Preparation and characterisation of PLGA-PEG-PLGA nanospheres prepared with a new thermogelling method for insulin delivery

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ABSTRACT

Biodegradable nanospheres based on a symmetric PLGA-PEG-PLGA triblock copolymer were prepared and investigated for insulin encapsulation and release. A PLGA-PEG-PLGA copolymer with a lactide (LA)-to-glycolide (GA) ratio of 3:1 was obtained by thering-opening polymerisation method and characterised via ^{1}HNMR and gel permeation chromatography. The gelation temperatures of different concentrations of copolymer solutions were measured using the inverted test tube method. Nanospheres were prepared in an aqueous solution of 6.6% w/v of copolymer and 1.33% w/v of insulin without using any organic solvent. This new encapsulation method utilised the thermogelling property of the copolymer in the procedure. Under optimum conditions, uniform insulin nanospheres, 230 nm in size, were obtained. Insulin release from the nanospheres was investigated using spectrofluorimetryat $\lambda_{excitation}=280$ nm and $\lambda_{emission}=303$ nm. The number average molecular weight of the copolymer was found to be 4743, and the sol-gel transition temperatures were in the 16–19°C range in terms of copolymer concentration. Drug loading within the nanospheres was about 20%. The insulin release profile exhibited an initial burst release (~30%) followed by a sustained release over three weeks (>85%). The obtained results suggest that nanospheres prepared with this new thermogelling method are a good candidate for encapsulation and insulin release.

Keywords: Biodegradable polymer, PLGA-PEG-PLGA, nanosphere, insulin

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INTRODUCTION

Protein drugs have emerged as potent medicines for various types of widespread human diseases, such as cardiovascular diseases, cancers, and diabetes [1-6].

It is well understood that diabetes has become one of the most serious fatal chronic diseases worldwide. Insulin is one of the most important and commonly used drugs to treat diabetes mellitus patients in clinics[7]. Insulin is a peptide hormone, produced by pancreas β-cells to decrease blood glucose levels[8].

There are some limitations to the pharmaceutical formulation of insulin, such as its short half-life and its degradation by proteolytic enzymes in the human body [9].

In recent decades, due to advancements in nanotechnology, hydrogels [10, 11], polymeric microparticles[12, 13], nano-polymersomes[14], liposomes [15], polyion complex micelles [16], and carbon nanotubes [17] have been established as carriers for systemic controlled protein delivery. These protein delivery applications, however, require the use of organic solvents, which might lead to protein denaturation, require the chemical modifications of proteins, have low protein-loading levels, and/or not be appropriate for clinical administrations.

For the first time, Kwon and Kim[18] encapsulated zinc crystalline recombinant insulin within biodegradable triblock PLGA-PEG-PLGA microspheres in aqueous medium without using an organic solvent. The prepared microspheres exhibited a sustained release pattern within 20 days.

In the present study, to avoid using organic solvent in the preparation of PLGA particles loaded with insulin, the insulin was encapsulated in PLGA-PEG-PLGA nanospheres using the sol-gel transition temperature of the copolymer dissolved in aqueous medium. The particle size was adjusted in the nano-range in order to tailor the drug release profile.

It should be mentioned that in the formulation of systemic controlled release particulate systems, an important pre-requisite is their escaping from macrophages. It has been shown that intermediate size particles (~2 µm) arephagocytosed more rapidly than smaller or larger particles[19-24]. To avoid from phagocytic uptake of particulate controlled-release drug delivery systems, in most studies microspheres larger than 10 µm have been fabricated [18]. But in another study, for the preparation of an insulin controlled release system, formulation of nano-sized polymerosomes in the range of 75-130 nm in size have been described to avoid the phagocytic uptake[20]. Regards to the easy administration and more controllable release profile of nano-range particulate systems, we have tried the PLGA-PEG-PLGA nanospheres for controlled release of insulin.

EXPERIMENTAL SECTION

General

Insulin was obtained from the Novo Nordisk representative in Tehran, Iran. Poly(ethylene glycol) M_n=1000, 3,6-dimethyl-1,4-dioxane-2,5-dione, glycolide and stannous octoatewere purchased from Sigma-Aldrich (USA).

All other chemical reagents and solvents were obtained from Merck (Germany) and used without further purification.

Synthesis of PLGA-PEG-PLGA

PLGA–PEG–PLGA with a lactide (LA)-to-glycolide (GA) ratio of 3:1 was synthesised using a ring-opening polymerisation method as described previously [25, 26].

Briefly, PEG 1000 (60 g) were heated to 150°C and stirred (250 rpm) in a stainless steel reactor under a vacuum (5 mmHg) for 3 h.

In the next stage, D,L-lactide (113.46 g) and glycolide (30.48 g) were added, and the mixture was heated and stirred at 150°C under a vacuum for 30 min. Then, stannous 2-ethylhexanoate (0.04 g) was added as a catalyst. The mixture was further heated at 160 °C under a vacuum for 8 h.

Purification and characterisation of synthesised copolymer

In order to purify the synthesised copolymer from impurities, the product was dispersed in deionised water at 80°C. Then, the temperature of the suspension was reduced to 4°C to dissolve the copolymer completely. The copolymer solution was heated again to 80°C to precipitate the triblock copolymer. The process was repeated three times to obtain a pure copolymer. The purified copolymer was freeze-dried, and the product was kept at -20°C until use.

The ^{1}H-NMR spectrum of the resulted PLGA-PEG-PLGA was recorded on a Bruker Ac-80 spectrophotometer in CDCl₃ at 25°C.

The number average molecular weight (M_n) and LA-to-GA ratio were estimated by integrating the signals pertaining to each monomer, such as the peaks from CH and CH₃ of lactide (LA), CH₂ of ethylene glycol, and CH of glycolide (GA), according to the method established by Jeong*et al.*[27] and as described previously [25].

The molecular weight (M_w) and polydispersity of the obtained copolymer was determined by gel permeation chromatography, using the Agilent GPC-Addon system and RID-A refractive index signal detector coupled to the PLgel columns. Tetrahydrofuran was used as an eluent (flow 1 ml/min), and the sample injection volume was 10 µl.

Evaluation of the phase transition temperature

Gelation temperature was measured using the inverted test tube method [28]. The three copolymer solutions (17%, 23%, 28%) in phosphate buffer pH 7.4 ($n = 3$) were submerged in a refrigerated bath circulator instrument (WISD P-22, South Korea) at 0°C, and then the temperature of the bath was increased 0.5°C min⁻¹ until gelation occurred. The transition temperature was determined by inverting the test tube after keeping the sample at a constant

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temperature for 2 min to allow the establishment of equilibrium. The contents of the tube do not flow after inverting once the hydrogel is formed and the temperature recorded.

Preparation of PLGA-PEG-PLGA nanospheres encapsulated with insulin

An aqueous solution of insulin (2 mg/mL) in phosphate buffer pH 7.4 was prepared at 4°C. PLGA-PEG-PLGA copolymer (100 mg) was added to 1.5 mL of the 2 mg/mL insulin solution. Then, 1 mL of Span 80 and Tween 20 (1:1 weight ratio) was dissolved in 50 Ml mineral oil. The aqueous solution, containing 6.6% w/v of copolymer and 3% w/v of insulin, was added to 50 ml of mineral oil and homogenized at 4°C for 1 min.

The resultant o/w emulsion was stirred at 1200 rpm, and then the temperature was raised to 38°C. The mixture was stirred at this temperature for 15 min. The nanospheres were collected by centrifugation at 19,000 rpm at 37°C for 15 min, followed by dispersion in 40 mL of phosphate buffer pH 7.4 at 37°C. Mannitol (4% w/v) was added to the resultant nanospheres suspension, flash-frozen with a dry ice–acetone mixture, and freeze-dried. The nanosphere powders were kept at −18° to −20°C for further use.

Characterisation of nanospheres

To determine the entrapment efficiency of insulin, 20 mg of freeze-dried nanospheres were dissolved in dichloromethane and then centrifuged. The precipitate was dried, dissolved in 1 mL of phosphate buffer pH 7.4, and assayed for its insulin content by the Bradford protein assay method [29].

The measurement of nanosphere size and size distribution was carried out by dynamic light scattering (DLS; Zetasizer, Malvern, UK). All measurements were performed in triplicate at 25°C.

Study of therelease profile of insulin from PLGA-PEG-PLGA nanospheres

Phosphate buffer pH 7.4 (600 µl) was added to each microtube containing 30 mg of nanospheres (blank or insulin-loaded). The microtubes were submerged in a reciprocal water bath (NBIOTEK NB -304, South Korea) (20 rpm) at 37°C. At specified time intervals, a 500-µl aliquot was withdrawn from each sample and replaced with 500 µl of fresh PBS. The amount of released insulin was determined by spectrofluorimetryat $\lambda_{excitation}$=280 nm and $\lambda_{emission}$=303 nm. The fluorescence of samples drawn from the blank nanosphere suspension was used for correcting the background fluorescence.

Investigation of insulin folding

The relative surface hydrophobicity of standard insulin and released insulin from nanospheres were estimated using 8-anilino-1-naphthalene sulfonic acid (ANS). ANS solutions with twice as much protein samples concentration were added to insulin solution. The samples were excited at 370 nm and the emission spectra were recorded between 400 and 600 nm using a spectrofluorometer (Shimadzu, Japan).

Data analysis

The results were reported as means ± SD (n ≥ 3). Data were analysed by one-way analysis of variance (ANOVA)**.** A probability value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Characterisation of copolymer

The PLGA-PEG-PLGA copolymer was successfully synthesised by the ring-opening polymerisation method. The ^{1}HNMR spectrum of the copolymer is illustrated in Figure 1. The LA-to-GA was determined to be 2.9 by ^{1}HNMR, which matched the initial ratio of monomers used in the copolymerisation. The spectrum was similar to that reported by Chen *et al.*[30], and it verified the correct synthesis of the triblock copolymer.

 The number average molecular weight (M_n), the weight average molecular weight (M_w), and molecular weight distribution of the copolymer were determined by GPC and ^{1}HNMR (Table 1).

The GPC chromatogram of the synthesised copolymer is shown in Figure 2. The GPC curve of the copolymer is symmetric and unimodal.

No shoulder peaks corresponding to the lactide and/or glycolidemacromers or their homopolymers were detected.

Figure 3 shows the gelation diagram of thetriblock copolymer (17%, 23%, 28%) aqueous solutions.

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The sol-gel transition temperature was found to be a function of the concentration of the triblock copolymer. The transition temperature decreased with increasing copolymer concentration, from 17 to 28 wt%.

The sol-to-gel transition of an aqueous solution of the triblock copolymer is probably due to micellar packing, which is promoted by an increase in aggregation number driven by hydrophobic forces and the weakness of the hydrogen bonding with water molecules [4, 31].

Entrapment efficiency
The amount of insulin encapsulated in the PLGA-PEG-PLGA nanospheres was determined by the Bradford method. About 20% insulin was loaded in the nanospheres using this new thermogelling method.

In vitro insulin release
The mean diameter of the prepared nanospheres and their polydispersityare illustrated in Figure 4. Nanospheres of an average particle size of 230 nm were used for an*in vitro* release study. Figure 5 show that PLGA-PEG-PLGA-based nanospheres controlled the release of insulin at a constant rate for four weeks. The insulin release from the nanospheres exhibited an initial burst release (~30%) followed by a sustained, continuous release for up to three weeks.

The release kinetics of insulin showed the best fit for the Higuchi model (R^2= 0.9948), followed by zero-order release kinetics (R^2= 0.9668) (Table 2). Our data indicated that the nanospheres released insulin according to the Higuchi model, especially in the first few hours of release, and that the main release mechanism was diffusion.

As drug release continued, the rate of release was greatly decreased, and polymer degradation became the main mechanism of release.

Therefore, when the drugs in the surface of the nanospheres were released, the main mechanism of drug release changed to polymer degradation. The burst release of insulin is thought to be due to the surface localisation of the drug.

Since insulin may lose its activity during formulation, it is important for the controlled delivery system to release insulin in its active form.

As it is understood, ANS is a hydrophobic site-responsive probe, which has negligible fluorescence in aqueous media.Binding of this probe to hydrophobic sites in proteins results in a several fold enhancement in its florescence with a shift in the emission maximum to a lower wavelength [32]. The ANS fluorescence emission spectra of both the standard and released insulin were approximately similar suggesting unaltered surface hydrophobicity due to this formulation (Figure 6). The slight shift in spectrum of released insulin was related to presence of low amount of copolymer in the sample.

Table 1. Copolymer characteristics determined by ^{1}HNMR and GPC

Copolymer	^{1}HNMR		GPC		
	$M_n{}^a$	LA/GA[b]	$M_n{}^c$	$M_w{}^d$	$M_w/M_n{}^e$
	1116-1000-1116	2.9	3290.3	4743.7	1.44

[a] Numberaverage molecular weight determined by[1]HNMR
[b] LA/GA ratio determined by ^{1}HNMR
[c] Numberaverage molecular weight determined by GPC
[d] Weightaverage molecular weight determined by GPC
[e]Polydispersity determined by GPC

Table 2.Kinetic release of insulin from PLGA-PEG-PLGA nanospheres

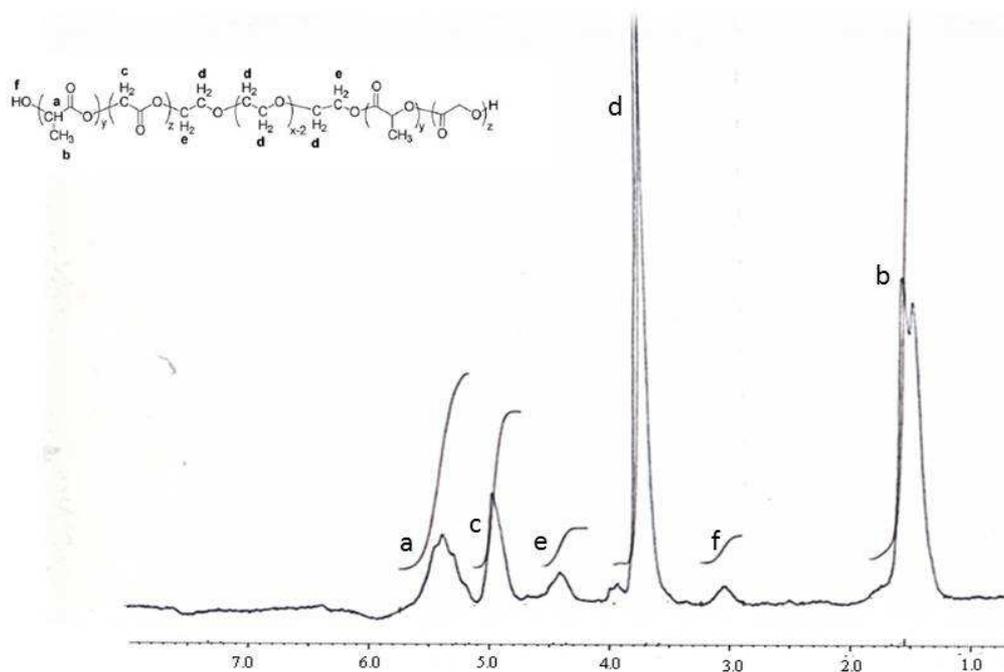| Drug (3% w/v)	Polymer (6.6% w/v)	Zero order		Higuchi	
		Slope	R^2	Slope	R^2
Insulin	PLGA-PEG-PLGA	2.6894	0.9668	3.1704	0.9948

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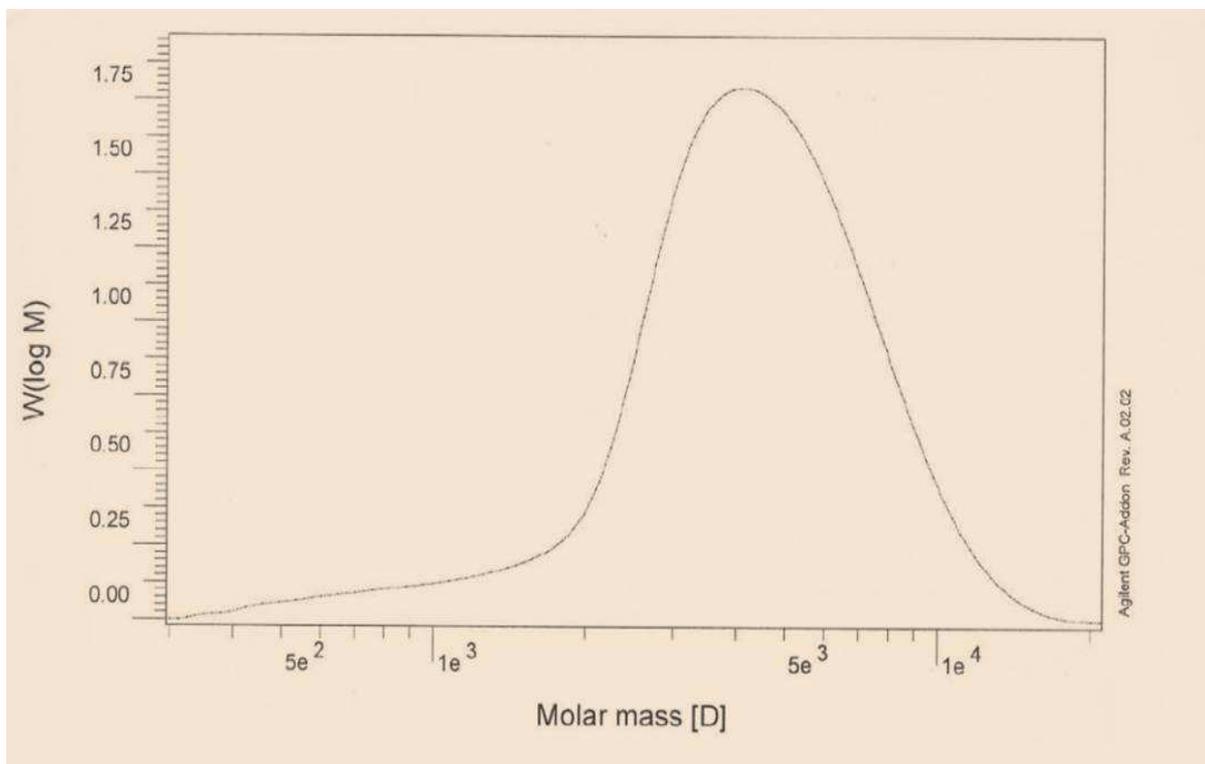**Fig. 1.¹HNMR spectrum of triblock PLGA-PEG-PLGA**

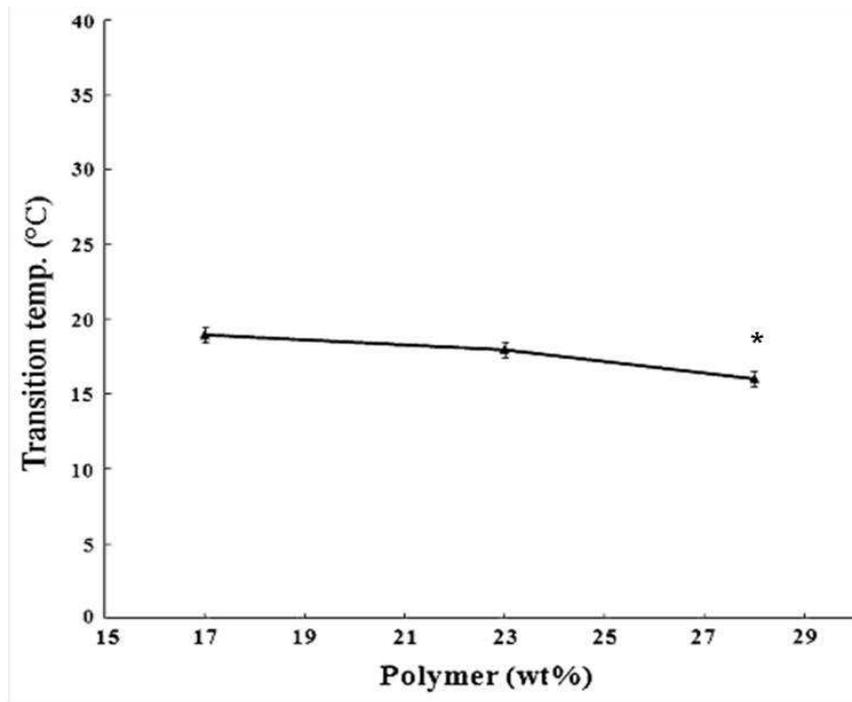**Fig. 2. GPC chromatogram of PLGA-PEG-PLGA**

Fig. 3.Gelation temperature diagram of the aqueous solutions of PLGA-PEG-PLGA copolymer at different concentrations
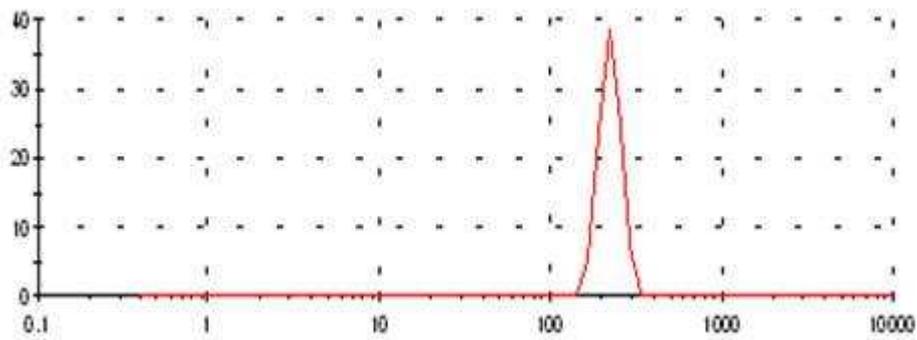*** p≤0.05**

Fig. 4.Insulin nanospheres were characterized for mean size by dynamic laser scattering
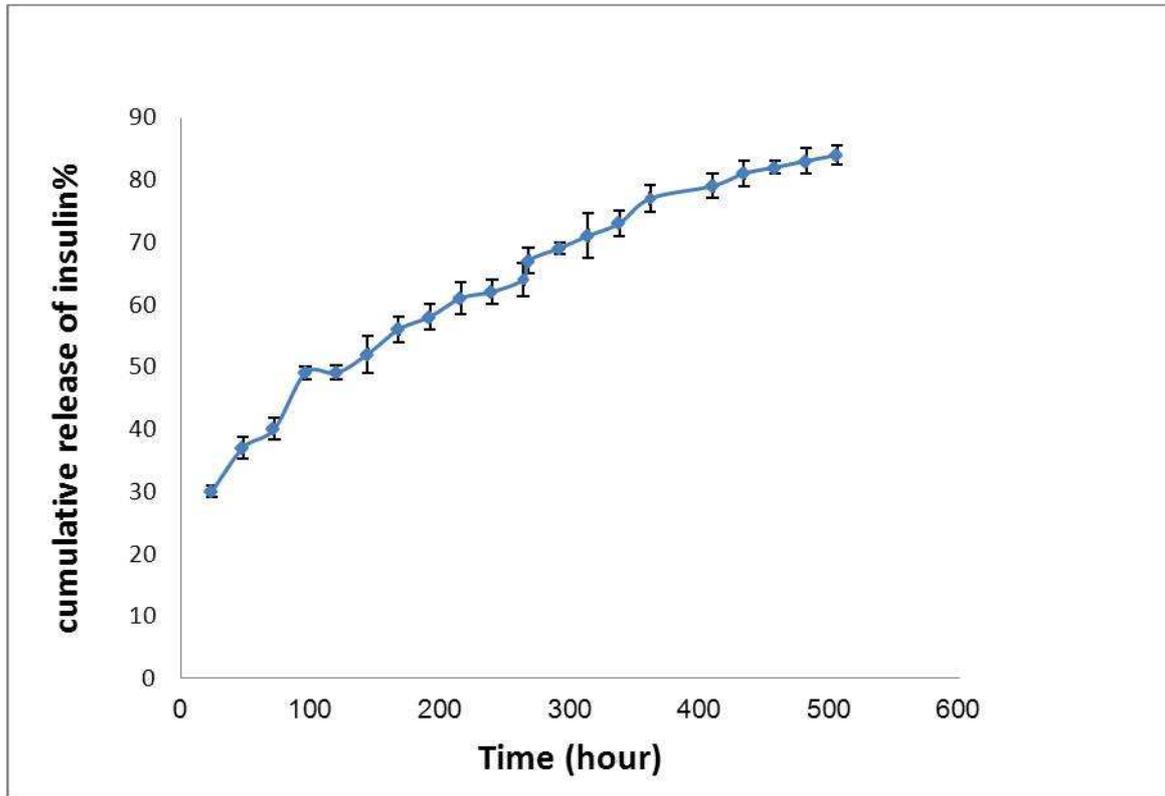
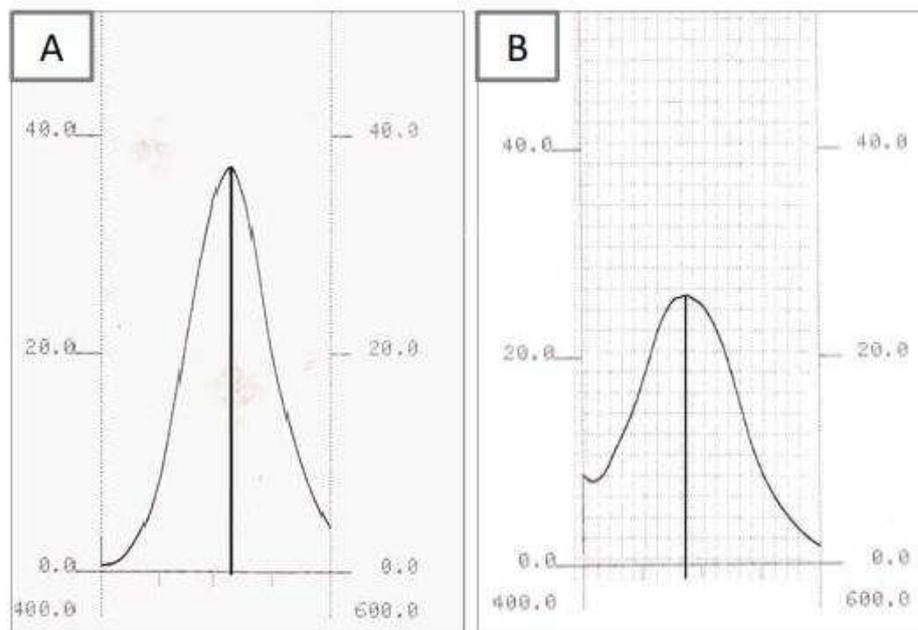**Fig. 5.Cumulative release of insulin from PLGA-PEG-PLGA nanospheres**

Fig. 6. ANS fluorescence spectra of standard insulin (A) and released insulin from nanosphares (B)

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CONCLUSION

The feasibility of using PLGA-PEG-PLGA nanospheres was investigated in order to achieve the goal of controlled release of insulin. The release of insulin was controlled by both drug diffusion and polymer degradation. The total release period lasted for three weeks, involving an initial (30%) burst release, followed by a sustained release of insulin (>85%). During the first hours of release, the drug release resulted from diffusion of insulin from the nanospheres, which was caused by localisation of insulin on the surface of the nanospheres. As the drug release continued, the encapsulated insulin was slowly and completely released by polymer degradation. The large molecules such as insulin do not diffuse easily through the small pores of the PLGA–PEG–PLGA matrix. Therefore, this drug is mainly released by copolymer degradation. However, according to our previous studies, it was indicated the drug caused copolymer erosion rate decreased as a result of a salting-out effect and a decrease in water activity[31].

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