparation of PLGA nanoparticles: a factorial design study. Int J Pharm. 2002;238:77–92.
34. Harisha RS, Hosamani KM, Keri RS, Shelke N, Wadi VK, Aminabhavi TM. Controlled release of 5-flurouracil from biomedical polyurethanes. J Chem Sci. 2010;122:209-216.
35. Adams LLA, Kodger TE, Kim S-H, Shum HC, Franke T, Weitz DA. Single step emulsification for the generation of multi-component double emulsions. Soft Matter. 2012;41;10719-10724.
36. Burapapadh K, Takeuchi H, Sriamornsak P. Pectin-based nano-sized emulsions prepared by high-pressure homogenization. Adv Mat Res. 2012;506:286-289.
37. Johnson OL, Jaworowicz W, Cleland JL, Bailey L, Charnis M, Duenas E, *et al*. The stabilization and encapsulation of human growth hormone into biodegradable microspheres. Pharm Res. 1997;14:730-735.