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PLGA-PEG-PLGA tri-block copolymers as an in-situ gel forming system for calcitonin delivery

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Summary

One of the main routes of peptide and protein delivery is parenteral. The need for daily or repetitive injection of these parenteral formulations is the main cause of patient non-compliance. The objective of this study was to prepare a controlled delivery system for peptide and proteins to increase patient compliance. Biodegradable triblock copolymers of PLGA and PEG (PLGA-PEG-PLGA) were synthesized and characterized using various content of glycolide. Solutions of PLGA-PEG-PLGA containing calcitonin as a model peptide were prepared and drug release from the sol-gel systems was evaluated in two different incubation conditions. Zero order release kinetics was achieved for up to 100 hours. No significant burst release effect was observed. It seemed that surface gelation was the main cause of drug release control. The release data fitted to Higuchi's modeling and showed good correlation. It can be concluded that in situ gelling system prepared in this study can be used for a weekly peptide delivery system.

Keywords

PLGA-PEG-PLGA, in-situ gelation, peptide delivery, thermo-responsive drug delivery, calcitonin, biodegradable polymers

Introduction

The use of peptides and proteins as therapeutic agents has increased considerably in recent years. Therefore different drug delivery systems have been investigated for the delivery of peptides and proteins [1]. Short half life of peptides and proteins as well as limited efficiency of non-parenteral route require daily injection or several injections per day, which results in decreasing patient compliance and treatment failure. In addition, efficacy of peptides and proteins in sustained release dosage forms is usually higher than that in immediate release dosage forms due to optimal blood concentration and lower adverse reactions [2]. Poor patient compliance and more adverse effect of immediate release peptides and proteins express the importance of controlled delivery systems.

Peptide and protein encapsulation using biodegradable polymers has been widely used as a controlled delivery system for peptides and proteins [3]. Polymers such as PLA, PLGA have been used to entrap proteins by using organic solvents. A main disadvantage of these systems is the decreased biological activity of peptides and proteins when organic solvents are used during the preparation process [4]. In addition, large-scale production problems such as sterilization have been reported as a major concern [5]. It has been also reported that the injection of microspheres causes acute tissue reactions [4].
The use of hydrogels as controlled delivery systems for peptides and proteins has been recently investigated. These polymers, also called smart polymers, dissolve in aqueous media and produce physically cross-linked hydrogels by stimulants such as pH, ion and temperature [6]. They have less manufacturing problems compared to microspheres and due to their homogenous dispersion in tissue, they have less tissue related side effects. Amongst the smart polymers, thermoresponsive polymers have attracted more attention [7,8]. Aqueous solution of thermoresponsive polymers produce low viscosity fluids, hence their handling during manufacturing process, formulation, filtration and filling would be as simple as conventional parenteral dosage forms preparations. In situ forming polymers when injected become gel as the temperature is increased to body temperature, above their gelling point [9]. Entrapped drug molecules are then released according to several mechanisms such as diffusion, polymer degradation, solubility and etc [10,11].
There are many types of thermoresponsive polymers such as copolymers composed of poly ethylene glycol (PEG) and poly lactide (PLA), poly glycolide (PGA), poly lactide-co-glycolide (PLGA), poly ε-caprolactane (PCL) and poloxamer series. Among them, copolymers of PEG/PLGA are more attractive due to their biocompatibility and biodegradability [9].
The objective of this work was to prepare a controlled delivery system using PLGA-PEG–PLGA triblock copolymer as an in situ gelling system with different percentage of glycolide mass to evaluate the release of calcitonin as a model protein. The effect of gelling time on the release profile was also studied.

Experimental

Materials

D, L-Lactide, Stannous 2-ethylhexanoate and tetra methyl ammonium hydroxide were purchased from Sigma Co (USA). Glycolide was purchased from Boehringer-Ingelheim Co (Germany) and calcitonin was purchased from Polypeptide Co (Denmark). All other chemicals such as acetonitril (HPLC grade), phosphoric acid, sodium acetate, were purchased in analytical grade from Merck Co (Germany) and used without any further purification.

Polymer synthesis

Triblock copolymers were synthesized by ring-opening method with a little change described by Zentner et al [12]. 6 g PEG 1500 was loaded to a stainless steel reactor and heated 2 hours at 150ºC at 5 mmHg vacuum in order to dry. Then specific amount of D, L-Lactide and Glycolide were loaded to reactor according to Table 1. The reactor heated at 150ºC under vacuum for 30 min. Stannous 2-ethylhexanoate (0.04g)

was added as catalyst and heating continued at 160ºC for 6 hours under 5 mmHg vacuum. After the reaction completed the copolymers were dissolved in cold water (4ºC) then heated to 80ºC to precipitate to remove water-soluble impurities and un-reacted material for purification. The purification process was done 3 times by dissolving in cold water and precipitating it. Purified copolymers were dried under 5-mmHg vacuum at 37ºC to get constant weight.

Table 1. The composition of copolymers synthesized in this study

	D, L-Lactide weight (g)	Glycolide weight (g)	Total weight (g)	Glycolide/PLGA ratio (%)	LA/GA molar ratio
Coplymer1	10.2	1.8	12	15	4.56
Coplymer2	9.6	2.4	12	20	6.28
Coplymer3	9.0	3.0	12	25	15.50

Polymer characterization

^{1}H NMR spectrum has been used for structure analysis and molecular weight estimation [13]. Spectra were recorded using a 400 MHz Bruker spectrometer (AVANCE model, Germany) at 25°C. The solvent used was deuterated chloroform ($CDCl_3$). Average molecular weight and lactide to glycolide ratio were determined by ^{1}H NMR by integration of the signals pertaining to each monomer such as the peaks from CH and CH_3 of Lactide, CH_2 of ethylene glycol and Glycolide.

The molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC). An Agilent 1100, 10 µm apparatus equipped with RI detector and three PLgel® columns in 10^2-10^6 ranges were used. The analyses were performed at 30ºC using THF as an eluent, at a flow rate of 1ml/min. GPC was calibrated with polystyrene standards.

PLGA-PEG-PLGA copolymers were dissolved in buffer acetate (pH 4) at 4ºC in 15, 20, 25 weight ratios. They were then evaluated incubated for 2 minutes in 37°C water bath. Sol-Gel transition was measured by test tube inverting method. Solution was poured in test tube and left for 2 minutes at 37±0.1ºC. The test tube was then removed and reversed to check if the content was viscous enough to be considered as gel.

Calcitonin stability

Stability of calcitonin was investigated to minimize calcitonin self-degradation during release period. 0.5 mg /ml calcitonin in various acetate and phosphate buffers (pH 3, 4, 7.4, 8) was kept at 37ºC for 12 days and the quantity of calcitonin was analyzed.

Preparation of delivery systems

Various PLGA-PEG-PLGA copolymers having 15, 20, 25% glycolide contents were dissolved in acetate buffer (pH 4) to make 25% (w/w) solution at 4ºC and then calcitonin was added to prepare 0.05% solution and mixed gently for 10 min. Viscosity of polymeric formulations were measured by Oswald tube in cold condition to evaluate their sol viscosity. Formulations were also loaded in 5ml syringes and pushed through 22G needles to investigate their injectability.

In-vitro drug release

Calcitonin formulations (2 ml) added to 10 ml vials (n=4) were transferred to water bath at 37±0.1ºC and incubated for 2 or 15 minutes to form gel. 5 ml acetate buffer (pH 4) was added to each vial as release media. The vials were kept in shaking water bath (20±2 rpm) at 37±0.1ºC for the entire period of study. At different intervals (2, 24, 48, 72, 96, 144, 168, 192±2 hours) aliquots of 1 ml was removed from each vial and 20µl was injected to HPLC. The residue was transferred back to vial and the volume was kept constant by replacing 20 µl fresh medium to each vial. The method has been reported before [14, 15].

The analysis was performed according to calcitonin monograph in BP 2004 [16]. Released calcitonin was determined by HPLC method consisted of a Knauer K-1001 pump, triathlon auto-injector, a Knauer K-2800 photodiode array detector, and Chromogate® software equipped with a 15 cm Novo-pack® C18 column at 65°C thermostated with a column oven. The mobile phase was prepared in liner gradient mode by buffer solution (A) (tetra methylamonium hydroxide 3.62 g in 1000 ml water, adjust pH 2.5 with Phosphoric acid) and acetonitril (B), starting from 76 (A)-24(B) to 64 (A)-36 (B) in 30 min, flow rate 1 ml per min and injection volume was 20 µl.

Data Analysis

The results were expressed as mean±SD (n=4). Statistical comparisons were made using student's t-test with the level of significance of $P<0.05$.

Results and discussions

The ^{1}H NMR spectrum of PLGA-PEG-PLGA copolymer containing 20% glycolide (polymer 2) with its chemical structure is presented in figure 1. The signals pertaining to PLGA-PEG-PLGA are found in δ=5.12 ppm (CH of LA, a), 1.52 ppm (CH3 of LA, b), 4.7 ppm (CH2 of GA, c), 3.65 ppm (CH2 of ethylene glycolide, d) and 4.10 ppm (CH2 of ethylene glycol, e). The molecular weights were calculated by ^{1}H NMR according to Jeong et al method to compare with GPC results [13].

The H NMR spectrum of PLGA-PEG-PLGA copolymer containing 20% Glycolide (polymer 2) presented in figure 1, is very similar to the reported spectrum and all the signals were assigned on the spectrum [16]. The complicated splits in the peaks were due to the random polymerization of the polymeric network. Despite Chen's report, there is no peak at 2.75 ppm that is considered as the terminal OH [5]. Although, existence of terminal OH was approved by infrared spectrometry, it was not seen in the NMR spectrum in this study, due to low intensity [5].

The molecular weight of polymers was determined using GPC method. The elution volume was 18-27 ml for copolymers according to their molecular weight. The other peaks in the chromatogram are considered to correspond to the solvents. The molecular weight of the polymers and their molecular weight distribution are tabulated in tables 2 and 3. The polydispersity of all copolymers was found to be about 1.14-1.19, which shows a symmetric peak and has a relative narrow molecular weight. Unimodal GPC with a low polydispersity confirms the formation of triblock copolymers. The molecular weight was controlled by adjusting the synthesis conditions in the ring opening polymerization step.

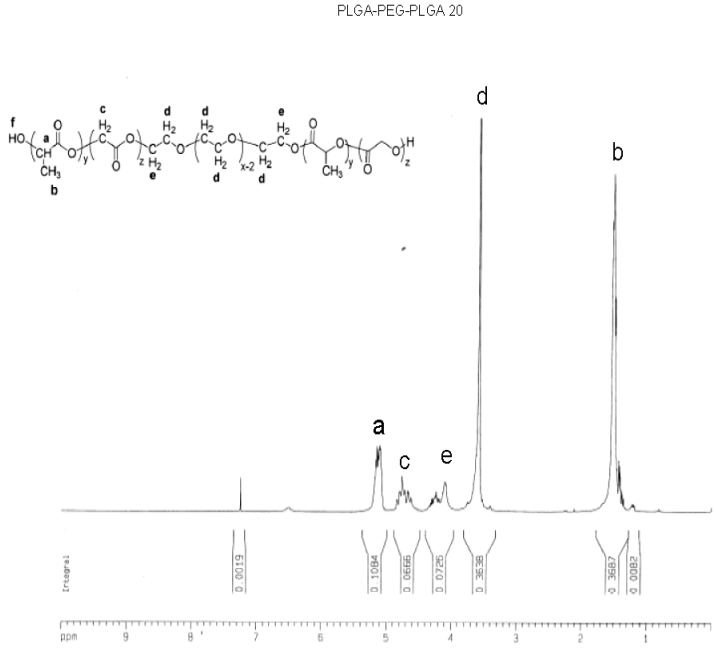

Fig. 1. ^{1}H NMR spectra obtained for PLGA-PEG-PLGA with 20% glycolide (polymer 2)

Table 2. Characteristics and molecular weight of PLGA-PEG-PLGA determined by GPC and ^{1}H NMR

	Mn[a]	Glycolide[b]	Mn[c]	Mw[d]	Mw/Mn[e]
Coplymer1	5151	16.2%	5387	6185	1.14
Coplymer2	5529	19.8%	5703	6547	1.14
Coplymer3	5604	23.1%	5254	6304	1.19

a) Number average molecular weight determined by HNMR
b) Mass percentage of glycolide to PLGA determined by HNMR
c) Number average molecular weight determined by GPC
d) Weight average molecular weight determined by GPC
e) Polydispersity determined by GPC

Table 3. Molecular weight distribution of PLGA-PEG-PLGA determined by GPC

	Copolymer 1	Copolymer 2	Copolymer 3
10%	3612	3812	3711
30%	4609	4805	4523
60%	6241	6659	6590
85%	8649	9201	9311
90%	9955	10125	9900

In this study, calcitonin was used as a model peptide. Calcitonin is used in osteoporosis and Paget's syndrome and is a polypeptide with 32 amino acids [10]. The stability of calcitonin in various pHs is very important for its therapeutic effect.

Figure 2 shows the calcitonin stability at pH 4 and 7.4 at 37ºC. It is assumed that calcitonin is stable for up to 10 days at pH 4 at 37ºC to study the release profile of the peptide.

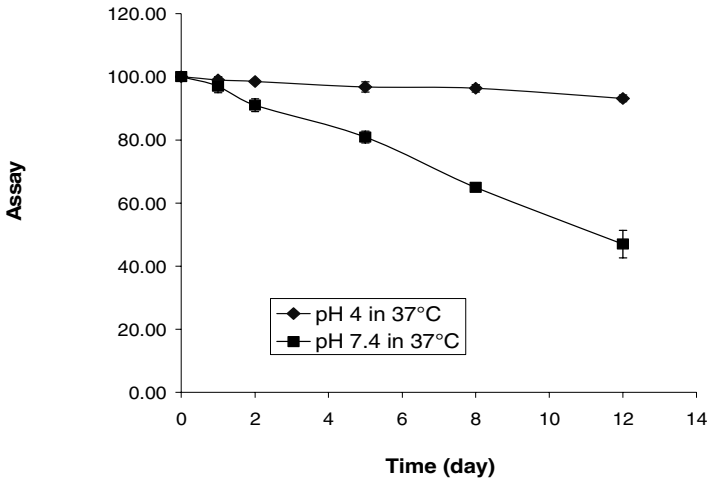

Fig. 2. Stability of calcitonin in pH 4 and pH 7.4

Calcitonin release from different polymers (15, 20, 25% Glycolide) plotted against time are shown in figure 3. As can be seen in this figure, there is no difference in peptide release from the gel systems when incubated at 2 or 15 minutes. Therefore, by increasing the incubation time no change in release profile can be expected. This can be attributed to the fast Sol-Gel transition phenomena in these copolymers, as 2 minutes incubation time, before starting the release test, was enough to produce a gel system similar to 15 minutes incubation time. This may be considered as an advantage of these copolymers since after injection, gel conversion is very fast.

No large burst release was observed, although 32±3, 8±2, 10±3 percent of calcitonin released in first 24 hours from copolymers 1, 2 and 3, respectively. This shows that peptide release from this system is mainly controlled by Sol-Gel transition at surface. However, they had a linear profile up to 100 hours. Chen et al also reported this kind of linear release in their investigations. They have worked on lyzozyme release profile from PLGA-PEG-PLGA copolymers with PEG1000 and PEGs with smaller molecular weight. They found a higher initial release followed by slower release after one day [5]. This copolymer can decrease burst release to a relatively low level compared to the large burst release (50 %) from microspheres formulations [18]. The burst release of drugs was considered to be due to surface located drug also according to Packhaeuser et al; those drugs associated with or dissolved in the hydrophobic core do not experience this push out effect [19]. It is assumed that with increasing hydrophilic properties in hydrophobic block, the slope of calcitonin may be decreased. The drug release data were fitted according to first-order (Eq. 1) and Higuchi (Eq. 2) kinetics models of drug release to evaluate if drug release is through bulk erosion or diffusion mechanism.

$$\text{Ln } M_t = Kt \qquad \text{Eq. 1}$$

$$M_t/M_\infty = Kt^{1/} \qquad \text{Eq. 2}$$

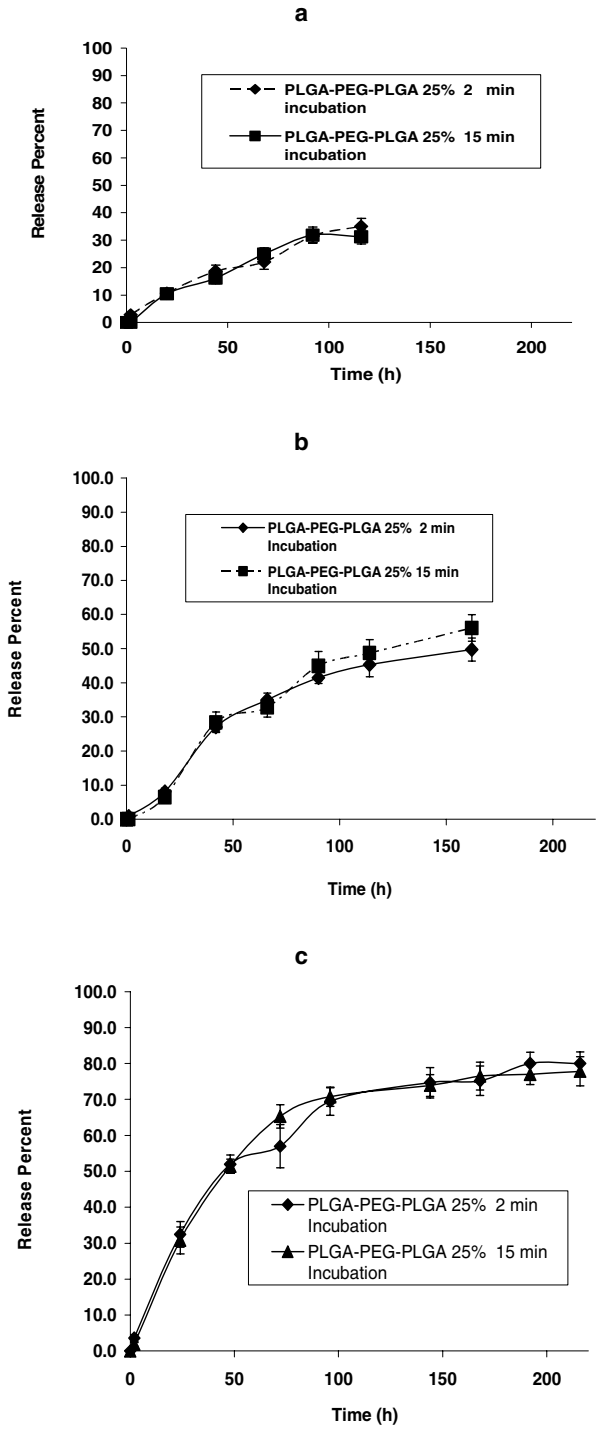

Fig. 3. Calcitonin release from in-situ gel system: a) 25% glycolide, b) 20% glycolide, c) 15% glycolide

As can be seen in table 4 the drug release from the system corresponds to diffusion mechanism, rather than first order (bulk erosion).

Table 4. Kinetic profile of calcitonin release from in-situ gel system

Lactide/glycolide ratio	First-order		Higuchi	
	Slope	R^2	Slope	R^2
15%	0.027	0.690	0.099	0.988
20%	0.019	0.597	0.073	0.972
25%	0.019	0.790	0.081	0.976

Figure 4 shows a sample of data fitted to Higuchi kinetics model. As shown in this figure, there is a good relation between release slope and the glycolide content of the copolymer.

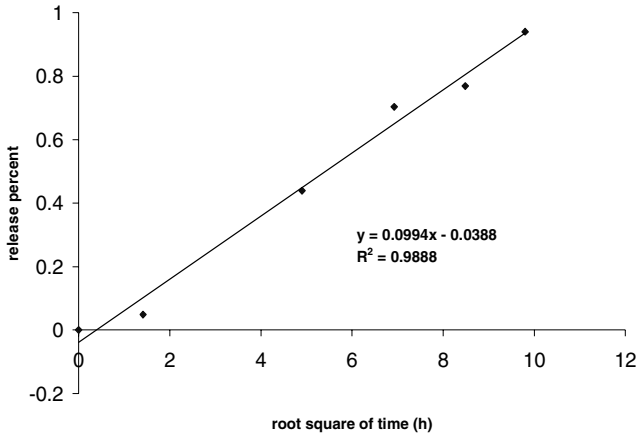

Fig. 4. Calcitonin release data fitted to Higuchi's modeling for 15% glycolide

When a triblock thermo-responsive copolymer is poured in water, hydrogen bonds will be created so the copolymer is dissolved in water like many other polymers. However, when the temperature is increased, hydrogen bonds will be broken and a three-dimensional physically cross-linked hydrogel is produced due to size, length and strength of hydrophobic blocks. All other dissolved material like drugs are entrapped in hydrogel and distributed between hydrophilic and hydrophobic compartments. Two mechanisms may be considered for drug release from the system: I) drug diffusion from hydrogel network and II) drug release as a consequence of polymeric surface erosion. The suggestion of surface erosion may be disregarded, as the system contains a high content of water molecules that may initiate the bulk erosion of the polymeric network. Figure 5 shows the effect of glycolide content of the block copolymer on the calcitonin release from the system.

As expected with the increase in the glycolide content of the block copolymer, the drug release slope is decreased. Glycolide is the hydrophilic part of PLGA section of the block copolymer. As hydrophilic section is enlarged, the hydrophilic drug is less released.

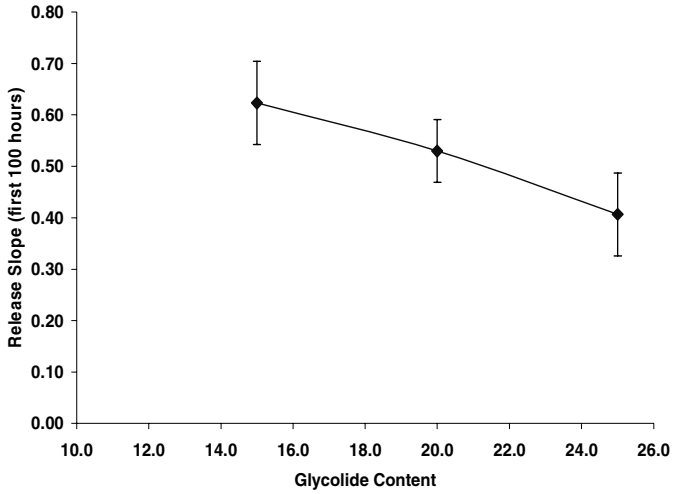

Fig. 5. The relation between glycolide content of copolymers and release slope for the first 100 h of drug release

Conclusion

Simple manufacturing process using aqueous solvents is a major advantage for the use of thermally reversible gels as controlled peptide and protein delivery systems. Gelation time does not affect the drug release profile of the system. Diffusion is the main mechanism for calcitonin release from these systems.

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