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Injectable thermosensitive PLGA-PEG-PLGA triblock copolymers-based hydrogels as carriers for interleukin-2

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Thermosensitive PLGA-PEG-PLGA triblock copolymers with the DL-lactide/glycolide molar ratio ranging from 6/1 to 15/1 were synthesized by bulk copolymerization of DL-lactide, glycolide and PEG1500. The resulting copolymers are soluble in water to form a freely flowing fluid at room temperature but become hydrogels at body temperature. The release of IL-2 from the copolymer-based hydrogel in the phosphate buffer (pH 7.2) was studied at 37 °C under agitation. IL-2 was released from the copolymer-based hydrogels over 20 days *in vitro* and the release rate decreased with increasing copolymer concentration. The change of DL-lactide/glycolide molar ratio in the PLGA block of the copolymer had little effect on the IL-2 release. The released IL-2 remained 57–90% of its original activity during the release period. To evaluate the anti-tumor effect of the IL-2 loaded copolymer, solutions were injected subcutaneously to H₂₂ tumor-bearing mice. IL-2 loaded copolymer hydrogel for *in vivo* use showed good anti-tumor effect. These results indicate that the thermosensitive PLGA-PEG-PLGA triblock copolymers could be a promising platform for sustained delivery of IL-2.

1. Introduction

Interleukin-2 (IL-2) was first used to activate the immune system against tumor cells transformed from normal cells in melanoma and metastatic renal cell carcinoma, and later in various cancers and immunotherapies (Rosenberg et al. 1986, 1985). Since IL-2 has a short half-life *in vivo*, frequent high-dose injections of IL-2 are required to obtain the anti-tumor efficacy (Lotze et al. 1985). A major limitation of this treatment is the severe toxicity caused by high doses of systemic delivery of IL-2 (Pardoll 1995). Although a variety of approaches have been investigated including microspheres (Hora et al. 1990; Liu et al. 1997), poloxamer hydrogels (Johnston et al. 1992), sustained delivery of IL-2 is still a big challenge.

Recently, injectable biodegradable copolymers of PLGA-PEG-PLGA (poly (DL-lactide-co-glycolide-ethyleneglycol-DL-lactide-co-glycolide), ReGel®) possessing reversible gelation properties have been reported (Zentner et al. 2001; Kim et al. 2001). ReGel® is the trademark of the PLGA-PEG-PLGA copolymers, of which the PEG has molecular weight of 1000 and DL-lactide/glycolide molar ratio ranging from 1.5 to 4. Aqueous solutions of the copolymers are a free-flowing sol below gelation temperature and undergo reversible sol-gel transition at the injection site, forming depots that slowly degrade over a period of four to six weeks (Jeong et al. 1999). In contrast to the chemically cross-linked hydrogels, this kind of hydrogel is held together by non-covalent forces avoiding the use of organic solvents or chemical reactions, which may be potentially deleterious to the loaded protein drugs. Furthermore,

the loading of such a system with a drug can be achieved by simple mixing of the drug with prepared copolymer solution and it can be easily injected with a syringe. However, the gelation temperature of the ReGel system is lower than room temperature, which may lead to difficulties in manufacturing, handling and administration. The thermosensitive PLGA-PEG-PLGA triblock copolymers with a more suitable gelation temperature (29.5 °C ~30 °C) have been synthesized by changing the ratio and length of the monomers (Qiao et al. 2005, 2006).

In this study, we developed thermosensitive PLGA-PEG-PLGA copolymer-based hydrogels for sustained release of IL-2. The *in vitro* release and *in vivo* anti-tumor effect of IL-2 from the hydrogels were investigated.

2. Investigations, results and discussion

2.1. Characteristics of thermosensitive PLGA-PEG-PLGA copolymers

The molecular weights, polydispersity indexes and DL-lactide/glycolide molar ratio of the copolymers are shown in Table 1. The aqueous solution (25%, w/w) of the PLGA-PEG-PLGA(6/1) copolymer flowed freely at room temperature, but became hydrogel at evaluated temperature such as 37 °C. For the thermosensitive copolymers, it is important to precisely control the gelation temperature in designing a drug delivery system. Gelation temperature means the temperature below which the copolymer is soluble in water and above which the copolymer undergoes phase transition to form a water-insoluble hydrogel. If the

Table 1: Molecular weights, compositions and polydispersity indexes of the copolymers

Copolymer	MW of copolymer		DL-Lactide/ glycolide ratio ^b	Polydispersity index
	M _w ^a	M _n ^a		
PLGA-PEG-PLGA(6/1)	4842	3824	5.7	1.27
PLGA-PEG-PLGA(10/1)	4584	3555	9.0	1.29
PLGA-PEG-PLGA(15/1)	5084	4067	14.8	1.25

^a Measured by GPC, relative to polystyrene standards^b Determined by ¹HNMR

gelation temperature is lower than room temperature, gelation will occur at room temperature leading to difficulties in manufacturing, handling and administration. If the gelation temperature is higher than body temperature, the formulation will maintain liquid at the injection site resulting in drug leakage. The gelation temperature of the ReGel[®] system is lower than room temperature (<30 °C), which needs to be improved. As shown in Fig. 1, the gelation temperatures of the copolymers at various concentrations ranged from 29.5 °C to 36.2 °C, which make them more suitable for drug delivery than ReGel.

2.2. *In vitro* release of IL-2 from the copolymer based hydrogels

In order to evaluate the ability of the thermosensitive copolymer based hydrogel to effectively sustained delivery of IL-2, *in vitro* release studies were performed in phosphate buffer (pH 7.2, 0.1 M) containing 0.1% SDS and 0.02% NaN₃ because IL-2 was found to maintain a good long term biological activity in this buffer (Cadée et al. 2002). The effect of the copolymer concentration on the release of IL-2 from the copolymer-based hydrogels is shown in Fig. 2. With increasing concentrations of the copolymer, a corresponding decrease in the release rate of IL-2 occurred. Figure 3 shows the effect of DL-lactide/glycolide ratio on the release of IL-2. IL-2 released from the hydrogels lasted for 20 days and the cumulative percentages of IL-2 were 65%, 68% and 72% for the copolymer with DL-lactide/glycolide molar ratio 6/1, 15/1 and 10/1 (data not shown). The DL-lactide/glycolide molar ratio had little effect on the release rate of IL-2.

It has been reported that only 3% of the encapsulated IL-2 was released from poly (lactide-co-glycolide) micro-

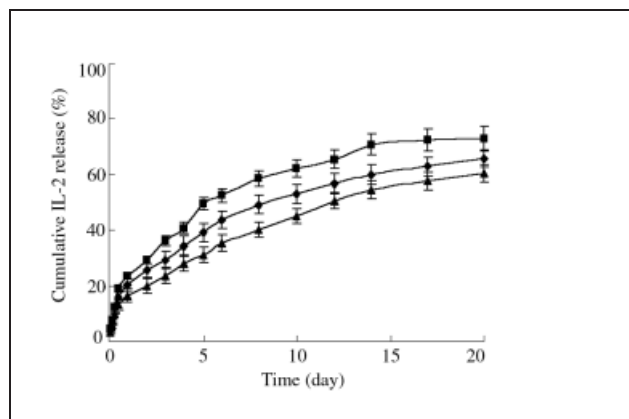


Fig. 2: Effect of PLGA-PEG-PLGA(6/1) copolymer concentration on the IL-2 release at 37 °C. (a): 15% (w/w), (b): 20% (w/w), (c): 25% (w/w). The results are represented as means \pm SD (n = 3)

spheres, but that co-encapsulation of IL-2 and human serum albumin (HSA) in the same carrier could increase the recovery rate of IL-2 to about 10% due to the porous structure in the polymer matrix created by the addition of HSA (Hora et al. 1990). Using the alginate/chitosan microspheres improve the recovery rate up to approximately 100%, but only provided a shorter release period of 5 days (Liu et al. 1997). Proteins encapsulated in microsphere systems can be damaged during formulation, storage and release, which can result in loss of biological activity. It has been reported that half of the biological activity of IL-2 is lost during the encapsulation process of the protein in microspheres due to the presence of organic solvents (Egilmez et al. 1998). For the thermosensitive PLGA-PEG-PLGA copolymers used in this study, the hydrogel formed by increasing the temperature, avoided the use of organic solvents or chemical reactions, which may be potentially deleterious to the loaded protein drugs. Moreover, the preparation of the IL-2 loaded hydrogel can be obtained by one step of gently mixing the IL-2 with copolymer aqueous solution, minimizing the possible biological loss in the formulation process. As shown in Table 1, IL-2 released from hydrogels has 57–90% of its original activity as evaluated by the CTLL-2 proliferation assay. The nature of the observed loss bioactivity is under present investigation.

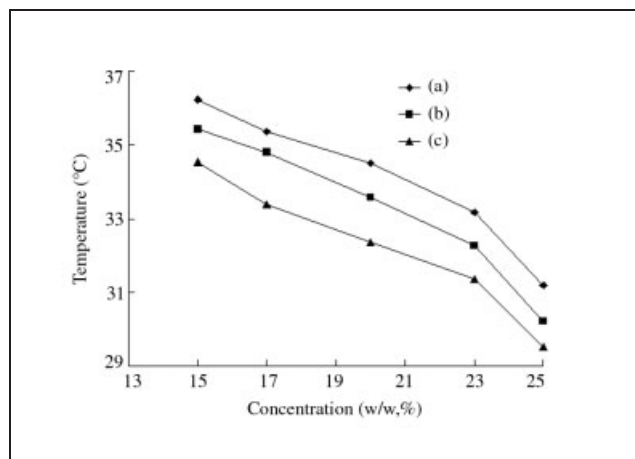


Fig. 1: The gelation temperatures of the PLGA-PEG-PLGA copolymers at various concentrations. (a): PLGA-PEG-PLGA(6/1); (b): PLGA-PEG-PLGA(10/1); (c): PLGA-PEG-PLGA(15/1). The results are represented as means \pm SD (n = 3)

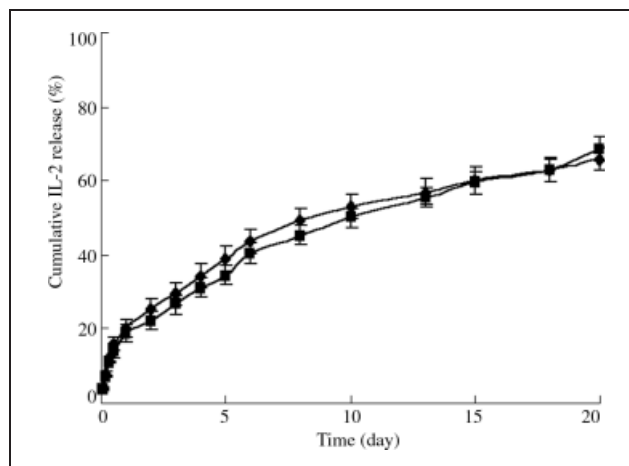


Fig. 3: Effect of DL-lactide/glycolide molar ratio on the IL-2 release at 37 °C. (a): PLGA-PEG-PLGA(6/1); (b): PLGA-PEG-PLGA(10/1); (c): PLGA-PEG-PLGA(15/1). The copolymer concentration was 20% (w/w). The results are represented as means \pm SD (n = 3)

In order to determine the release mechanism of BVP from the copolymer-based hydrogel, the release data of IL-2 from the copolymer-based hydrogels were evaluated by model-dependent method. According to the Higuchi model, $M_t/M_\infty = k_H t^{1/2}$, where M_t is the amount of drug released at time t , M_∞ is the quantity of drug released at infinite time, and k_H is the Higuchi dissolution constant, a straight line is expected for the plot of M_t/M_∞ versus the square root of time if drug release from the matrix is based on Fickian diffusion mechanism. For evaluation of the release data by the described models, the portion of the release curve where $M_t/M_\infty < 0.6$ was used as described in the literature (Higuchi 1963). Good correlation coefficients ($r > 0.9925$) were obtained according to the Higuchi model, suggesting that IL-2 release from the hydrogels was consistent with Fickian diffusion mechanism.

2.3. In vivo anti-tumor effect of IL-2 loaded hydrogel

In vivo anti-tumor activity of IL-2 loaded hydrogel was evaluated using Kunming male mice bearing H₂₂ transplanted hepatocellular carcinoma on right flank region. The tumor weight changes in mice after injection treatments of the groups A–G are shown in Fig. 4. There were no significant differences between groups A and 3, indicating that the blank copolymer based hydrogel rarely affected tumor growth. The cyclophosphamide treatment group (group B) showed the highest tumor inhibition rate of 62.8% compared to the group A. Among the three IL-2 loaded hydrogel treatment groups (groups E–G), significant differences were observed compared to the group C and the tumor inhibition rate ranged from 37.0% to 45.9%. Significant difference ($P < 0.05$) in tumor weight was observed between group D and group E (low IL-2 dose loaded hydrogel treatment group). In the case of groups E and G (medium and high dose of IL-2 loaded hydrogel treatment group), there were no significant differences ($P > 0.05$) compared to group D. The body weight of tumor-bearing mice in seven groups slightly increased during the period and no significant difference in body weight was noted among all groups. This indicated that the copolymer-based hydrogel could effectively sustained IL-2 and suppressed the tumor growth.

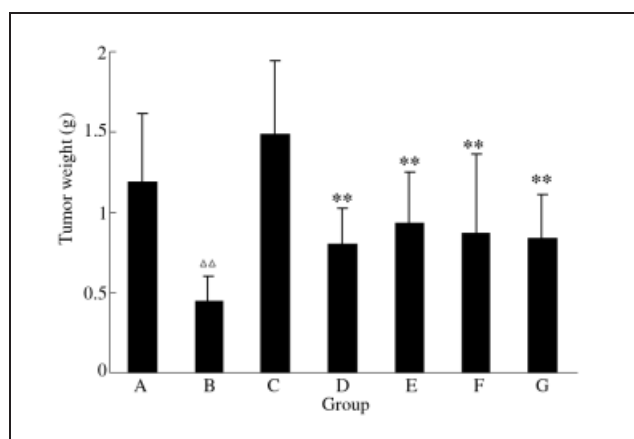


Fig. 4: *In vivo* anti-tumor activity of IL-2 loaded copolymers after subcutaneous administration. The results are represented as means \pm S.D. ($n = 10$). A: Negative control. B: Positive control. C: Blank hydrogel. D: IL-2 solution treated group (750×104 U/kg). E: Low dose of IL-2 sustained formulation (375×104 U/kg). F: Medium dose of IL-2 sustained formulation (750×104 U/kg). G: High dose of IL-2 sustained formulation (1500×104 U/kg).

** $P < 0.01$, the IL-2 solution and sustained formulation group vs. blank hydrogel group. △△ $P > 0.01$, the positive control vs. negative control

Table 2: Relative biological activity of IL-2 released from the PLGA-PEG-PLGA(6/1) copolymer (20%, w/w) based hydrogels at different time points

Release time (Day)	Biological activity (%) ^a
1	90
3	83
7	80
10	68
14	57

^a The biological activity is related to freshly reconstituted IL-2 samples. The values are the mean of two measurements that deviated 5 ~ 15%

3. Experimental

3.1. Materials

Polyethylene glycol (PEG 1500) was purchased from Shanghai Pudong Gaonan Chemical Corporation. DL-Lactide and glycolide were purchased from China Rehabilitation Research Center CONAN Polymer R&D Center and used without further purification. Stannous 2-ethylhexanoate was obtained from Sigma (St. Louis, MO, USA). Interleukin-2 was supplied by Shenyang Sunshine Pharmaceutical Co., Ltd. (P.R. China). Enzyme linked immunosorbent assay (ELISA) kits for quantitative determination of IL-2 were obtained from the Genzyme Corp (Boston, MA, USA). All other chemicals were of reagent grade purchased from the market.

3.2. Synthesis of PLGA-PEG-PLGA triblock copolymers

The synthesis of thermosensitive PLGA-PEG-PLGA copolymers, copolymer characterization and sol-gel transition behaviors were described previously (Qiao et al. 2005). Briefly, ring-opening polymerization in the bulk state was carried out at 150 °C for 8 h with different molar ratio of DL-lactide/glycolide (6/1 and 15/1) and the weight ratio of PEG was adjusted to 30% (w/w). Stannous (II) 2-ethylhexanoate (0.2%, w/w) was used as catalyst. After polymerization, the product was dissolved in water and the copolymer solution was heated to 80 °C to precipitate the copolymer. The resulting copolymer was dried under vacuum at room temperature until constant weight. The copolymer nomenclature was designated PLGA-PEG-PLGA (X/Y), where X/Y is the molar ratio of DL-lactide/glycolide.

The DL-lactide/glycolide molar ratio of the resulted copolymer was determined by ¹H NMR (Bruker ARX-300). The molecule weights and molecular weight distributions of the PLGA-PEG-PLGA copolymers were monitored by a GPC system with a Shimadzu LC-10AD HPLC pump, Shimadzu RID-6A refractive index detector (Kyoto, Japan) and Hewlett Packard Plgel columns. The molecular weights of the copolymer were determined relative to polystyrene standards.

3.3. Measurement of gelation temperature (Yong et al. 2001)

A 20 ml transparent vial containing a 2.6 g magnetic bar (cylinder, 10 × 5 mm i.d.) and 10 g water solution of PLGA-PEG-PLGA copolymer was placed in a water bath. The solution was heated at a constant rate of 2 °C per minute with constant stirring (200 rpm). When the magnetic bar stopped stirring due to gelation of the solution, the temperature read from the thermometer was determined as the gelation temperature.

3.4. Recombinant IL-2 assay

An enzyme linked immunosorbent assay (ELISA) was used to determine the concentration of IL-2. Briefly, the IL-2 assay was a solid-phase enzyme immunoassay, which employed a multiple antibody sandwich principle. Samples of IL-2 were bound by the biotin-labeled capture antibody and peroxidase-conjugated detection antibody by incubated at 37 °C. This complex bound via the biotin-labeled antibody to the streptavidin-coated 96-wells microliter plate. After washing, the peroxidase bound in the complex was developed by tetramethylbenzidine and the reaction was stopped by acidification. The absorbance was measured at 450 nm to calculate the concentration of IL-2.

3.5. Release of IL-2 from hydrogel in vitro

The PLGA-PEG-PLGA copolymers were dissolved in the phosphate buffer (pH 7.2, 0.1 M) to form various concentrations of copolymer solution (15%, 20%, 25%, w/w). The copolymer solution (0.4 ml) was placed into the test tube and mixed with IL-2 (1×10^6 IU). The tubes were incubated for 5 min at 37 °C. Four milliliter of phosphate buffer (pH 7.2) containing 0.02% (w/v) Na₃N and 0.1% (w/v) SDS were added to the formed hydrogel and gently shaken at 20 rpm. At sampling times, the release medium was replaced with the same amount of fresh buffer. The amount of IL-2 in the release medium was determined by IL-2 ELISA kit as described above.

3.6. IL-2 bioassay

The biological activity of IL-2 was determined by using a IL-2 dependent murine tumor-specific cytotoxic T cell line (CTLL-2). Cells were cultured in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum in a humidified atmosphere of 5% CO₂ in air at 37 °C. The samples were diluted in steps of 1 in 10 in a range of 30 ng/ml to 0.03 ng/ml in a 96-wells plate. Each dilution was made in three replicates. To each well 50 µl of CTLL-2 cell suspension (1×10⁴ cells) was added and incubated for 2 days at 37 °C. Then, 50 µl of XTT solution (5 mg/ml) was added to each well and after 4 h incubation the proliferation was measured by using a Cell Proliferation Kit II.

3.7. In vivo antitumor activity of IL-2 loaded copolymer-based hydrogels

Kunming male mice weighing 18 ~ 22 g were acclimatized for 7 days after arrival. Approximately, 2 × 10⁶ H₂₂ tumor cells were injected subcutaneously into hind right flank region of the mice and the tumors allowed the growth. The thermosensitive PLGA-PEG-PLGA(6/1) copolymer was prepared to 20% concentration for the *in vivo* test. The mice were randomly divided into 7 treatment groups of ten each. The control group (group A and C) was subcutaneously injected with saline (0.01 ml/g) and blank copolymer solution (0.01 ml/g), respectively. The treatment groups (group B and D) received 0.01 ml/g cyclophosphamide (equivalent to 0.02 mg/g dose) and IL-2 aqueous solution (equivalent to 7.5 × 10³ U/g IL-2 dose) subcutaneously injected into the hind left flank region (opposite site of tumor) for five consecutive days. The IL-2 loaded hydrogel treatment groups (group E–G) received 0.01 ml/g copolymer solutions (equivalent to 3.75 × 10³, 7.5 × 10³ and 1.5 × 10⁴ U/g IL-2 dose) subcutaneously injected into the hind left flank region (opposite site of tumor) on treatment day 0. The animals were sacrificed 24 h after the final administration of the IL-2 aqueous solution and the tumor inhibition rate was calculated as follows:

$$\text{Tumor inhibition rate (\%)} = \frac{a - b}{a} \times 100\%$$

where, a represents the tumor weight of control group and b represents tumor weight the treatment group.

The experiments were carried out under approval of the ethics committee of the Faculty of Veterinary Sciences of the Shenyang Pharmaceutical University. The protocol complies with the recommendations of the ethics committee and approval of the protocol was obtained.

3.8. Statistical analysis

Data are presented as the mean ± SD. The differences between the groups were examined using Student's t-test. A p value of less than 0.05 was considered statistically significant.

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