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Preparation of protein-loaded PLGA-PVP blend nanoparticles by nanoprecipitation method: entrapment, Initial burst and drug release kinetic studies

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ABSTRACT:

Objective(s): Despite of wide range applications of polymeric nanoparticles in protein delivery, there are some problems for the ûeld of protein entrapment, initial burst and controlled release profile.

Materials and Methods: In this study, we investigated the influence of some changes in PLGA nanoparticles formulation to improve the initial and controlled release profile. Selected parameters were: pluronic F127, polysorbate 80 as surfactant, pH of inner aqueous phase, L/G ratio of PLGA polymer, volume of inner aqueous phase and addition of polyvinylpyrrolidone as an excipient. FITC-HSA was used as a model hydrophilic drug. The nanoparticles were prepared by nanoprecipitation.

Results: Initial release of FITC-HSA from PLGA-tween 80 nanoparticles (opt-4, 61%) was faster than control (PLGA-pluronic) after 2.30 h of incubation. Results showed that decrease in pH of inner aqueous phase to pI of protein can decrease IBR but the release profile of protein is the same as control. Release profile with three phases including a) initial burst b) plateau and c) final release phase was observed when we changed volume of inner aqueous phase and L/G ratio in formulation. Co-entrapment of HSA with PVP and pluronic reduced the IBR and controlled release profile in opt-19. Encapsulation efficiency was more than 97% and nanoparticles size and zeta potentials were mono-modal and -18.99 mV, respectively.

Conclusion: In this research, we optimized a process for preparation of PLGA-PVP-pluronic nanoparticles of diameter less than 300 nm using nanoprecipitation method. This formulation showed a decreased initial burst and long lasting controlled release profile for FITC-HSA as a model drug for proteins.

Keywords: *Controlled release profile, FITC-HSA, Initial burst release, PLGA nanoparticles, Protein delivery*

INTRODUCTION

Nanoparticles have wide range applications in medicine and pharmacy, because of their specific properties [1]. In particular; biopolymeric nanoparticles have become increasingly attractive due to biocompatibility and biodegradability [2]. The design of nano-sized biodegradable polymeric nanoparticles to target tissues has certain unique advantages in drug delivery, pharmacology and tissue engineering [3, 4]. These novel formulations reduce therapeutic dosage, related toxicity and side effects [5-7]. Polymeric nanoparticles have wide applications in biomedicine [8] and especially for the ûeld of nanobiotechnology, nanoparticles composed of poly (D, L-lactide-co-glycolide) (PLGA) have shown several technological advantages such as sustained and controlled release and safety [9, 10]. PLGA is a copolymer of poly (D, L-lactide-co-glycolide) and is an ideal candidate of biodegradable polymers for

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formulation into nanoparticles [11-13]. The PLGA based colloidal systems are stable in biological fluid and are suitable for protein encapsulation due to their long half-life [14]. In the recent years, proteins have been proved to be very effective candidates for therapeutic use in a large number of human diseases [15, 16]. Proteins are hydrophilic drugs and thereby are unstable and degradable in the body system and therefore need effective drug delivery devices to complete protection from enzymes degradation and loss of their activity [17]. Polymeric nanoparticulated systems such as PLGA nanoparticles can control protein release kinetics and ease of administration in addition of increasing protein stability and activity in the biological systems [18]. However, the bulk core of PLGA nanoparticles is hydrophobic and can dissolve only hydrophobic drugs and hence nanoparticles formulated using PLGA polymers, demonstrate low proteintaceus drug encapsulation efûciency and rapid release [19]. In this research we demonstrated a new strategy by using of polyvinylpyrrolidone (PVP) to enhance encapsulation of FITC- HSA as a model hydrophilic drug into the hydrophobic core of PLGA nanoparticles. PVP or povidone, is soluble in water and polar solvents and made from monomeric unite of vinylpyrrolidone [20]. PVP was synthesized in 1939 after development of acetylene chemistry at BASF in the 1920 by Walter Reppe [21]. The U.S. Food and Drug Administration (FDA) has approved PVP.PVP was first as a substitution and expander for blood plasma. Its wide range solubility in conventional solvents, biocompatibility and non-toxicity characteristics gave it numerous applications in medicine, pharmacy and cosmetics. Some of advantageous characteristics of PVP are shown in Table 1. PVP has a long, proven history of pharmaceutical applications as delivery systems of poorly soluble drugs [22]. It also possesses a high degree of biocompatibility with the possibility to control the rate of drug release so as to improve the *in vivo* pharmacokinetics [23]. PVP has also been shown to inhibit drug crystallization in solution as well as in the solid state and protects against degradation in solution [24].The aim of this work was to solubilize water-soluble drug molecules in polymeric organic solution and make the homogeneous dispersion of protein with PLGA chains for nanoparticle formation during nanoprecipitation methods.We have investigated the effects of various formulation parameters on encapsulation efficiency and initial burst release (IBR) of FITC-HSA in comparison with PLGA-PVP blend nanoparticles.

MATERIALS AND METHODS

PLGA (poly(D,L-lactide-co-glycolide)) with 50:50 monomer ratio and inherent viscosity midpoint of 0.4 dl/g was purchased from purac-Hollandunder the commercial name of Purasorb®PDLG 5004. The pluronic F-127, polyvinylpyrrolidone (PVP) with molecular weights of K10 and fluorescein isothiocyanate conjugated to human serum albumin (FITC-HSA) were obtained from Sigma Aldrich. PVP with molecular weights of K15 and K25 were purchased from Fluka. Other chemicals and solvents used were of the highest grade commercially available.

Preparation of FITC-HSA loaded PLGA nanoparticles

Nanoparticles were formulated by a nano-precipitation technique [25]. FITC-HSA solution in water (1 mg/200 µl and pH=7.0) was emulsified into PLGA-Pluronic solution in organic solution (ratio of 50/50 mg in 2 ml dichloromethane). The primary emulsion was mixed by vortexing (3000/min, Vibromix) for 30s and then was poured onto a polar phase (25ml ethanol) under moderate magnetic stirring leading to immediate polymer nanoprecipitation in the form of nanoparticles. This nanoparticle suspension was immediately diluted with 25ml water and stirred using a magnetic stirrer for 10 min. Solvent was evaporated under vacume at 30°C (BUCHI, Rotavapor R-114, Germany). Nanoparticles were reco-vered by centrifugation (1 h, 8,000 × g, 15°C, model) and resuspended in water or PBS.

Preparation of different formulations for protein entrapment and release kinetic studies

In order to evaluate the effect of the different formulations on the entrapment efficiency and kinetic of drug release, some variables or modification in formulation were investigated (the effect of pH, L/G ratio, volume of internal aqueous phase, tween 80 instead of pluronic and PVP as excipient with/without pluronic. Individual exper ments for preparation of PLGA nano-particles are summarized in Table 2.

Preparation of PLGA-PVP and PLGA-PVP-pluronic blend nanoparticles

PLGA-PVP with/without pluronic blend nanoparticle was prepared by nano-precipitation method. Briefly,

an amount of PLGA (50/50 ratio) and PVP (K10, 15 and 25) with/without pluronic were dissolved in 2 ml of dichloromethane. This organic solution was mixed by magnet stirrer and poured in polar phase (25 ml ethanol). Nanoparticles solution was diluted with distilled water and solvent was evaporated under vacuum at 30°C. Nanoparticles were collected by centrifugation and resuspended in water or PBS. The method for preparation of FITC-HSA loaded PLGA-PVP nano-particles was the same as method for preparation of HSA loaded PLGA nano-particle.

Characterization of nanoparticles

Size and zeta potential determination

Particle size analyzing for determination of dimension was performed by means of a Coulter LS230 Laser Diffraction Particle Size Analyzer equipped with small volume module plus. 30% obscuration of PIDS detector was used for nanoparticle suspension size analysis with three runs on each sample. Deionized water was used as background. Zeta potential of the nanoparticles was determined by Beckman coulter delsa™ nano zeta potential and submicron particle size analyzer. Nanoparticles were dispersed in deionized water and were subjected to zeta potential analysis at room temperature.

Scanning electron microscopy (SEM)

SEM experiments were carried out on a JEOL LSM5600LV scanning electron microscopy. The nanoparticles samples were puriûed by centrifugation. The pellets were frozen at -20°C, freeze-dried in a 5Pascal Lio 5P lyophilizator. These powders were used for gold sputtering and SEM analysis.

Determination of FITC-HSA entrapment efficiency and yield of nanoparticle production

Protein entrapment efficiency was calculated from total FITC-HSA added in formulation and the amount of FITC-HSA present in the supernatant samples recollected after centrifugation of nanoparticle suspension. Total HSA content was calculated as the sum of FITC-HSA amount both in the supernatant and entrapped in nanoparticles. The amount of FITC-HSA present in the supernatant was determined by fluorescence spectroscopy (excitation/emission wave-lengths of 495/518 nm) using spectro-photometer. Drug entrapment efficiency was calculated as the percent of the total HSA added in formulation. Standard curve was obtained by plotting the fluorescence intensity of FITC-HSA versus the concentration of HSA (mg/ml). To measure yield of nanoparticle production, nano-particle suspension in pre-weight falcon tube was centrifuged and the pellet was frozen at -20°C, freeze-dried in a 5Pascal Lio 5P lyophilizator. Yield of nanoparticle (%w/w) was calculated as the percent of the total polymers (PLGA, PVP and pluronic) were used in formulation.

In vitro release kinetic studies

In vitro release kinetic of nanoparticle encapsulated drug was determined during one month. Nanoparticles pellet was re-dispersed in 25 ml phosphate buffered saline (PBS, pH=7.4) after centrifugation. The nanoparticle suspension was incubated at 37°C with horizontal shaking (100 rpm). At determined time intervals, 1 ml of medium was centrifuged and the amount of FITC-HSA present in the supernatant was determined by fluorescence spectroscopy. Drug concentration in the release medium and cumulative release profiles were calculated using calibration curve.

Cell culture and in vitro cell cytotoxicity assessment

The human fibroblast cell line was obtained from national cell bank of Iran (Pasteur institute of Iran, Tehran) and cultured in DMEM medium supplemented with 10% heat-inactivated FBS and 100U/mL pen-icillin and 100 µg/mL streptomycin at 37ºC in humidiûed incubator with an atmosphere of 5% CO_2. The cell cytotoxicity of PLGA-PVP blend nanoparticles was evaluated by MTT assay. Briefly 2×10^4 cells/mL were seeded in 96-well culture plates containing 200 µl of medium in triplicate, allowed to attach, grow for 24 h and subsequently the culture medium was replaced by fresh medium containing nanoparticles (0.5-10 mg/mL), then incubated for 48 h. The wells without drug and nanoparticle were used as control. Negative control cells were treated with DMSO. After treatment, media were carefully removed and 20 µl of MTT (5 mg/ml) is added to each well. Microplates were incubated for 4 h at 37ºC and then the medium was removed; formazan was dissolved in 200 µl of acidic isopropanol (0.04-0.1 N HCl in absolute isopropanol). Formazan absorbance was assessed at 490 nm using a microplate reader. A wavelength of 630 nm was used as the reference. The results are given as relative values to the negative control in percentage, whereas the untreated (positive) control is set to be 100% viable. The data were analyzed using Student's t-test with significant difference at $P < .05$.

Table 1. Some advantageous characteristics of polyvinylpyrrolidone

Broad specterum solubility in hydrophilic and hydrophobic solvents
Good affinity to different kinds of polymers and substrates
High hygroscopicity
Good adhesiveness to various substrates and formation of stable complex with active substances
Formation of chelate or complex
Improve the release rate, bioavailability and solubility of drugs
Reduction of drugs toxicity
Stabilization of suspension
Homogeneous distribution of substances in solution or in the coating

Table 2. Individual experiments with different formulations for protein encapsulation and release

Experiments	PLGA (50/50) ratio (mg)	PLGA (75/25) ratio (mg)	Pluronic (mg)	PVP (mg)	Tween 80 (µl)	aqueous phase volume (µl)	aqueous phase pH
Control [a]	50	-	50	-	-	200	7.0
Opt-1	50	-	50	-	-	100	7.0
Opt-2	-	50	50	-	-	200	7.0
Opt-4 [b]	50	-	-	-	50	200	7.0
Opt-5	50	-	50	-	-	200	3.0
Opt-6 [c]	50	-	-	50 (K25)	250	200	7.0
Opt-7	50	-	-	50 (K10)	250	200	7.0
Opt-8	50	-	-	50 (K15)	250	200	7.0
Opt-11	50	-	25	25 (K25)	-	200	7.0
Opt-17 [d]	25	-	41	13 (K15)	-	200	7.0
Opt-18 [e]	25	-	41	13 (K10)	-	200	7.0
Opt-19 [f]	25		41	13 (K25)	-	200	7.0

RESULTS

Entrapment efficiency and in-vitro release kinetic studies

All of entrapment efficiencies for different formulations were higher than 90%. The entrapment efficiency was increased more than 97% by adding of PVP in nanoparticles formulation (Table 3, opt-6 and opt-17, 18, 19). A nanoprecipitation method was used for preparation of PLGA nanoparticles with different formulations.

The release of FITC-HSA from PLGA nanoparticles was monitored in PBS (pH=7.4), 100 rpm at 37°C for more than one month. Percentage of protein that had been released was shown in Figs. 1, 2 and 3. FITC-HSA loaded PLGA-pluronic blend nanoparticles were considered as control formulation in release kinetic studies. Nanoparticles were prepared as noted in materials and methods and monitored for protein release in PBS. In this research, our aims were decreasing of IBR and increasing duration of the release profile. For these resions, we changed some of factors in control formulation (e.g tween 80 as surfactant, pH of inner aqueous phase, L/G ratio of PLGA polymer, volume of inner aqueous phase and addition of PVP with different molecular weight as excipient). IBR was measured after 2.30 h of incubation. IBR in control formulation (Fig. 1) is about 58% of the nanoparticle bounded FITC-HSA after 2.30 h. The release profile was increased and continued with constant slope during 10 days and more than 80% of total entraped protein, released to the PBS solution. Addition of tween 80 instead of pluronic in organic phase (opt-4) and change of pH of inner aqueous phase (opt-5) increased and decreased IBR of protein, respectively (Fig. 1). IBR in opt-4 is the same as control formulation, but release was dramatically increased and more than 85% of entraped protein released into the PBS after 24 h of incubation. These data indicate that the rate of release of HSA is enhanced due to use of tween-80. Change in pH of inner aquoes phase (opt-5) considerably decreased IBR. A slow IBR was observed in PLGA-pluronic nanoparticles when pH of aquoes phase containing of HSA was decreased below pH of isoelecteric point of HSA protein (pH=3.0<4.5). In this formulation, 37% of total proteins were released during 2.30 h of incubation in PBS (pH=7.4) at 37°C. But the release profile was the same as control and opt-4 during

10 days. Release profiles from formulations with change in volume of inner aquoes phase (opt-1), L/G ratio (opt-2) and addition of PVP as excipient with/without pluronic in formulation (opt-6, opt-19) were completely different from release profile of control formulation (Figs. 2 and 3). IBR is 50% for opt-1 after 2.30 h of incubation (Fig. 2). An slow release profile was observed to 10% of total drug loaded in nanoparticles between 1-13 days and continued by a final release up to 85% between 13 to 18 days of incubation. The same profile was observed for opt-2 formulation. IBR is 46% after 2.30 h. This phase was continued and 66% of proteins were released during 2 days of incubation. The release profile showed a constant slop with slow increasing of protein release till 18 days of incubation. The significant influence of addition of PVP instead of pluronic on IBR was observed in PLGA-PVP nanoparticles. Lower IBR was observed in opt-6 and there was 37% of HSA released during the 2.30 h of incubation and 58% of total drugs was released during 2 days of incuation. The plateau phase was observed between 2-10 days of incubation and continued by a final release of 20% of total drug during 10-18 days. Addition of PVP in formulation with pluronic (opt-19) resulted in change in IBR and duration (Fig. 3). IBR and duration were decreased and increased in opt-19 formulation (with pluronic as surfactant), respectively. 35% of total drugs were released during 2.30 h of incubation and there is a plateau phase between 1-16 days of incubation. No significant release was not observed during this phase. Final release was observed after 16 days and reached to 75% of total protein. In this formulation, the quantity of PLGA, PVP and pluronic are optimized and nanoparticle size is 297±52 nm with mono-modal distribution (Table 3).

Nanoparticles characterization: SEM, nanoparticle size and distribution

Nanoparticles morphology was investigated by SEM. All nanoparticles showed a swell-defined spherical shape with nano-size (Figs. 5 and 6). The size of nanoparticles were depended on the formulation conditions. Decreases of volume and pH of inner aquoeus phase (opt-1 and 5 respectively) and use of tween 80 instead of pluronic in organic phase (opt-4) resulted in decreases of nanoparticle size to 132, 124 and 149 nm with mono-modal distribution, respectively (Table 3).The nanoparticle size was increased and ranged between 250-297 nm, when formulations were changed in amount of pluronic and PVP. The nanoparticles size was increased when 50 mg of PVP was used instead of pluronic in opt-6 formulation.The average size of PLGA-PVP nanoparticles was 250±32 nm with tri modal-distribution (Fig. 4). The other two populations (3 and 200 µm) were observed with very low number. Average size of nanoparticles ranged 169±34 nm in control formulation (Fig. 5). Opt-19 is the optimized formulation (Fig. 6). In these formulation, PVP was used in combination with pluronic to improve of nanoparticles size and distribution, initial burst release and stability. All of nanoparticles were mono-odal and very stable. Nanoparticles size is 297±52 nm with mono-modal distribution.

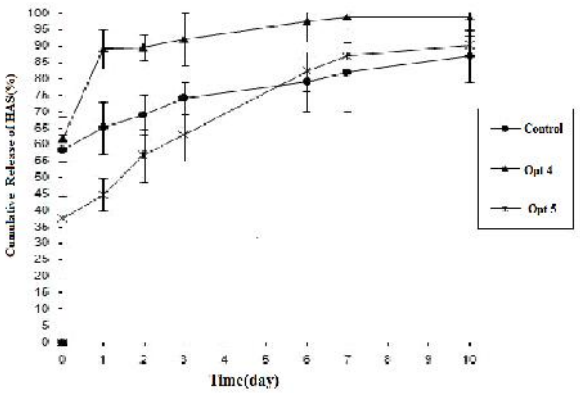

Fig. 1. The influence of the tween-80 (²%), pH (×) and pluronic (control, Ï%) on the FITC-HSA in vitro release from PLGA nanoparticles incubated in PBS (pH=7.4) at 37 °C and 100 rpm

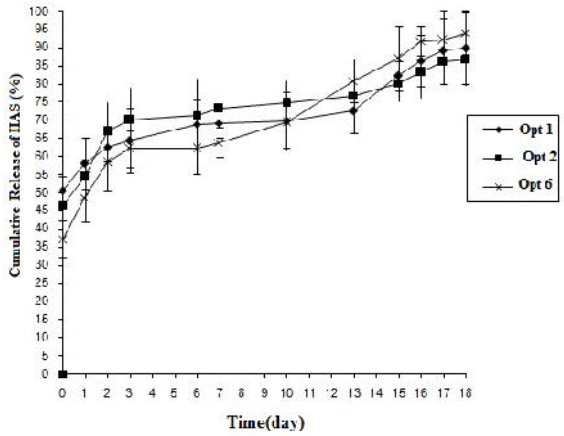

Fig. 2. The influence of the inner water volume (f&), L/G ratio (%) and PVP instead of pluronic (×) on the FITC-HSA in vitro release from PLGA nanoparticles incubated in PBS (pH=7.4) at 37 °C and 100 rpm

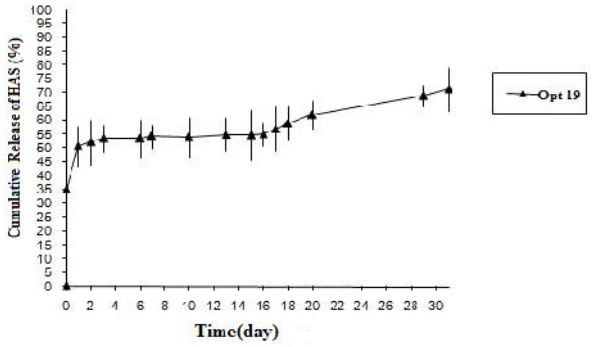

Fig. 3. The influence of the inner water volume (f&), L/G ratio (%) and PVP instead of pluronic (×) on the FITC-HSA in vitro release from PLGA nanoparticles incubated in PBS (pH=7.4) at 37 °C and 100 rpm

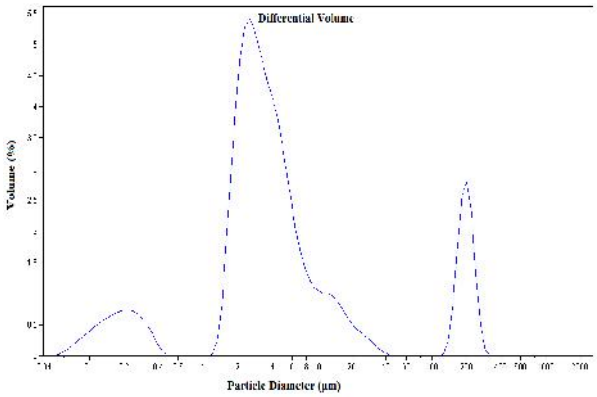

Fig. 4. Particle size distribution of FITC-HSA-loaded PLGA-PVP nanoparticles (Opt 6) prepared by nanoprecipitation method

Homogeneous distribution of FITC-HAS

We observed two different regions with different colors in nanoparticles pellet after nanoprecipitation. Aqueous suspension of FITC-HSA is in yellow color. In general, hydrophilic properties of HSA prevent it to dissolve homogeneously in organic phase (DCM phase). Heterogeneous dispersion of HSA was observed in PLGA nanoparticle in control formulation after addition of first emulsion (water phase in organic solution) into the ethanol phase. Two regions were observed in nanoparticles pellet (Fig. 7-A). The region R_1 is in white color and region R_2 is in yellow color. Homogeneous distribution of FITC-HSA molecules was observed within the nanoparticles pellet when PVP was used without/ with of pluronic in fromulations of opt-6 and opt-19 respectively (Fig. 7-C, B). The united yellow color was observed in whole nanoparticles pellet in both formulations.

Yield of PLGA nanoparticles

The effect of concentration of PVP on the yield of lyophilized PLGA nanoparticles is shown in Table 4. The yield of control formulation based on polymers and surfactant used in formulation is 39%. The yield of PLGA nanoparticles is increased and reached a value of about 60%, when 50 mg of PVP was used instead of pluronic (opt-6). We used tween 80 as surfactant in ethanolic phase (1% v/v) in this formulation. The percentage of yield decreased in opt-19 to a value of 33% in comparision with control formulation, when w/w ratio of Pluronic/PLGA increased from 1 to 1.65 and w/w ratio of PVP/PLGA decreased from 1 to 0.5. As a result, the yield of nanoparticle formations was increased as the concentration of PVP increased. The yild in PLGA-Pluronic nanoparticle (control formulation) was low in comparision of formulation with PVP addition (opt-6). The zeta potential and mobility of opt-19 nanoparticles were -18.99 mV and -1.483e-004 Cm^2/Vs, respectively and nanoparticles were stable in suspension.

In vitro cytocompatibility assay

Cytocompatibility of prepared PLGA-PVP nanoparticles were assessd by MTT test. The viability of fibroblast cells treated with 0.5, 1, 2, 4 and 6 mg /mL of nanoparticles was 98%, 87%, 63%, 11% and 1.8%, respectively. Results showed PLGA-PVP nanoparticles have good cytocompatibility (IC_{50}=2.4 mg/ml).

DISCUSSION

Low protein encapsulation efficiency and rapid initial burst release (IBR) are major obstacles for application of nanoparticles in nanomedicine (26). Thereby, hydrophilic molecules of proteins with short physiological half-life need to the delivery systems to enhance entrapment, their half-life and controlled release after administration (27, 28). The main objects of this research were to investigate different formulations of PLGA nanoparticles for enhanced entrapment efficiency, controlled initial burst and *in vitro* release of FITC-HSA as a model proteinaceous drug. It had been shown that PLGA:pluronic

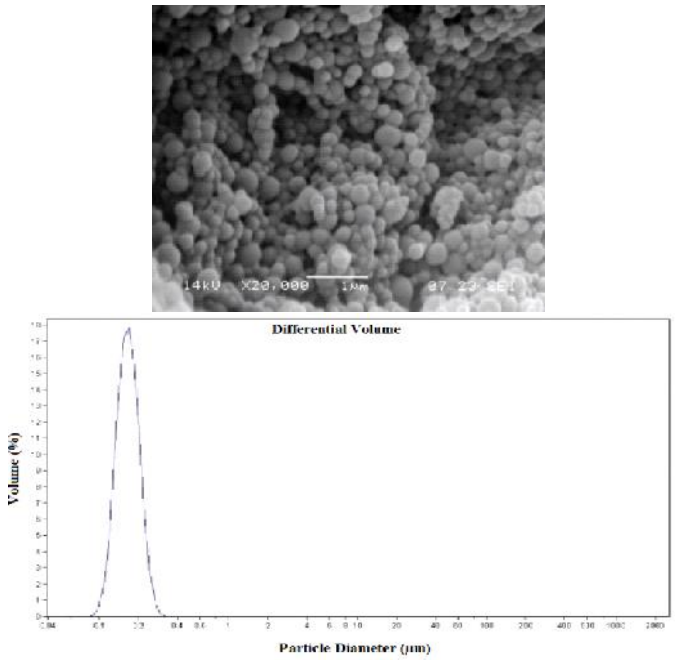

Fig. 5. Scanning electron micrograph and particle size distribution of FITC-HSA-loaded PLGA-pluronic nanoparticles prepared by nanoprecipitation method. Average size of nanoparticles ranged 169±34 nm (control formulation)

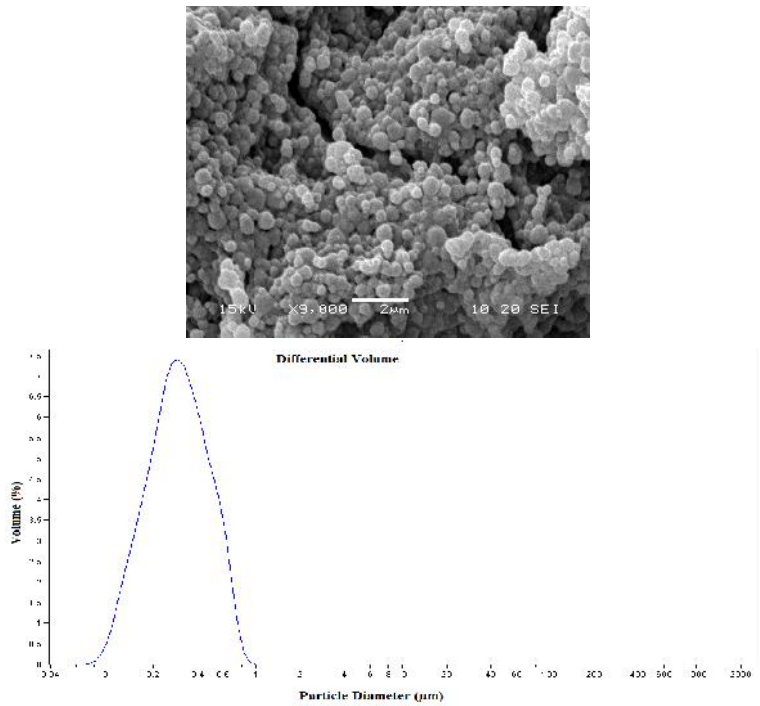

Fig.6. Scanning electron micrograph and particle size distribution of FITC-HSA-loaded PLGA-PVP-pluronic nanoparticles prepared by nanoprecipitation method (Opt-19). Nanoparticles size is 297±52 nm with mono-modal distribution

Table 3. Entrapment efficiency (%) and initial burst release of PLGA nanoparticles: effect of volume of aqueuse phase, L/G ratio, surfactant (pluronic and tween 80), polyvinylpyrrolidon and effect of pH of aqueouse phase

Experiments	Entrapment efficiency (%)	Size (nm)	Initial burst release (%) after 2.30 h	Size distribution
Control	92	169±34	58%	Mono-modal
Opt-1	95	132±52	50%	Mono-modal
Opt-2	91	174±47	46%	Mono-modal
Opt-4	93	149±33	61%	Mono-modal
Opt-5	95	124±25	37%	Mono-modal
Opt-6	96	250 nm,3 µm, 200 µm	37%	Tri-modal
Opt-7	ND	600 nm,2 µm, 5 µm, 15 µm	ND	Tetra-modal
Opt-8	ND	2 µm	ND	Mono-modal
Opt-11	>97%	155 nm, 3 µm	45%	Bi-modal
Opt-17	>97%	271±42	34%	Mono-modal
Opt-18	>97%	263±58	38%	Mono-modal
Opt-19	>97%	297±52	35%	Mono-modal

Table 4. Yield of nanoparticle production with various formulations via nanoprecipitation method

Factors (mg)	Control	Opt-6	Opt-19
PLGA (mg)	50	50	25
PVP (mg)	---	50	13
Pluronic (mg)	50	---	41
PLGA+PVP+pluronic (mg)	100	100	79
PVP/PLGA ratio (w/w)	---	1	0.52
Pluronic/PLGA ratio (w/w)	1	---	1.65
Nanoparticle (mg)	39±2	60±3	26±1
Yield (based on PLGA+PVP +pluronic)	39±2%	60±3%	33±2%

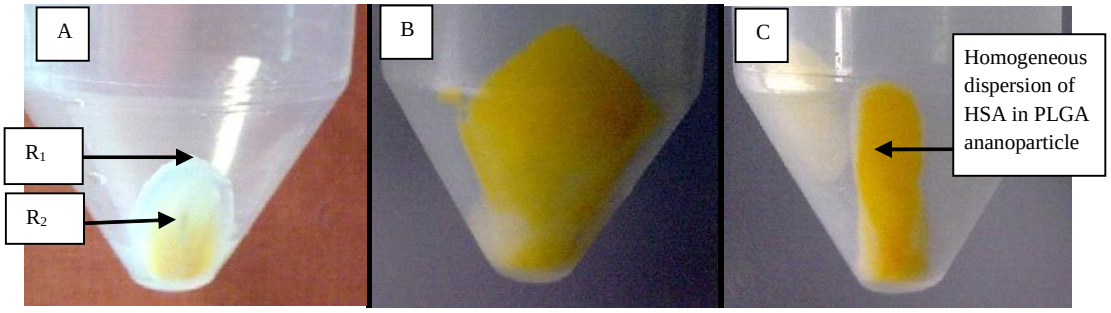

Fig. 7. The effect of different concentrations of polyvinylpyrrolidon on dispersion of FITC-HSA in nanoparticles pellet. A) Heterogeneous dispersion of HSA in control formulation. Two regions were observed with white and yellow colors. B) Homogeneous dispersion of HSA in PLGA nanoparticle (opt-6) with formulation of of PLGA 50 mg, polyvinylpyrrolidon 50 mg and HSA-FITC 1 mg/200 µl water. Tween 80 (1%) was used as surfactant in ethanol phase, only one region was observed in nanoparticle pellet in yellow color. C) Homogeneous dispersion of HSA in PLGA nanoparticle (opt-19) with formulation of PLGA 25 mg, pluronic 41 mg, polyvinylpyrolidon 13 mg and HSA-FITC 1 mg/200 µl water, only one region was observed in nanoparticle pellet in yellow color

nanoparticles encapsulate hydrophilic proteins with minimum loss in their biological activity [29, 30] and protect them against degradation by acidic condition [10]. But, IBR is one of the main problems that still associated to PLGA nanoparticles as protein delivery system [31]. Initial drug release and long time release during incubation time are dependent to composition of nanoparticles formulation. There were two separate release phases based on release kinetic for opt-4, opt-5 and control formulations. The release profiles were

biphasic. The first phase is acceleration or initial burst release during 2.30h of incubation. The main reasons for IBR are deficiently protein entrapment and weakly attachment on the surface of the PLGA nanoparticles. This release profile was continued by a constant release with increasing slop during 10 days in opt-4, 5 and control. This phase named as final release phase or secound phase. This phase is related to protein release from nanoparticles matrics. The release of HSA from PLGA-tween 80 nanoparticle formulation (opt-4) was faster than control (PLGA-pluronic). The results showed that PLGA nanoparticles prepared in opt-4 formulation have a higher initial burst (61%) than those prepared by control formulation reached up to 85% after 1 day. Tween 80 instead of pluronic not only increased the IBR, but also final release was ocurred during 1 day of incubation. The most of HSA was released during acceleration phase and only less than 10% was release during final release phase. The weak interaction between HSA and PLGA polymer or inhibitory effect of tween 80 on adsorbtion of protein to the polymer chains are probable resions for fast release of drug in the first phase. The results showed that the most of protein molecules are attached weakly on the surface of PLGA nanoparticles not inside of core of nanoparticles. The use of tween-80 as a surfactant instead of pluronic reduced the affinity of HSA to entrap inside of PLGA nanoparticles. The results suggested that tween 80 has an inhibitory role in protein entrapment. These results could be explained by the reduced HSA–PLGA interaction caused by the presence of tween 80, as a surfactant. Results showed that decreases of pH of inner aqueous phase to pI of protein can decrease IBR but not duration of protein release. Proteins are stable in pH 6.5-7.0, but protein refolding, aggregation and formation of proteins fibrils take place at a pH close to pI of proteins [32]. Change of pH in opt-5 formulation can affect physic-chemical stabilities of proteins. There is a new phase in addition of IBR and final release phases in opt-1, 2, 6 and 19. Initial burst release or acceleration phase was continued by a deceleration phase and then there is a plateau phase before final release phase. The slow release or plateau phase observed when we changed volume of inner aqueous phase, L/G ratio and when we used PVP without or with pluronic in formulation. PLGA with L/G ratio of 75:25 is less hydrophilic than copolymer with a 50:50 ratio of L/G [33]. This hydrophobic nature is due to lactic acid monomers. So, this kind of PLGA degrades in a longer time in comparison of more hydrophilic PLGA [34, 35]. Results indicate that, in the absence of pluronic, HSA was very efficiently encapsulated in the opt-6 formulation. The results showed that the FITC-HSA initial burst release from opt-6 and 19 is lower than nanoparticles prepared by formulations without PVP. This could be interpreted to the enhanced entrapment of the protein molecules inside of the nanoparticles matrix by application of PVP in formulations. Opt-6 wasn't the best formulation for nanoparticle preparation and some works were needed to find the optimized formulation for nanoparticles size, mono-modal distribution and long time release. However, the best formulation for protein encapsulation and release was opt-19 (PLGA-PVP-pluronic). On the other hand, the mono-dispersed nanoparticles size and long time release of protein were observed in opt-19 formulation by the incorporation of the PVP and pluronic together. PVP has wide range solubility in conventional solvents and has a long, proven history of applications as delivery systems for poorly soluble drugs (20). PVP inhibits and protects drug crystallization and degradation in solution [24]. We did not analyze inner structure of nanoparticles in opt-19 which could affect the release of the encapsulated Protein, but the size of nanoparticles is bigger than other formulations. As shown in Table 3, the size of the nanoparticles in opt-6 and opt-19 formulations was highly affected by introduction of PVP in formulation with or without pluronic. The increase in EE% and decrease in IBR maybe due to the stabilizing property of PVP. Stabilizers and their concentration are important factors in nanoparticles size, homogeneous or heterogeneous distribution of nanoparticles, entrapment efficiency and release rate profile [33]. Coombes et al [36] used PVP as stabilizer for ovalbumin (OVA) loaded microparticles. Their results showed that PVP enhanced entrapment efficiency and release rate profile. Nanoparticles based on PLGA-pluronic (control formulation) showed faster onset of initial and final release than nanoparticles based on PLGA-PVP-pluronic.

Hydration property of nanoparticles and formation of water channels inside of nanoparticles structure increase by pluronic and thereby resulted in initial

burst and faster final release [10, 29, 30]. Amphiphilic property of PVP make this polymer soluble in water and many organic solvents [37]. Addition of PVP in formulation changes hydrophobic or hydrophilic properties of nanoparticles. Its potential as a hydrophilizing agent for biomaterials, reduce hydrophobic interactions in the surface and matrix of PLGA nanoparticles [21, 38]. In the other hand, hydrophilic property of protein and its interaction with polymer chains and nanoparticles matrix determine protein entrapment and IBR [31, 39]. The best interaction between proteins molecules and PLGA matrix resulted in entrapment of protein inside of nanoparticles with high efficacy. It has been reported that conjugation of PVP with proteins prevents them from autolysis, degradation and enhances half life of proteins [40-43]. This conjugation can control initial burst and release profile as tailored made nanoparticle [44]. Applications of PVP nanoparticles in drug delivery have been shown.This kind of nanoparticles can evade fro reticoloendothelial system and have long time circulation in blood stream [22, 23]. These results showed that PVP as a nanoparticle, conjugate with proteins or as a polymeric modifier in combination with other biomaterials (e.g., PLGA and pluronic) is a good candidate for delivery of protein and other hydrophilic therapeutic agents like as plasmid DNA [45]. In this paper we optimized a process for preparation of PLGA-PVP-pluronic nanoparticles of diameter less than 300nm using nanoprecipitation method. This formulation showed an decreased initial burst and long time controlled release profile for FITC-HSA as a model drug for proteins. These results showed that the release of HSA or proteinaceus hydrophilic drugs from different formulation based nanoparticles is not dependent only to polymer degradation. There are effective factors such as protein properties, surfactants, inhibitors (for inhibition of protein interaction with polymer chains) and stabilizing agants that can affect protein entrapment and release from nanoparticles.

CONCLUSION

In this research, optimized formulation of PLGA-PVP-pluronic nanoparticles were prepared by nano-precipitation method for improvement of initial release and kinetic of hydrophilic model drug. Initial burst release was decreased in this formulation and controlled release profile was observed in a long time for FITC-HSA as a model drug for proteins. In conclusion, PLGA-PVP nanoparticles have good cytocompatibility and can be used as a nanocarrier for controlled release of protein and peptide drugs.

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CONFLICTS OF INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

SS and CE performed the experiments and collected data. RR, GE and EC: Supervisors and designed the experiment and interpreted results. FC and CE: Advisors and interpreted results. SS prepared the manuscript. All authors read and approved the final manuscript.

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