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Novel thymopentin release systems prepared from bioresorbable PLA–PEG–PLA hydrogels

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ABSTRACT

Copolymers were synthesized by ring opening polymerization of L- or D-lactide in the presence of dihydroxyl PEG with molar mass of 6000, 12,000 and 20,000, using zinc lactate as catalyst. Bioresorbable hydrogels were obtained by mixing PLLA–PEG–PLLA and PDLA–PEG–PDLA aqueous solutions due to stereocomplexation between PLLA and PDLA chains. Rheological measurements show that the hydrogels present typical viscoelastic behaviors, although degradation could occur during the gelation process. Thymopentin was taken as a model drug to evaluate the potential of PLA–PEG–PLA hydrogels as carrier of hydrophilic drugs. Various parameters such as copolymer concentration, drug load, copolymer composition and the difference between sol and gel were considered. The release profiles are characterized by an initial burst followed by slower release. Higher copolymer concentration leads to slower release rate and less burst effect due to more compact structure which disfavors drug diffusion. Similarly, higher molar mass of the copolymers disfavors the release of TP5, and hydrogels composed of both PLLA/PEG and PDLA/PEG present slower release rates than single copolymer solutions. In contrast, drug load exhibits little influence on the release profiles due to the high water solubility of TP5. In all cases, nearly 80% of TP5 is released. In vivo studies proved the potential of TP5 containing hydrogels, especially those with a concentration of 25%. Both the $CD4^+/CD8^+$ ratio and the morphology of thymus indicate the immunization efficacy of the TP5 release systems based on PLA/PEG hydrogels.

1. Introduction

Drug delivery systems (DDS) have drawn great interest for decades because DDS present many advantages compared with conventional drug delivery routes. First, blood drug concentration may be maintained at a proper level for prolonged time periods, thus avoiding repeated drug administration. Second, drug bioavailability can be improved by protecting drugs, especially protein-like drugs in vivo. The most widely investigated DDS include micro-/nanoparticles, micelles, hydrogels, etc. Among them, hydrogels appear most attractive for their excellent biocompatibility due to the presence of large amounts of water.

Hydrogels consist of a crosslinked, three-dimensional hydrophilic polymeric network which swells by imbibing water without dissolution (Lin and Metters, 2006). Early in 1954, Wichterle and Lim reported the first synthetic hydrogel (Wichterle and Lim, 1954). Since then, hydrogels have attracted growing interest in the field of biomedical and pharmaceutical applications (Kashyap et al., 2005). The high water content and water insolubility endow hydrogels with outstanding biocompatibility, hydrophilicity, low adsorption of proteins and special physicochemical properties such as similar mechanical properties as soft tissues.

Biodegradable polymers have shown great potential in the biomedical field (Holland et al., 1986). Among them, polylactide (PLA) has been investigated worldwide due to its good degradability and biocompatibility. In fact, its final degradation product, lactic acid, is a metabolite and can be easily eliminated from the human body via the Kreb's cycle. However, PLA is highly hydrophobic, which considerably restricts its applications as a biomaterial. Introduction of hydrophilic polyether blocks into degradable polyester chains is a means to modulate the properties of the parent polymers. Poly(ethylene oxide) (PEO) or poly(ethylene glycol) (PEG) is often used as macro-initiator to prepare block copolymers with polyesters (Kricheldorf, 2001). PEG presents unique physicochemical and biological properties including biocompatibility, low immunogenicity, water solubility, and can be eliminated from an animal's body when the molar mass is below 30,000 (Hu et al., 2003). Some PEG-based materials have been approved by the Food and Drug Administration (FDA) for biomedical uses.

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PLA/PEG copolymers exhibit enhanced hydrophilicity and degradation rate, reduced acidity of degradation products as compared to PLA (Li et al., 2002). They have been extensively investigated as DDS in the form of micro-/nanoparticles, micelles and hydrogels (Matsumoto et al., 1999; Govender et al., 2000; Molina et al., 2001; Ruan and Feng, 2003). Recently, we reported on PLA/PEG hydrogels formed by stereocomplexation, a well-known phenomenon for optically active PLA. A stereocomplex can be obtained from co-precipitation of poly(L-lactide) (PLLA) and poly(D-lactide) (PDLA) in solution, through cooling from a melt of both polymers, or during hydrolytic degradation of poly(DL-lactide) (Krouse et al., 1987; Li and Vert, 1994; Brizzolara and Cantow, 1996). When PLLA/PEG is mixed with PDLA/PEG in an aqueous solution, interactions between PLLA and PDLA blocks can lead to stereocomplexation and formation of a hydrogel (Fujiwara et al., 2001; Li, 2003; Li and Vert, 2003; Li et al., 2005). Compared with other DDS such as micro-/nanoparticles or implants, PLA/PEG hydrogels present a tremendous advantage, i.e. facile fabrication without using organic solvents or heating which may denature protein drugs. Drug release is generally driven by two mechanisms: diffusion-based mechanism and degradation-based mechanism (Li, 2003; Li et al., 2005).

Goldstein et al. reported in 1975 the isolation of a 49 amino acid polypeptide from human thymus tissue (Goldstein, 1975; Schlesinger and Goldstein, 1975). Since then, the synthetic pentapeptide, thymopentin or TP5 (Arg-Lys-Asp-Val-Tyr) has been well known for its activity as an immuno-modulating drug, and clinical efficiency in the treatment of autoimmune diseases such as chronic lymphocytic leukemia, Sezary's syndrome, decreased immune, atopic dermatitis, especially in rheumatoid arthritis as well as the competency in elder surgical patients (Weaver et al., 1984; Bernengo et al., 1988; Colle et al., 1988; Faist et al., 1988). In fact, TP5 has the function of inducing differentiation of T-cell and accelerating the development of T lymphocyte. The structure of TP5 is shown in Scheme 1.

TP5 exhibits a very short half-life in plasma of about 30 s due to enzymatic degradation, poor membrane permeability, and extensive metabolism in the gastrointestine. Repeated injections or intravenous infusions of TP5 are thus necessary to reach the efficient blood drug concentration to stimulate the $CD8^+$ cell. All these considerably restrict its clinical applications (Parikh and Jones, 1965). Sustained releasing systems such as nanoparticles and microparticles have been reported (Morel et al., 1996; Yin et al., 2006). Morel et al. prepared TP5 containing lipid nanoparticles from O/W or W/O/W microemulsions by dissolving TP5 in the aqueous internal phase. The incorporation of TP5 was 5.2 and 1.7%, respectively. In both cases, the in vitro release of TP5 followed a pseudo-zero-order kinetics. About 10% TP5 was released during the whole release period of 400 min (Morel et al., 1996). Yin et al. evaluated lectin-conjugated PLGA nanoparticles for oral delivery of TP5 which were prepared by a double emulsion-solvent evaporation technique. The drug entrapment efficiency was below 33%. Less than 50% of TP5 was released during the whole release period up to 100 h (Yin et al., 2006). The results show that lectin-conjugated PLGA nanoparticles can effectively improve the oral absorption of thymopentin compared with conventional TP5 nanoparticles and TP5 solution. Both systems present the same disadvantages, i.e. low drug entrapment efficiency and low released amount of TP5 (<50%). Recently, He et al. reported TP5 loaded nanoparticles prepared from poly-butylcyanoacrylate (PBCA) by nanoprecipitation method. The entrapment efficiency was 92%. In vitro release showed that less than 60% TP5 was released from lyophilized nanoparticles, while 80% TP5 was released from colloidal nanoparticles in 48 h (He et al., 2008). However, PBCA nanoparticles are not biodegradable under human body conditions.

TP5 based hydrogel systems have not been reported, so far. In this work, PLA/PEG copolymers were synthesized by ring opening polymerization of L- or D-lactide in the presence of dihydroxyl PEG with Mn = 6000, Mn = 12,000 and Mn = 20,000 using low toxic zinc lactate as catalyst. Hydrogels were prepared by mixing both PLLA/PEG and PDLA/PEG copolymers in water. TP5 was incorporated in the hydrogels before gelation. Release of TP5 was performed in phosphate buffered saline (PBS) at 37 °C and monitored by using high performance liquid chromatography (HPLC). The results are reported herein in comparison with the literature data.

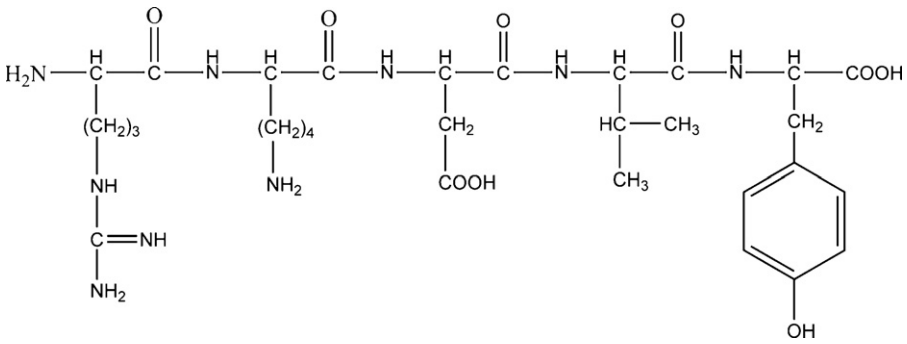
2. Materials and methods

2.1. Materials

L-Lactide and D-lactide from Purac (Gorinchem, The Netherlands) were recrystallized from ethyl acetate. PEG with molar mass of 6000, 12,000 and 20,000 was obtained from Fluka (Steinheim, Germany) and used as received. TP5 was supplied by Shanghai Soho-Yiming Pharmaceuticals Co., Ltd. (Shanghai, China). Zinc lactate from Merck (Darmstadt, Germany) was dried under vacuum before use.

Copolymers were synthesized by ring opening polymerization of L- or D-lactide in the presence of dihydroxyl PEG using zinc lactate as catalyst. Briefly, predetermined amounts of PEG and lactide were introduced into a polymerization tube. The initial molar ratio of ethylene oxide to lactate repeat units (EO/LA) was 3/1 or 5/1. Zinc lactate (0.1 wt%) was then added. After degassing, the tube was sealed under vacuum, and polymerization was allowed to proceed at 140 °C. After 24 h, the product was recovered by dissolution in dichloromethane and precipitation in diethyl ether. Finally, the product was washed and dried under vacuum up to constant weight.

Typically, predetermined amounts (0.15, 0.25 or 0.40 g) of PLLA/PEG and PDLA/PEG triblock copolymers were separately dissolved in 1 ml of deionized water. After swelling overnight, both



Scheme 1. Structural formula of thymopentin (TP5).

fluids were mixed. Thereafter, gelation was allowed to proceed at 37 °C for various time periods to yield hydrogels with different concentrations (15, 25, or 40%). Thymopentin-containing hydrogels were prepared under similar conditions. TP5 was incorporated in the copolymer mixture before gelation which lasts 2 days at 37 °C. The drug load was 20, 40 or 80 mg for each hydrogel sample.

2.2. In vitro release

Protein-containing hydrogel samples were placed in a dialysis bag (MWCO = 25,000), and immersed in 4 ml of pH = 7.4 phosphate buffered saline (PBS) containing 0.02% of NaN_3. In vitro release was preformed at 37 °C. At preset time intervals, the buffer solution was taken out, and replaced by 4 ml fresh buffer. The release was regularly monitored by HPLC, using calibration curves obtained from standard solutions.

2.3. In vivo experiments

2.3.1. Experiment animals

Wistar rats (160–200 g) obtained form Shanghai Xipu'er-bikai Experimental Animal Co., Ltd., were used for in vivo experiments. All of them were maintained on a light/dark cycle. All care and handling of animals were performed according to the National Act on the use of experimental animals (People's Republic of China).

2.3.2. Immunosuppression model and administrations

Briefly, 49 normal female Wistar rats were randomly divided in 7 groups, of which 6 were used for experimentation and 1 for control. The rats of experimentation groups were treated with cyclophosphamide (CTX) (35 mg/kg day) by intraperitoneal injection 3 days before in vivo experiments. All rats had free access for food and water. On the first day of in vivo experiment, the administration was done as shown in Table 1.

2.3.3. Samples of blood and excision of tissues

Blood samples (0.5 ml) of each rat were collected into tubes which were precovered with heparin on the1st, 7th and 21st days. 1.5 ml cell lysis solution was added. The samples were gently mixed, allowed to stand for 10 min at 4 °C, and centrifuged. The supernatant was removed. 200 μl of PBS solution containing corresponding antibody were then added into the tubes and homogenized. After 30 min at room temperature in the absence of sunlight, the samples were analyzed by using flow cytometry (BD FACSAria, Becton Dickinson Cop., USA). The $CD4^+/CD8^+$ ratio of each group was calculated from the FCM charts.

Table 1
Administration of the six experimentation groups on the first day.

No.	Group	Dosage (mg/kg)
1	CTX	0
2	TP5-1[a]	7
3	TP5-2[b]	2 × 4
4	15% gel-1[c]	7
5	15% gel-2[d]	14
6	25% gel[e]	7

[a] Administrated with TP5 of 7 mg/kg by hypodermic injection (i.h.).
[b] Administrated with TP5 of 2 mg/kg (i.h.); the rats were treated with TP5 of 2 mg/kg every 2 days and the total administration amount was 8 mg/kg.
[c] Administrated with 15% TB6K3L/TB6K3D hydrogel containing 40 mg TP5 (i.h.), the total TP5 amount injected was 7 mg/kg.
[d] Administrated with 15% TB6K3L/TB6K3D hydrogel containing 80 mg TP5 (i.h.), the total TP5 amount injected was 14 mg/kg.
[e] Administrated with 25% TB6K3L/TB6K3D hydrogel containing 40 mg TP5 (i.h.), the total TP5 amount injected was 7 mg/kg.

After 7 days, three rats in each group were sacrificed by cervical vertebra disjointedness. Incisions were made in the abdomen. The thymus was immediately excised and made into paraffin sections which were then observed by using optical microscope to examine the morphology of thymus.

2.4. Measurements

^1H Nuclear magnetic resonance (NMR) was performed at room temperature with a Bruker A VAVCE-DMX 500 spectrometer operating at 500 MHz by using $CDCl_3$ as solvent.

Differential scanning calorimetry (DSC) was conducted with a Perkin Elmer DSC 6 calorimeter, the heating rate being 10 °C/min. 10 mg of product were used for each analysis.

Rheological properties were determined on a Carri-Med CSL2 Rheometer of TA Instruments. For all the experiments, a cone-plate measuring geometry was used (steel, 4 cm diameter with an angle of 2°, gap 56 μm). A solvent trap was used to prevent water evaporation. Measurements were realized in the linear viscoelastic range.

The amount of released TP5 was determined by using high performance liquid chromatography (HPLC) equipped with a LC-10A apparatus (Shimadzu), a UV detection (SPD-10A, Shimadzu) and a Diamonsil column (4.6 mm × 250 mm, pore size 5 μm, C18, Dikma, China). All samples and the mobile phase were filtered through 0.22 μm filter before injection. The detection wavelength was 275 nm, and the flow rate 1.0 ml/min. The mobile phase was prepared by mixing 85% (volume) buffered saline (NaOH 0.0291 mol/l, KH_2PO_4 0.0896 mol/l) and 15% methanol. The calibration curve was linear in the range of 0.01–20 mg/l with a correlation coefficient of $R^2 = 0.999996$.

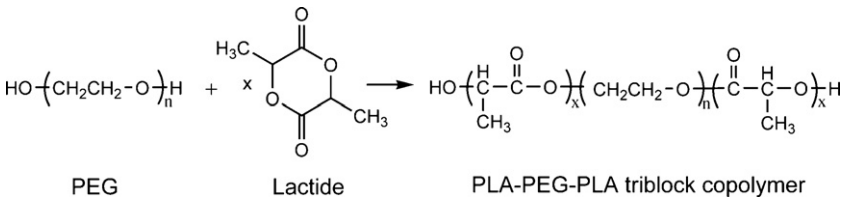
3. Results and discussion

3.1. Characterization of triblock copolymers

Triblock copolymers were synthesized by ring opening polymerization of L- or D-lactide in the presence of PEG (Scheme 2). The initial EO/LA feed ratio was 3/1 or 5/1 in order to obtain water soluble copolymers with sufficiently long PLA blocks. Zinc lactate was used as catalyst instead of stannous octoate or other catalysts which are more or less cytotoxic (Tanzi et al., 1994; Schwach et al., 1997).

The resulting triblock copolymers were characterized by using ^1H NMR and DSC. ^1H NMR allows to determine the structural characteristics such as EO/LA ratio, number average degree of polymerization (DP), and number average molar masses (M_n) (Table 2). The copolymers are named as TB6K3L, TB6K3D, TB12K3L, TB12K3D, TB20K5L and TB20K5D, where TB designates triblock, 6K, 12K and 20K the molar mass of PEG, 3 and 5 the EO/LA feed ratio, L or D the PLLA or PDLA block, respectively. The EO/LA ratios of both copolymers are higher than the feed ratio. This finding can be assigned to the fact that the conversion of lactide was not complete and unreacted lactide was eliminated during the purification procedure, as previously reported (Li and Vert, 2003). The DP of PEG block is 136, 272 and 455, for PEG6000, PEG12000 and PEG20000, respectively, while DP_{PLA} ranges from 35.2 to 67.0. All the copolymers are water soluble as expected. The NMR-derived Mn of the copolymers ranges from 8521 to 23,986.

DSC was used to evaluate the thermal properties, including melting temperature (T_m), melting enthalpy (ΔH_m), glass transition temperature (T_g), and cold crystallization temperature (T_c). All the copolymers are semicrystalline, with T_m ranging from 47.4 to 58.4 °C and ΔH_m from 80.1 to 113.5 J/g. T_g was detected in the −36.4 to −50.5 range, and T_c in the −28.6 to −43.6 °C range.



Scheme 2. Ring opening polymerization of L- or D-lactide in the presence of dihydroxyl PEG using zinc lactate as catalyst.

Table 2
Structural characteristics and thermal properties of PLA/PEG triblock copolymers.

Copolymer	EO/LA[a]	DP_{PEG}[b]	DP_{PLA}[c]	M_{nNMR}[d]	T_m (°C)[e]	ΔH_m (J/g)[e]	T_g (°C)[f]	T_c (°C)[f]
TB6K3L	3.86	136	35.2	8521	51.4	98.5	−36.4	−28.6
TB6K3D	3.72	136	36.6	8616	47.4	113.5	−36.4	−28.6
TB12K3L	4.06	272	67.0	16,792	54.2	81.3	−46.4	−30.3
TB12K3D	4.43	272	61.4	16,389	55.2	80.1	−43.3	−34.2
TB20K5L	8.26	455	55.1	23,986	56.9	91.9	−50.5	−43.6
TB20K5D	9.89	455	46.0	23,332	58.4	85.7	−50.0	−42.9

[a] Calculated from the integration of NMR bands belonging to PEG blocks at 3.6 ppm and to PLA blocks at 5.2 ppm.
[b] $DP_{PEG} = M_{nPEG}/44$.
[c] $DP_{PLA} = DP_{PEG}/(EO/LA)$.
[d] $M_{nNMR} = DP_{PEG} \cdot 44 + DP_{PLA} \cdot 72$.
[e] Obtained by DSC (first heating).
[f] Obtained by DSC (second heating).

3.2. Rheological measurements

When equal amounts of PLLA–PEG–PLLA and PDLA–PEG–PDLA were mixed in an aqueous solution, hydrogels were gradually formed by stereocomplexation between PLLA and PDLA blocks (Li, 2003; Li and Vert, 2003; Li et al., 2005). These PLA/PEG hydrogels are of particular interest for controlled release of proteins because the gelation process proceeds under smooth conditions, without heating or use of organic solvents which could denature proteins.

Fig. 1 shows the evolution of storage modulus (G') and loss modulus (G'') of a 15% TB6K3L/TB6K3D fluid as a function of frequency from 0.01 to 10 Hz. The storage modulus G' is higher than the loss modulus G'' from the very beginning, indicating that a hydrogel was immediately formed after mixture of 15% TB6K3L and TB6K3D aqueous solutions. In other words, a tri-dimensional network was formed due to interactions between PLLA and PDLA blocks. A modulus increase is observed as a function of frequency, G' increasing from 310 to 1954 Pa and G'' from 185 to 896 Pa in the 0.01 to 10 Hz range, which is characteristic of a viscoelastic behavior.

Rheological property changes of the 15% TB6K3L/TB6K3D sample were followed for longer periods of time up to 7 days. Fig. 2 presents the evolution of G' at 37 °C as a function of frequency at different time intervals. After 1 day's gelation, a large increase is observed for the whole frequency range. At 10 Hz, for example, G' increased from initial 1954–3025 Pa at $t = 1$ day. This finding shows that the hydrogel became more consistent due to stereocomplexation between PLLA and PDLA blocks. After 4 days, however, the modulus slightly decreased as compared to values at 1 day, which can be assigned to the partial degradation of the copolymers. In fact, the hydrogel is a dynamic and evolutive system, gelation and degradation occurring simultaneously as previously reported (Li, 2003; Li and Vert, 2003; Li et al., 2005). After 7 days, the modulus further decreased due to more pronounced degradation. Nevertheless, the system remained a hydrogel as G' was higher than G'' (data not shown).

Similar behaviors were obtained in the case of 25 and 40% TB6K3L/TB6K3D samples, 15% TB12K3L/TB12K3D and 15% TB20K5L/TB20K5D samples.

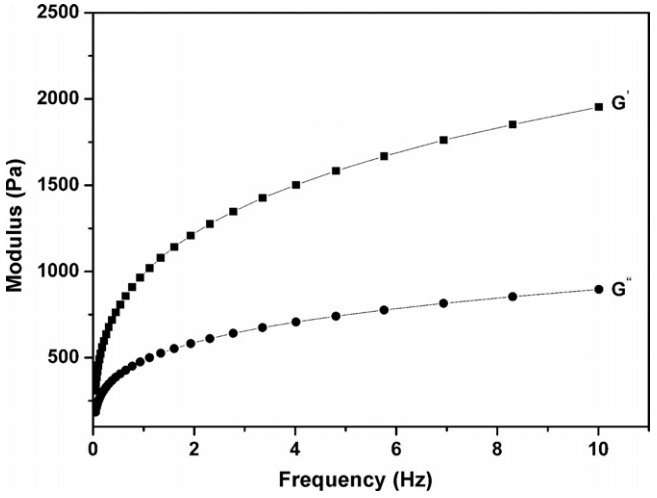


Fig. 1. Variation of storage modulus (G') and loss modulus (G'') of a 15% TB6K3L/TB6K3D fluid as a function of frequency at 37 °C.

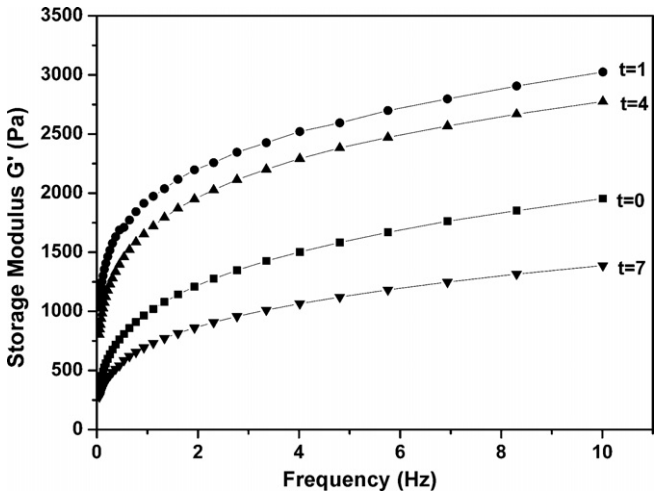


Fig. 2. Storage modulus (G') variation of a 15% TB6K3L/TB6K3D sample as a function of frequency after $t = 0$, 1, 4 and 7 days at 37 °C.

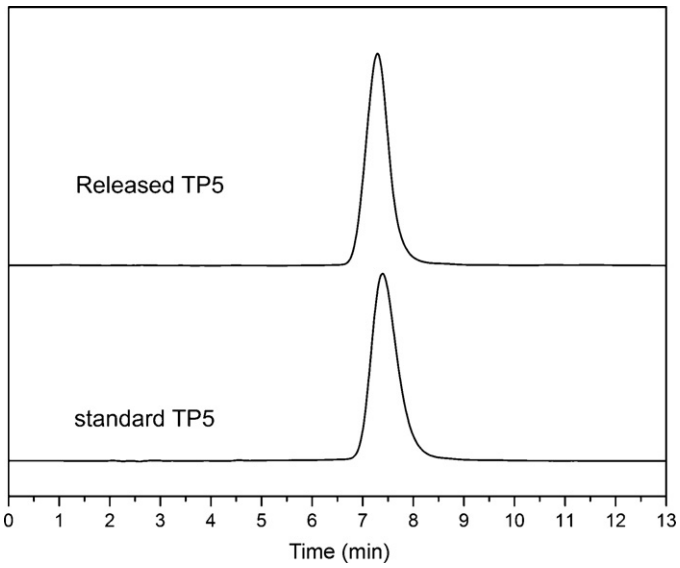


Fig. 3. HPLC chromatograms of thymopentin released from the formulations and original thymopentin.

3.3. In vitro release

TP5 was incorporated in the copolymer solution before gel formation. A gelation time of 2 days was employed so as to obtain hydrogels of good consistency without excessive degradation. Various TP5 containing hydrogels were prepared to elucidate the drug release behaviors, considering the effects of copolymer concentration, drug load, copolymer composition and stereocomplexation.

Fig. 3 presents the HPLC chromatograms of TP5 released from the formulations in comparison with the standard TP5. It appears that the two profiles are very similar. TP5 released from the hydrogels exhibits the same peak time as the standard TP5. Similar findings were obtained at the end of the release experiments, indicating that TP5 is stable in the release medium.

Polymer concentration of hydrogels plays a major role in determining the drug release rate. Fig. 4 shows the drug release profiles of TB6K3L/TB6K3D hydrogels with copolymer concentration ranging from 15 to 40% containing 40 mg of TP5. The 15% hydrogel exhibits a parabolic release profile. A burst release is initially observed. Nearly 36, 54, 65 and 80% of TP5 is released after 2, 4, 6, and 10 h, respectively. This is in agreement with the very high water solubility of TP5 (>10 g/l). The burst release could be considered as beneficial because it allows to rapidly attain the efficient blood concentration. Beyond the burst period, the release rate slows down. After 80 h, nearly 90% of TP5 is released from the hydrogel. A similar release profile is detected for the 25% hydrogel. However, the release rate is lower than that of the 15% hydrogel, 22, 38, 50 and 72% of TP5 being released after 2, 4, 6, and 10 h, respectively. In the case of the 40% hydrogel, the release appears much slower than the two others. In the first 10 h, less than 40% of TP5 is released and the whole release percent is over 80% within 80 h. These findings can be assigned to the fact that release of TP5 molecules is disfavored with increasing copolymer concentration because higher concentration may lead to a more compact structure.

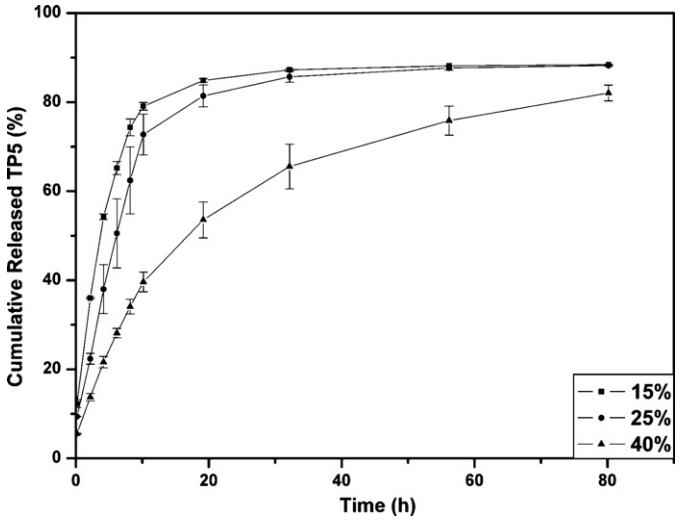


Fig. 4. In vitro release profiles of TP5 from TB6K3L/TB6K3D hydrogels with various copolymer concentrations: 15% (■), 25% (●) and 40% (▲).

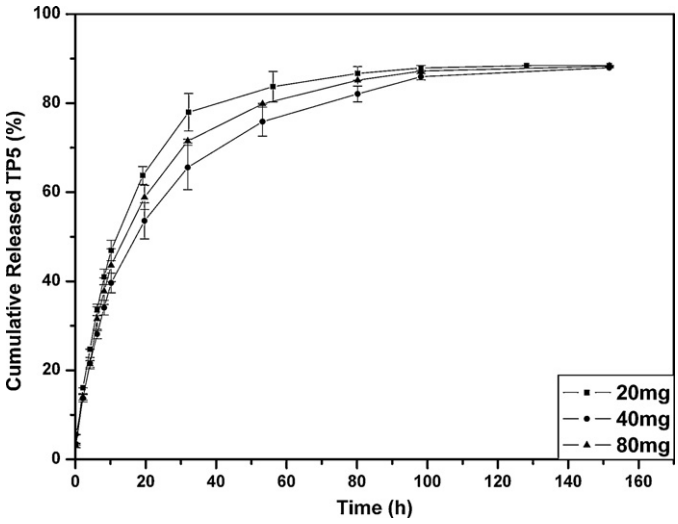


Fig. 5. In vitro release profiles of TP5 from 40% TB6K3L/TB6k3D hydrogels with different TP-5 loads: (■) 20 mg, (●) 40 mg and (▲) 80 mg.

The influence of drug load on the release behavior was also examined. Fig. 5 shows the drug release profiles of 40% TB6K3L/TB6K3D hydrogels containing 20, 40 and 80 mg of TP5. Almost the same release profiles are observed: an initial burst followed by slower release. Nearly 90% of TP5 is released after 152 h. This finding can also be assigned to the very high water solubility of TP5. In fact, with the same copolymer concentration (40%), the release of TP5 is diffusion and degradation controlled. Rheological measurements have shown that degradation did occur during the first 7 days (168 h), but the system remained as a hydrogel (Fig. 2).

Another important parameter in drug delivery systems is the composition of the copolymers. Fig. 6 shows the drug release profiles of 15% TB6K3L/TB6K3D, TB12K3L/TB12K3D and TB20K5L/TB20K5D hydrogels containing 40 mg of TP5. The TB6K sample exhibits a strong burst release, followed by slower release as mentioned above. In contrast, TB12K and TB20K hydrogels present smoother release or slower release rate. In the first 20 h, for example, the release percentage attains 85, 70 and 60% for the TB6K, TB12 and TB20K hydrogels, respectively. These findings are assigned to the different molar masses of the copolymers. In fact, higher molar mass leads to more compact structure of hydrogels at the same concentration, and as a consequence, to lower release rate of TP5. It is also noted that the 3 release curves tend to converge beyond 80 h.

Drug release from TB6K3L single solution was compared to that from TB6K3L/TB6K3D hydrogel in order to elucidate the effect of stereocomplexation. Fig. 7 shows the release profiles of TP5 from TB6K3L solution and TB6K3L/TB6K3D hydrogel, both containing 40 mg of TP5 with a copolymer concentration of 40%. The initial

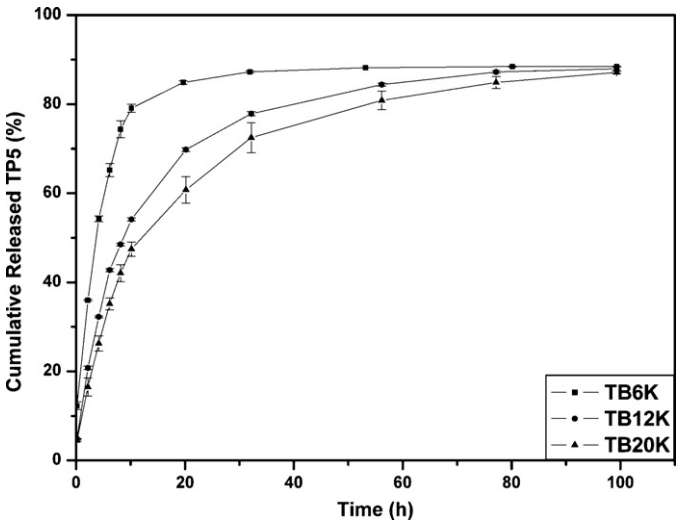


Fig. 6. In vitro release profiles of TP5 from 15% hydrogels prepared from various copolymers: TB6K3L/TB6K3D (■), TB12K3L/TB12K3D (●) and TB20K5L/TB20K5D (▲).

TP5 release appeared much faster for TB6K3L solution than for TB6K3L/TB6K3D hydrogel during the first 90 h. This phenomenon can be assigned to the fact that gelation leads to more consistent structure which not only restricts TP5s diffusion, but also decreases copolymer' degradation rate.

The effect of the dialysis bag on the release behavior was evaluated because it could affect the diffusion of TP5 across the dialysis membrane. A dialysis bag containing 1 ml of 10 mg/ml TP5 solution was immersed in 2 ml of PBS. At an interval of 1 min, all the buffer solution outside the dialysis bag was taken out and replaced by 2 ml of PBS. HPLC analysis showed that over 80% of TP5 had been released in 6 min only, indicating that TP5 can freely diffuse across the dialysis bag into the external medium. However, about 20% TP5 were not released during the release experiments, probably imbibed in the dialysis bag.

In the literature, TP5 containing nanoparticles have been reported as mentioned above (He et al., 2008; Morel et al., 1996; Yin et al., 2006). These systems present some disadvantages including low drug entrapment efficiency, low released amount of TP5, or nondegradability. In our work, the entrapment efficiency can be considered as 100% since TP5 was totally mixed in the hydrogel. Nearly 80% of TP5 was released during the release period up to 150 h. Therefore, the PLA–PEG–PLA hydrogels present many advantages compared to nanoparticle systems, including much easier formulation without organic solvents or surfactants involved, higher drug entrapment efficiency, total bioresorbability, and high release ratio of TP5.

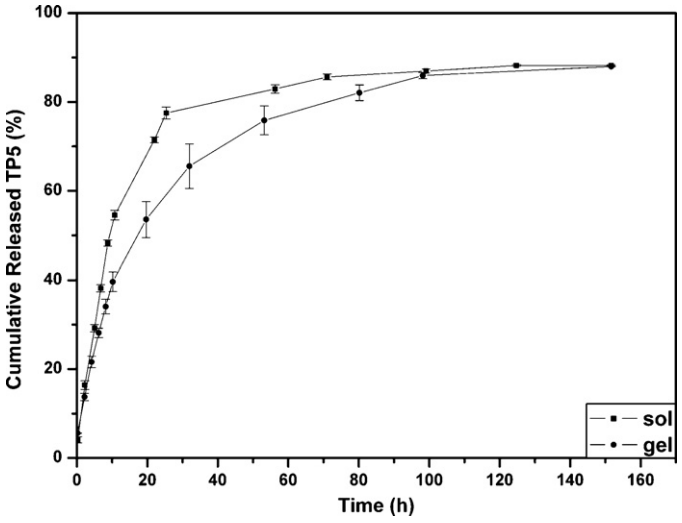


Fig. 7. In vitro release profiles of TP5 from 40% TB6K3L solution (■) and TB6K3L/TB6K3D hydrogel (●).

3.4. In vivo release

The concentration of TP5 cannot be determined with HPLC under in vivo conditions because of the extremely short half-life (about 30 s). As an immuno-modulating drug, TP5 has the function of inducing and promoting differentiation of T-cells, accelerating the development and activating of T-lymphocytes (Gonser et al., 1999). Once absorbed through injection or oral administration, TP5 will rapidly interact with special receptor so that its effect will last several days. The $CD4^+/CD8^+$ ratio and the morphology of thymus are indicators of the immunization efficacy of TP5.

After intraperitoneal injection of CTX, the rats appeared physiologically weaker and weaker during 3 days. Table 3 presents the $CD4^+$, $CD8^+$ data and corresponding $CD4^+/CD8^+$ ratios of the CTX and control group ($P < 0.001$). Both the $CD4^+$ value and $CD4^+/CD8^+$ ratio of the CTX group are much lower than those of the control group, indicating the occurrence of immunosuppression.

Fig. 8 presents the $CD4^+/CD8^+$ ratios of all the experimentation groups at the 1st, 7th and 21st day, in comparison with those of the control group. At the 1st day, the $CD4^+/CD8^+$ ratios of the experimentation groups are much lower than that of the control due to immunosuppression. At the 7th day, the control group, TP5-1 group and 25% gel group exhibit slightly higher $CD4^+/CD8^+$ ratios than the other groups. TP5-2, 15% gel-1 and 15% gel-2 groups present comparable $CD4^+/CD8^+$ ratios as the CTX group since the immunomodulating effect of TP5 is not obvious at this time due to the prolonged interactions between TP5 and the receptor and to the fact that the rats need time to recover from immunosuppression.

At the 21st day, all groups which were treated with TP5 present much higher $CD4^+/CD8^+$ ratios than the CTX group. The con-

Table 3
$CD4^+$, $CD8^+$ data and $CD4^+/CD8^+$ ratio of the control and CTX groups 3 days after intraperitoneal injection.

Group	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
Control	0.27	0.16	1.69
CTX	0.04	0.18	0.22

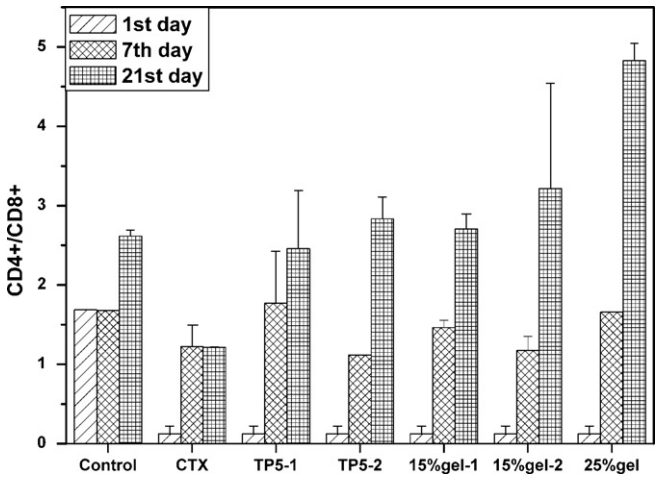


Fig. 8. Variation of $CD4^+/CD8^+$ ratio at the 1st, 7th and 21st day of the control and experimentation groups.

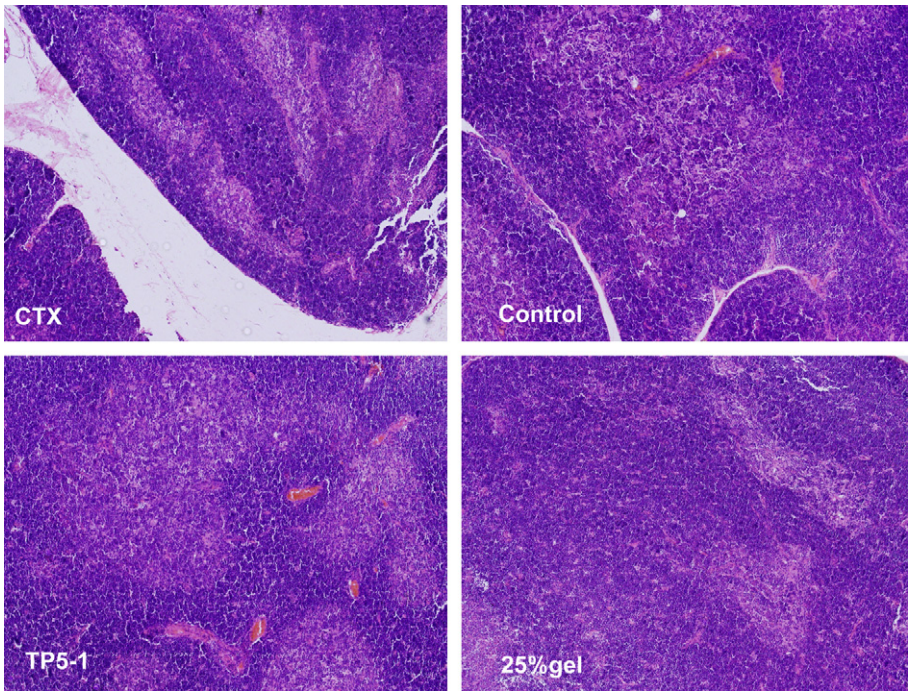


Fig. 9. Optical micrographs (HE 100×) of the thymus of the CTX, control, TP5-1 and 25% gel groups 21 days after the treatment (the light and dark colored parts correspond to medulla and cortex, respectively).

trol group also presents higher $CD4^+/CD8^+$ ratio than the CTX group ($P < 0.02$). This indicates that the rats cannot totally recover themselves from immunosuppression even at the 21st day, and the increase of $CD4^+/CD8^+$ ratio should be assigned to both the immuno-modulating effect of TP5 and to the immune system of the rats. The $CD4^+/CD8^+$ values of all groups treated with TP5 are close to or higher than that of the control group, indicating that the injection of TP5 allowed the rats to recover from immunosuppression. On the other hand, the $CD4^+/CD8^+$ values of 15% gel-2 and 25% gel groups are higher than those of the TP5-1 and TP5-2 groups, especially the 25% gel group with a $CD4^+/CD8^+$ value of 4.83, in agreement with enhanced immunization. Therefore, it can be concluded that the hydrogel drug delivery systems exhibit controlled release behavior. In fact, free TP5 molecules in the TP5-2 and TP5-1 groups can be metabolized, thus losing the immunomodulating effect, whereas those in the hydrogels remain efficient for longer periods of time. The 25% gel group showed the best-controlled release effect because of the more compact structure and slower release rate, as will be confirmed by the morphology of thymus.

The morphology of the thymus was examined by using optical microscope. The lobules of thymus of the CTX group appear much atrophied after 21 days, leaving large zones unoccupied (white part). Both cortex and medulla parts are observed (Fig. 9). In contrast, the lobules of thymus of the control group are well developed with almost no unoccupied zones, the cortex appearing thickened. The TP5-1 and 25% gel groups present similar morphologies as the control group, indicating that both groups well recovered from immunosupression. However, the cortex and medulla parts of the 25% gel group seem better developed than the TP5-1 group, in agreement with the controlled release behavior of the hydrogel systems.

4. Conclusion

Bioresorbable hydrogels are obtained from water soluble PLA–PEG–PLA triblock copolymers through stereocomplexation between PLLA and PDLA blocks. Rheological measurements show that the hydrogels present typical viscoelastic behaviors, although degradation could occur during the gelation process. A water soluble pentapeptide—TP5, was successfully incorporated into the hydrogels. Various parameters such as copolymer concentration, drug load, copolymer composition and the difference between sol and gel were considered. The release profiles are characterized by an initial burst followed by slower release. Higher copolymer concentration leads to slower release rate and less burst effect due to more compact structure which disfavors drug diffusion. Similarly, higher molar mass of the copolymers disfavors the release of TP5, and hydrogels composed of both PLLA/PEG and PDLA/PEG present slower release rates than single copolymer solutions. In contrast, drug load exhibits little influence on the release profiles due to the high water solubility of TP5. In all cases, nearly 80% of TP5 is released. In vivo studies proved the potential of TP5 containing hydrogels, especially those with a concentration of 25%. Both the $CD4^+/CD8^+$ ratio and the morphology of thymus indicate the immunization efficacy of the TP5 release systems based on PLA–PEG–PLA hydrogels.

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