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In vitro and in vivo protein delivery from in situ forming poly(ethylene glycol)–poly(lactide) hydrogels

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Abstract

Previous studies have shown that stereocomplexed hydrogels are rapidly formed in situ by mixing aqueous solutions of eight-arm poly (ethylene glycol)-poly(L-lactide) and poly(ethylene glycol)–poly(D-lactide) star block copolymers (denoted as PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$, respectively). In this study, *in vitro* and *in vivo* protein release from stereocomplexed hydrogels was investigated. These hydrogels were fully degradable under physiological conditions. Proteins could be easily loaded into the stereocomplexed hydrogels by mixing protein containing aqueous solutions of PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$ copolymers. The release of the relatively small protein lysozyme (d_h = 4.1 nm) followed first order kinetics and approximately 90% was released in 10 days. Bacteria lysis experiments showed that the released lysozyme had retained its activity. The relatively large protein IgG (d_h = 10.7 nm) could be released from stereocomplexed hydrogels with nearly zero order kinetics, wherein up to 50% was released in 16 days. The *in vitro* release of the therapeutic protein rhIL-2 from stereocomplexed hydrogels also showed nearly zero order kinetics, wherein up to 45% was released in 7 days. The therapeutic efficacy of stereocomplexed hydrogels loaded with 1×10^6 IU of rhIL-2 was studied using SL2-lymphoma bearing DBA/2 mice. The PEG–$(PLLA)_8$/PEG–$(PDLA)_8$/rhIL-2 mixture could be easily injected intratumorally. The released rhIL-2 was therapeutically effective as the tumor size was reduced and the cure rate was 30%, whereas no therapeutic effect was achieved when no rhIL-2 was given. However, the cure rate of rhIL-2 loaded stereocomplexed hydrogels was lower, though not statistically significant, compared to that of a single injection with 1×10^6 IU of free rhIL-2 at the start of the therapy (cure rate = 70%). The therapeutic effect of rhIL-2 loaded stereocomplexed hydrogels was retarded for approximately 1–2 weeks compared to free rhIL-2, most likely due to a slow, constant release of rhIL-2 from the hydrogels.
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1. Introduction

Recombinant human IL-2 (rhIL-2) is a broadly acting T cell-derived cytokine with proven anti-tumor activity, especially after local administration, and is produced by recombinant DNA technology [1]. Local IL-2 therapy is most effective against cancer when injected intratumorally [2]. In a clinical phase II trial, patients with advanced nasopharyngeal carcinoma were treated with combined radiotherapy and local rhIL-2 immunotherapy. The patients received 15 injections of rhIL-2 (3 times 5 daily injections in weeks 2, 4 and 6). After five years 63% of the patients were tumor-free, whereas treatment with only radiotherapy resulted in 8% tumor-free patients [3]. To avoid frequent and painful injections, a long acting protein delivery system is required.

Hydrogels have been used extensively as carriers for proteins, since their high water content renders them compatible with

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incorporated proteins and living tissue [4]. Injectable, in situ forming hydrogels are particularly interesting, because they allow easy and homogeneous loading of proteins [5]. Hydrogels can be formed by chemical and physical crosslinking. In situ forming physically crosslinked hydrogels have been prepared by a variety of noncovalent interactions, including self-assembly through hydrophobic interactions of poly(ethylene glycol) based block copolymers [6–8] or poly(N-isopropylacrylamide) (PNIPAAm) (co)polymers [9–11]. Crosslinking by physical interactions proceeds under milder conditions as compared to chemical crosslinking, which requires the use of photo-irradiation, organic solvents, auxiliary crosslinking agents and/or other reactive molecules that may damage the proteins to be incorporated. Recently, hydrogels have been prepared in situ from water-soluble poly(L-lactide) (PLLA) and poly(D-lactide) (PDLA) based block copolymers, in which the physical crosslinks are provided by stereocomplexation between the enantiomeric PLLA and PDLA blocks [12–18]. De Jong et al. have prepared stereocomplexed hydrogels from dextran–lactate graft copolymers [14] and Li et al. have prepared stereocomplexed hydrogels based on PLA–PEG–PLA triblock copolymers [15]. These stereocomplexed hydrogels have many advantages, e.g. they can be formed in situ at physiological conditions (37 °C, pH 7.4) by simply mixing two aqueous enantiomer solutions, the gelation process is very mild in which both temperature and pH do not change, and they are biodegradable. Nevertheless, dextran–PLA stereocomplexed hydrogels require involved synthesis of dextran–PLA graft copolymers, and stereocomplexed hydrogels based on PLA–PEG–PLA triblock copolymers exhibit relatively slow gelation and low mechanical strength.

The use of hydrogels for the release of rhIL-2 has been investigated [17–19]. Hanes et al. prepared rhIL-2 loaded microspheres by crosslinking of gelatin and chondroitin sulphate with gluteraldehyde. Release experiments *in vivo* using a brain tumor mice model showed a cure rate of 40% [19]. De Groot et al. prepared rhIL-2 loaded dextran-(hydroxyethyl)methacrylate (dex-(HE)MA) hydrogels by redox initiated polymerization [17]. When these hydrogels were used *in vivo* in SL2-lymphoma bearing DBA/2 mice, cure rates of 62% were obtained. Bos et al. studied release of rhIL-2 *in vivo* from stereocomplexed hydrogels based on dextran-L-lactate and dextran-D-lactate copolymers in this SL2-DBA/2 tumor mice model [18]. The therapeutic effect of rhIL-2 loaded hydrogels was at least comparable to injection of an equal dose with free rhIL-2 (cure rate of 60%).

We have previously reported on stereocomplexed hydrogels based on eight-arm PEG–PLA star block copolymers (PEG–$(PLA)_8$) [13,20]. The PEG–$(PLA)_8$ copolymers could readily be prepared with controlled compositions. Upon mixing aqueous solutions of PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$ copolymers, hydrogels with a high physical crosslinking density were rapidly formed. Rheological experiments showed that the hydrogel storage modulus increased with increasing PLA block length and polymer concentration, thus indicating a higher crosslinking density and a smaller hydrogel mesh size at higher PLA block length and higher polymer concentration. In this paper, the *in vitro* release of two model proteins with different hydrodynamic diameters, lysozyme and immunoglobulin G (IgG), were studied, as well as the release of the therapeutic protein rhIL-2. The therapeutic efficacy of rhIL-2 loaded stereocomplexed hydrogels was studied using the SL2 tumor mice model.

2. Materials and methods

2.1. Materials

Eight-arm PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$ star block copolymers were prepared as reported previously [13]. Lysozyme (from hen egg white) was purchased from Fluka (Buchs, Switzerland) and bovine immunoglobulin G (IgG, fraction II) was purchased from ICN Biochemicals BV (Zoetermeer, The Netherlands). Recombinant human interleukin-2 (rhIL-2) was purchased from Chiron BV (Amsterdam, The Netherlands). When the white lyophilized powder is reconstituted with 1.2 ml of water each vial contains per ml solution: 1 mg (18×10^6 IU) of rhIL-2, 50 mg (5% w/v) of mannitol, and 0.2 mg (0.02% w/v) of SDS, buffered with sodium phosphates to a pH of 7.5 (range 7.2–7.8).

2.2. Critical gel concentration

PEG–$(PLA)_8$ star block copolymer solutions were prepared with concentration increments of 2.5% w/v, by dissolving the polymers overnight. Subsequently, solutions of equimolar amounts of PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$ star block copolymers were mixed and equilibrated overnight. The critical gel concentrations were determined by inverting the vials. When the sample showed no flow within 20 s, it was regarded as a gel.

2.3. Hydrogel degradation/swelling tests

Stereocomplexed hydrogels (0.5 ml) containing equimolar amounts of PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$ star block copolymers were prepared by mixing aqueous solutions of both polymers in HEPES buffered saline (pH 7.0, 100 mM, adjusted to 300 mOsm with NaCl, 0.02 wt.% NaN_3) and equilibration overnight. Subsequently, 3 ml of HEPES buffered saline was applied on top of the hydrogels and the hydrogels were allowed to swell at 37 °C. The swelling experiment was performed in triplicate. The swollen hydrogels were weighed at regular time intervals after removal of excess buffer. After each weighing the buffer was refreshed. Similar degradation/swelling studies were performed at pH 5.0 using an ammonium acetate buffer (100 mM, adjusted to 300 mOsm with NaCl). The swelling ratio of the hydrogels was calculated from the initial hydrogel weight after preparation (W_0) and the swollen hydrogel weight after exposure to buffer (W_t):

$$\text{Swelling ratio} = \frac{W_t}{W_0}$$

2.4. In vitro release of model proteins

For the *in vitro* release of the model proteins lysozyme and IgG, 20 µl of a concentrated protein solution was added to both

PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$ solutions in HEPES buffered saline (pH 7.0) to a final protein concentration of 1 wt.%. Stereocomplexed hydrogels (0.5 ml) were prepared by mixing the solutions of equimolar amounts of PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$. After equilibration overnight, the hydrogels were transferred to cylindrically shaped vials with a flat bottom and a diameter of 8.8 mm, only exposing the upper surface of the hydrogel (device described in ref. [21]). Subsequently, 3 ml of HEPES buffered saline was applied on top of the gels and the system was kept at 37 °C. Samples of 0.5 ml of the supernatant buffer were taken at regular time intervals (the first days after 30 min, 1 h, 2 h, 4 h, 8 h and 24 h, and subsequently after 1 to 3 days) and replaced by an equal volume of fresh buffer. Similar release experiments were performed at pH 5.0 using ammonium acetate buffered saline. The concentrations of lysozyme and IgG in the release samples were determined using the BCA® Protein assay [22]. Standard protein solutions (concentration range 0.01–2 mg/ml) were prepared to generate calibration curves. Release samples (25 μl) were pipetted into a 96-microwells plate and 200 μl of working reagent (BCA reagent A: BCA reagent B, 50:1 v/v) was added. The plates were incubated for 30 min at 37 °C and then cooled to room temperature. Finally, the absorbance at 550 nm was determined with a Microplate Manager (Bio-Rad Laboratories, Hercules, CA, USA).

The enzymatic activity of lysozyme was determined for a few release samples. The assay is based on the lysis of the outer cell membrane of *Micrococcus lysodeikticus*, resulting in solubilization of the affected bacteria and consequent decrease of light scattering [23]. The release samples were diluted to a concentration of 50–100 μg/ml and 10 μl of the sample was added to 1.3 ml of the bacteria suspension (0.2 mg/ml, HEPES buffered saline, pH 7.0). The decrease in turbidity was measured at 450 nm and the percent remaining enzymatic activity was determined by comparing the activity of the sample with that of a freshly prepared reference lysozyme solution (0.1 mg/ml).

2.5. In vitro release of rhIL-2

For the *in vitro* release of rhIL-2, 20 μl of a concentrated rhIL-2 solution was added to both PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$ solutions in HEPES buffered saline (pH 7.0) to a final concentration of 12×10^6 IU of rhIL-2 per 0.5 ml of solution. Stereocomplexed hydrogels (0.5 ml) were prepared by mixing these solutions of equimolar amounts of PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$. After equilibration overnight, the hydrogels were transferred to cylindrically shaped vials with a flat bottom and a diameter of 8.8 mm, only exposing the upper surface of the hydrogel. Subsequently, 3 ml of PBS (pH 7.2, 100 mM, adjusted to 300 mOsm with NaCl, 0.02 wt.% NaN_3) was placed on top of the gels and the system was kept at 37 °C. PBS contained 0.01 wt.% SDS to prevent precipitation of rhIL-2 [24]. The concentration of rhIL-2 in the release samples was determined by reversed phase high-performance liquid chromatography (RP-HPLC) using a LC Module I system (Waters™) with an analytical column (Jupiter, 5 μm C4 300 A, 150 × 4.6 mm, including a SecurityGuard™ cartridge system with Widepore, C4, 4 × 3 mm). The rhIL-2 samples were centrifuged for 1 min (13,000 g) and 100 μl of the supernatant was applied on the column. A linear gradient was run from 40% A (water/acetonitrile 95:5 w/w; 100 mM sodium perchlorate ($NaClO_4$); 10 mM perchloric acid ($HClO_4$)) and 60% B (water/acetonitrile 5:95 w/w; 100 mM $NaClO_4$; 10 mM $HClO_4$) to 100% B in 10 min. The flow rate was set at 1.0 ml/min and the column oven was set at 30 °C. UV detection at a wavelength of 205 nm was applied or the fluorescent emission at 300 nm (excitation wavelength of 295 nm) was measured. Peak areas were determined with Millennium 2010V.2.15 software (Waters Associates Inc.). The total amount of oxidized and native rhIL-2 was calculated by using a rhIL-2 calibration curve over the range of 1.10^3–92.10^3 IU of rhIL-2.

2.6. Animals and tumor cells

Inbred female DBA/2 mice (age 6–8 weeks) were obtained from Charles River France (Saint Aubin les Elbeuf, France) and were housed in filter-top cages. SL2 lymphosarcoma cells, originally arisen as a spontaneous tumor in DBA/2 mice, were propagated by intraperitoneal injection. After 7 days, tumor cells were harvested by peritoneal lavage with 5 ml of RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml neomycin sulphate. The cells were spun down and resuspended in medium after removal of the supernatant.

2.7. Animal model

1×10^5 SL2 cells in 0.1 ml of medium were injected subcutaneously in DBA/2 mice and the tumors were allowed to grow for 11 days. Four different treatment groups were chosen. Two negative controls, group A (HEPES buffered saline, pH 7.0) and group B (PEG–$(PLA_{12})_8$ hydrogels without rhIL-2), consisting of 10 and 4 mice, respectively, and one positive control, group C (free rhIL-2), consisting of 7 mice. The experimental group D (rhIL-2 loaded PEG–$(PLA_{12})_8$ hydrogels) consisted of 10 mice. At day 0, group A was injected intratumorally with 400 μl of HEPES buffered saline and group B with 400 μl of empty PEG–$(PLA_{12})_8$ hydrogel. Group C was injected intratumorally with 400 μl of a rhIL-2 solution in HEPES buffered saline (1×10^6 IU/400 μl) and group D with 400 μl of rhIL-2 loaded hydrogel (1×10^6 IU/400 μl). A single injection with 1×10^6 IU rhIL-2 was chosen for both the free rhIL-2 control and the rhIL-2 loaded hydrogel groups, as this dose is effective in SL2-lymphoma bearing DBA/2 mice [2,35]. Using this experimental setup, the therapeutic efficacies of rhIL-2 slowly released from the gel and free rhIL-2 administered by a single injection were compared. Stereocomplexed hydrogels were prepared by mixing rhIL-2 containing aqueous solutions of PEG–$(PLLA_{12})_8$ and PEG–$(PDLA_{12})_8$ in HEPES buffered saline. The mixture was injected intratumorally within 5 min of mixing. The therapeutic efficacy was measured by the reduction in the tumor size and the survival rate of the mice. When treated animals survived for more than 60 days without visible signs of tumors, they were considered to be cured [2].

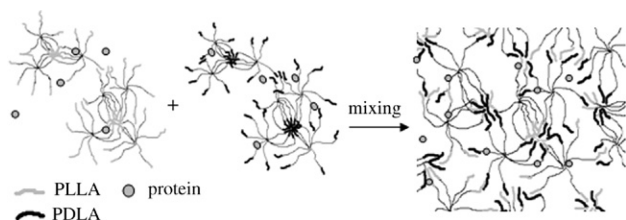

Fig. 1. Preparation of protein loaded stereocomplexed hydrogels by mixing protein containing aqueous solutions of PEG–(PLLA)$_8$ and PEG–(PDLA)$_8$ star block copolymers.

2.8. Ethics

The protocol of the animal experiments was approved by the ethics committee of the Faculty of Veterinary Sciences of the Utrecht University.

3. Results and discussion

3.1. Star PEG–PLA stereocomplexed hydrogels

Our previous studies showed that hydrogels were rapidly formed under physiological conditions upon mixing aqueous solutions of eight-arm poly(ethylene glycol)–poly(L-lactide) and eight-arm poly(ethylene glycol)–poly(D-lactide) star block copolymers (denoted as PEG–(PLLA)$_8$ and PEG–(PDLA)$_8$, respectively) via stereocomplexation of the PLLA and PDLA blocks [13,20]. In this study, PEG–(PLLA)$_8$ and PEG–(PDLA)$_8$ star block copolymers ($M_{n,\ \mathrm{PEG}}$=21.8 kDa) with 12, 14 and 15 lactyl units per PLA block were prepared. Stereocomplexed hydrogels were formed in situ by mixing aqueous solutions in HEPES buffered saline (pH 7.0) of equimolar amounts of PEG–(PLLA)$_8$ and PEG–(PDLA)$_8$ star block copolymers, when the polymer concentration was above the critical gel concentration (CGC). The CGCs for PEG–(PLA)$_8$ copolymers with 12, 14 and 15 lactyl units per PLA block were 7.5, 5 and 5% w/v, respectively (as determined by vial tilting). Degradation/swelling tests, performed at 37 °C and pH 7.0, showed that the swelling of the stereocomplexed hydrogels increased over a period of 2 days (results not shown). After 2 days, the swelling could not be determined accurately, since the stereocomplexed hydrogels became too fragile to effectively remove all excess buffer and to subsequently weigh the stereocomplexed hydrogels. After 3 weeks a clear solution was obtained, showing that the stereocomplexed hydrogels fully degraded into water-soluble degradation products. In contrast, at pH 5.0 PLA degradation is substantially retarded [25] and the stereocomplexed hydrogels remained intact for 3 weeks, showing negligible swelling (results not shown). This indicates that the stereocomplexed hydrogels are initially physically stable and that the loss of the hydrogel integrity is associated with PLA degradation.

3.2. Release of model proteins in vitro

The release of two model proteins, lysozyme (hydrodynamic diameter of 4.1 nm [26]) and immunoglobulin G (IgG, hydrodynamic diameter of 10.7 nm [27]) was studied at 37 °C and pH 7.0. Proteins could be easily loaded into the stereocomplexed hydrogels by mixing protein containing aqueous solutions of PEG–(PLLA)$_8$ and PEG–(PDLA)$_8$ copolymers (Fig. 1).

In Fig. 2a and b the release profiles of lysozyme from stereocomplexed PEG–(PLA)$_8$ hydrogels are shown as a function of polymer concentration and PLA block length, respectively. The release is proportional to the square root of time up to a cumulative release of approximately 80% irrespective of the polymer concentration or PLA block length, indicating that he release kinetics are first order (inserts in Fig. 2a and b). Although this release profile suggests a typical diffusion-controlled release of a compound from a hydrogel [28], which has reached equilibrium swelling, the actual situation is more complex. The stereocomplexed hydrogels degrade in time, caused by removal of physical crosslinks, leading to increased swelling and final disintegration of the network. All these factors influence the release behavior of the

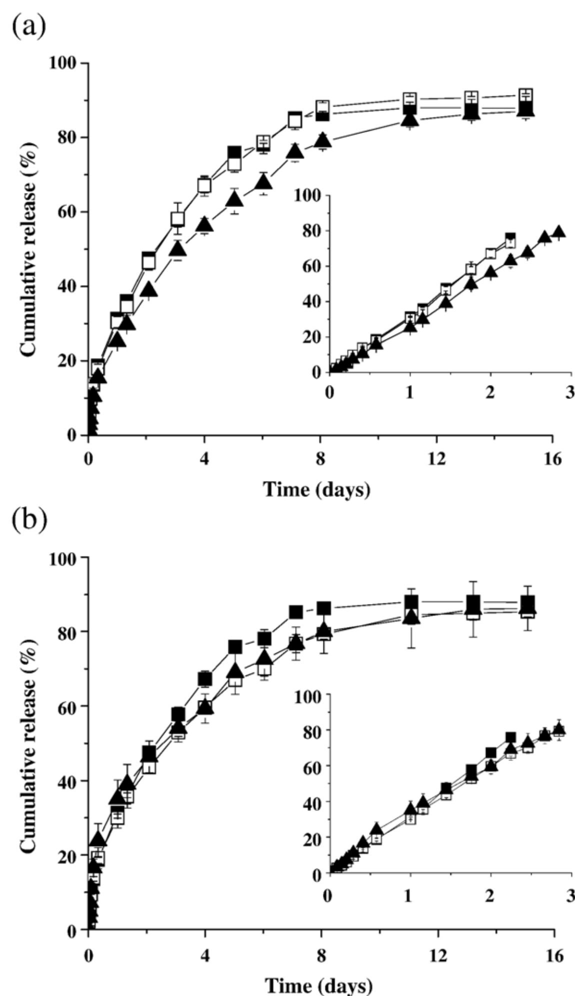

Fig. 2. Cumulative release profiles of lysozyme from stereocomplexed PEG–(PLA)$_8$ hydrogels at 37 °C (average ± S.D., n=3). (a) PEG–(PLA$_{12}$)$_8$ hydrogels at pH 7.0 and initial polymer concentrations of 10 (■), 12.5 (□) and 15% w/v (▲); (b), PEG–(PLA$_{12}$)$_8$ (■), PEG–(PLA$_{14}$)$_8$ (□) and PEG–(PLA$_{15}$)$_8$ (▲) hydrogels at pH 7.0 and 10% w/v initial polymer concentration. The inserts show the cumulative release (%) as a function of the square root of time (days$^{1/2}$).

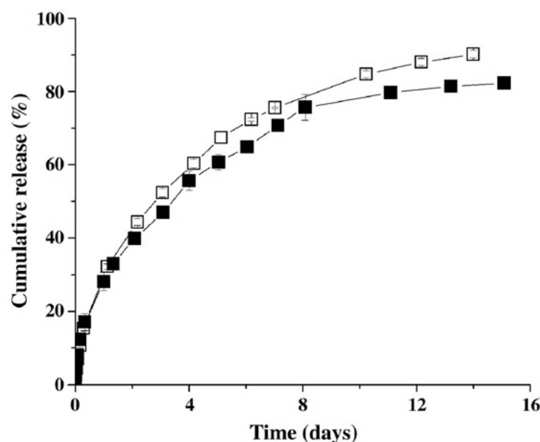

Fig. 3. Cumulative release profiles of lysozyme from stereocomplexed PEG–$(PLA_{14})_8$ hydrogels at 12.5% w/v initial polymer concentration and 37 °C, at pH 7.0 (■) or pH 5.0 (□) (average ± S.D., $n = 3$).

protein. Lysozyme was released approximately 90% in 10 days. The release of lysozyme is hardly influenced by the polymer concentration and PLA block length, which indicates that the pores in the hydrogel are substantially larger than the hydrodynamic diameter of the protein (Fig. 2a and b). Bacteria lysis experiments showed that the released lysozyme retained its activity (results not shown). This emphasizes the protein-friendly preparation process of the stereocomplexed hydrogels. The release profile at pH 7.0 was similar to that at pH 5.0 (Fig. 3), indicating that the release is mainly determined by diffusion rather than degradation of the hydrogel matrix. At pH 5.0 the hydrogel showed negligible swelling and degradation over the release period. In contrast, at pH 7.0 the hydrogels completely degraded over the release period. Most likely, the initial mesh size of the hydrogel is larger than the hydrodynamic diameter of lysozyme (4.1 nm) and the release at pH 5.0 is diffusion-controlled.

Fig. 4a shows that stereocomplexed PEG–$(PLA_{12})_8$ and PEG–$(PLA_{14})_8$ hydrogels at 12.5% w/v polymer concentration release IgG with nearly zero order release kinetics during the first 16 days. Stereocomplexed PEG–$(PLA_{14})_8$ hydrogels with a polymer concentration of 7.5% w/v show a biphasic release. The release kinetics were nearly zero order up to 5 days, whereafter the release was accelerated, and the release kinetics became close to first order. It should be noted that the acceleration in the release was observed only for hydrogels formed at a lower polymer concentration of 7.5% w/v. The acceleration in the release is probably caused by partial disintegration and/or fragmentation of the network due to PLA degradation. The lower release rate at 12.5% w/v polymer concentration compared to 7.5% w/v polymer concentration is most likely due to a smaller initial hydrogel pore size as well as a slower degradation of the hydrogel at 12.5% w/v polymer concentration. The lower release rate at higher polymer concentration is in line with previous rheological experiments, which showed increased hydrogel storage moduli at increased polymer concentration [20]. The release of IgG, using corresponding hydrogels, was much slower than the release of lysozyme. After 16 days up to 50% and approximately 60% IgG was released from stereocomplexed hydrogels at 12.5 and 7.5% w/v polymer concentration, respectively. The slow, constant release of IgG is most likely due to a combination of diffusion and degradation/swelling. It should be noted that after 3 weeks, IgG was not completely retrieved. This may be due to interaction with hydrophobic domains and partial denaturation during the release experiment [29]. At pH 5.0 a small burst effect is observed for the release of IgG, while at pH 7.0 the initial release is almost linear in time (Fig. 4b). Surprisingly, a faster release of IgG is observed at pH 5.0 compared to pH 7.0, despite the fact that PLA degrades slower at pH 5.0 compared to pH 7.0. IgG is known to destabilize at pH values that deviate from neutral due to conformational changes [30]. Our release data suggest that at pH 5.0 smaller, more compact IgG structures are formed compared to pH 7.0. Vermeer et al. have observed the formation of small, compact IgG structures at pH 2.0 [31].

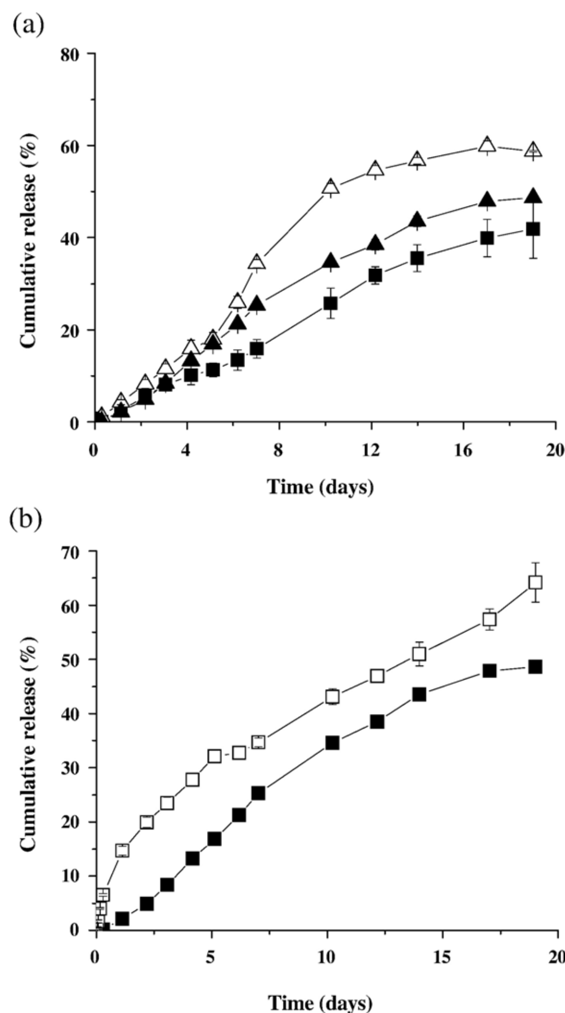

Fig. 4. Cumulative release profiles of IgG from stereocomplexed PEG–$(PLA)_8$ hydrogels at 37 °C (average ± S.D., $n = 3$). (a) PEG–$(PLA_{12})_8$ hydrogel at 12.5% w/v initial polymer concentration (■) and PEG–$(PLA_{14})_8$ hydrogel at initial polymer concentrations of 7.5 (△) and 12.5% w/v (▲) at pH 7.0; (b) PEG–$(PLA_{14})_8$ hydrogels at 12.5% w/v initial polymer concentration and pH 7.0 (■) or pH 5.0 (□).

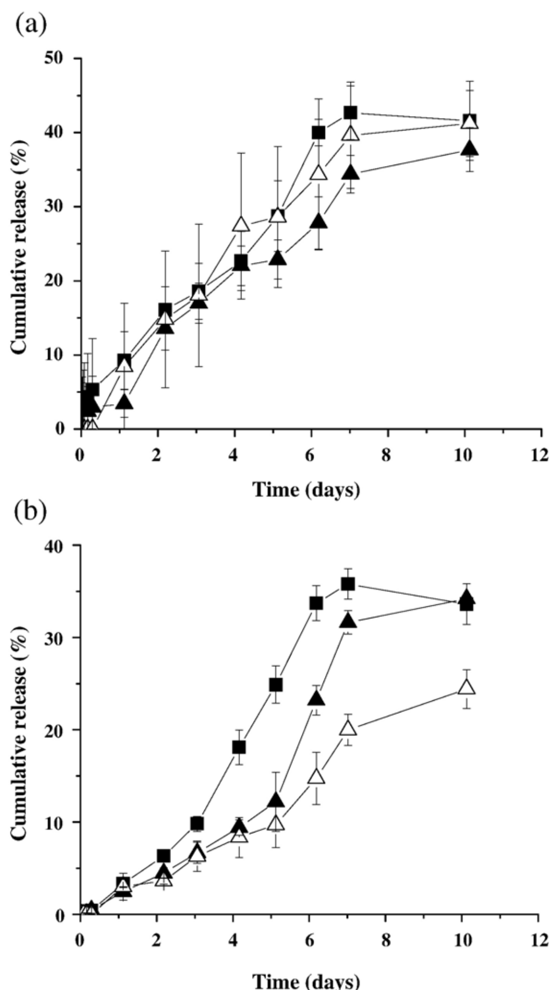

Fig. 5. Cumulative release profiles of rhIL-2 from stereocomplexed PEG–(PLA)$_8$ hydrogels at 37 °C and pH 7.2 (average ± S.D., $n=3$). (a) PEG–(PLA$_{12}$)$_8$ hydrogels at initial polymer concentrations of 10 (■), 12.5 (▲) and 15% w/v (△); (b) PEG–(PLA$_{14}$)$_8$ hydrogels at initial polymer concentrations of 7.5 (■), 10 (▲) and 12.5% w/v (△).

3.3. Release of rhIL-2 in vitro

RhIL-2 was released *in vitro* from stereocomplexed PEG–(PLA$_{12}$)$_8$ and PEG–(PLA$_{14}$)$_8$ hydrogels. The amount of rhIL-2 in the release samples was determined with HPLC using both UV and fluorescence detection. Fig. 5a shows that the incorporated rhIL-2 was released with almost zero order kinetics from stereocomplexed PEG–(PLA$_{12}$)$_8$ hydrogels up to 7 days, independent of the polymer concentration. Lysozyme and rhIL-2 have similar molecular weights (14.6 kDa and 15.3 kDa, respectively) and therefore similar release kinetics were expected for these proteins. The difference in release kinetics of rhIL-2 and lysozyme may be due to differences in hydrophobicity as well as formation of rhIL-2 dimers and/or larger hydrodynamic size of rhIL-2 due to SDS interaction [24]. The release of rhIL-2 from stereocomplexed PEG–(PLA$_{12}$)$_8$ hydrogels is not influenced by the polymer concentration, which is most likely because rhIL-2, similar to lysozyme, is substantially smaller than the hydrogel mesh size. The cumulative release profiles measured with UV and fluorescence detection were similar up to 6 days. However, fluorescence detection showed approximately 5–10% higher release at later time points, due to a higher sensitivity (results not shown), resulting in a cumulative release of approximately 45 and 50% in 7 and 10 days, respectively. The cumulative release from stereocomplexed PEG–(PLA$_{14}$)$_8$ hydrogels was approximately 5–20% lower compared to stereocomplexed PEG–(PLA$_{12}$)$_8$ hydrogels and increased with decreasing polymer concentration (Fig. 5b, measured with UV detection). RhIL-2 is a relatively hydrophobic protein. The longer and more hydrophobic PLA blocks may therefore have caused increased interaction with rhIL-2, especially at increased polymer concentration. Generally, the cumulative release of rhIL-2 did not reach 100% when all hydrogel material had been dissolved, which may be due to interaction with hydrophobic domains and partial denaturation during the release experiment. Low retrieval of rhIL-2 was also reported by Bos et al., who obtained a cumulative release of approximately 65% for stereocomplexed dextran–lactate hydrogels [18].

3.4. Animal model

DBA/2 mice were injected subcutaneously with 1×10^5 SL2 lymphosarcoma cells and the tumors were allowed to grow for 11 days before starting the experiments. At day 0, immunotherapy was started on mice bearing tumors of 44–176 mm^2 (average 100 mm^2, corresponding to 4% of the body weight). Stereocomplexed PEG–(PLA$_{12}$)$_8$ hydrogels with 10% w/v polymer concentration were selected for the further *in vivo* release study because they showed a close to zero-order release of rhIL-2 in vitro and a higher cumulative release of rhIL-2 in vitro as compared to PEG–(PLA$_{14}$)$_8$ hydrogels. RhIL-2 loaded (1×10^6 IU) stereocomplexed hydrogels or a solution of free rhIL-2 (1×10^6 IU) in HEPES buffered saline (pH 7.0) were injected intratumorally. The stereocomplexed hydrogels were prepared by mixing rhIL-2 containing solutions of PEG–(PLLA$_{12}$)$_8$ and PEG–(PDLA$_{12}$)$_8$ in HEPES buffered saline and intratumorally injected within 5 min of mixing. This time was considered optimal, since the mixtures still had a low viscosity, which allowed easy injection, while after 5 min the injection became increasingly difficult. The therapeutic efficacy of rhIL-2 loaded stereocomplexed PEG–(PLA$_{12}$)$_8$ hydrogels was measured by the reduction in the average tumor size (Fig. 6) as well as by the survival rate of the mice (Fig. 7). All *in vivo* data was analyzed by Kaplan–Meier statistics. Fig. 6 shows that the tumors of the negative control groups (administered HEPES buffered saline or empty 10% w/v PEG–(PLA$_{12}$)$_8$ hydrogel) grew rapidly compared to the positive control group (administered free rhIL-2 in HEPES buffered saline) and the experimental group (administered rhIL-2 loaded hydrogel). The size of the tumors of the free rhIL-2 treated group stabilized at day 3 and 1, 3 and 5 out of 7 mice were tumor-free after 10, 17 and 24 days of treatment, respectively (data not shown). The size of the tumors of the rhIL-2 loaded stereocomplexed hydrogels stabilized around day 17 and 2 and 3 out of 10 mice were tumor-free after 24 and 31 days of treatment, respectively (data not shown). This difference in timing and number of mice

becoming tumor-free was statistically significant ($p=0.01$, data not shown). In Fig. 6 the small increase in the average tumor size between day 31 and 45 in the rhIL-2-loaded stereocomplexed hydrogels treated group is caused by a single mouse with progressive disease after a partial regression of tumor growth prior to day 31. At day 45 this mouse died, and the other mice of this group remained tumor-free.

The Kaplan–Meier survival curves show a significantly higher cure rate in the experimental group (30% cures) and positive control group (70% cures) compared to the negative controls (0% cures), wherein most mice died after 6 to 13 days (Fig. 7). The hazard ratios of rhIL-2 loaded hydrogel and free rhIL-2 are 0.30 and 0.14, respectively. The difference in cure rate of the rhIL-2 loaded stereocomplexed PEG–$(PLA_{12})_8$ hydrogels and free rhIL-2 is not statistically significant ($p=0.15$). These results show that rhIL-2 loaded stereocomplexed hydrogels as well as free rhIL-2 have a therapeutic effect on SL2 tumor bearing mice. Both treatments reduce tumor size, induce tumor regression and increase the cure rate. The data on tumor size and survival (Figs. 6 and 7) indicate that the therapeutic effect of rhIL-2 loaded stereocomplexed hydrogels, though clearly present, is significantly retarded compared to free rhIL-2. Remarkably, rhIL-2 loaded stereocomplexed hydrogels have a similar therapeutic effect in the SL2-lymphoma bearing mice model as 5 subsequent daily injections with 1×10^5 IU of free rhIL-2 (unpublished results). The *in vitro* release experiments showed that during the first 5 days a similar amount of rhIL-2 is released every day (Fig. 5a). Therefore, the retardation of the therapeutic effect of rhIL-2 loaded stereocomplexed hydrogels compared to free rhIL-2 is most likely due to a slow, constant release of rhIL-2 from the hydrogels. Bos et al. showed that the therapeutic efficacy of rhIL-2 loaded stereocomplexed dextran–lactate hydrogels is at least equal to free rhIL-2 [18]. However, *in vitro* release studies showed that these stereocomplexed dextran–lactate hydrogels released 50% of the rhIL-2 within a few hours. In the SL2 mice model the tumor grows rapidly and most of the mice of the negative control groups (no rhIL-2) had already died before the rhIL-2 released from the stereocomplexed hydrogels started to stabilize tumor growth at day 10. Fast growing tumors in humans and veterinary animals do not grow as fast as the SL2 tumor in mice. Therefore, patients may benefit from a slow release system giving a prolonged therapy compared to a single injection of free rhIL-2. The results obtained on the stereocomplexed PEG–$(PLA_{12})_8$ hydrogels suggest that treatment may be improved by one injection of free rhIL-2 followed by slow release of rhIL-2 from the PEG–$(PLA_{12})_8$ stereocomplexed hydrogel. Further study is needed to optimize the dose of rhIL-2 in the hydrogel.

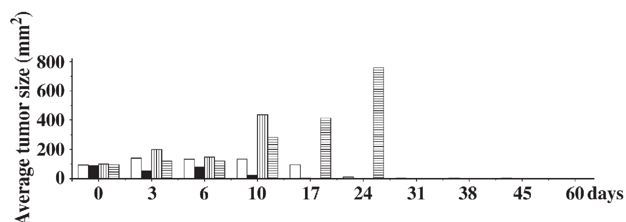

Fig. 6. Average absolute tumor size by group of SL2 tumor bearing mice injected intratumorally with □, an in situ forming stereocomplexed PEG–$(PLA_{12})_8$ hydrogel at 10% w/v initial polymer concentration loaded with 1×10^6 IU of rhIL-2 ($n=10$); ■, a solution of 1×10^6 IU of rhIL-2 in HEPES buffered saline ($n=7$); ◫, an in situ forming, empty stereocomplexed PEG–$(PLA_{12})_8$ hydrogel at 10% w/v polymer concentration ($n=4$); ⊟, HEPES buffered saline ($n=10$).

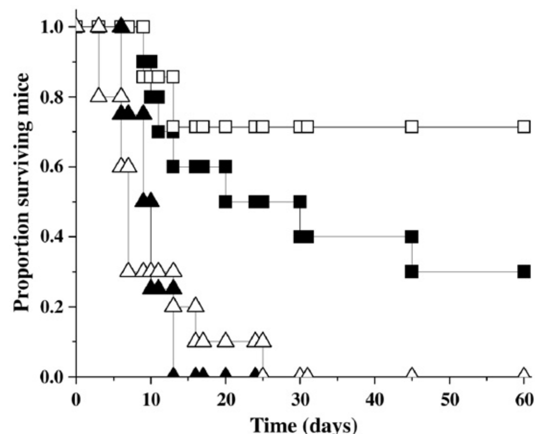

Fig. 7. Kaplan–Meier survival curves by groups of SL2 tumor bearing mice intratumorally injected with in situ forming stereocomplexed PEG–$(PLA_{12})_8$ hydrogels at 10% w/v initial polymer concentration loaded with 1×10^6 IU of rhIL-2. RhIL-2 loaded hydrogel ($n=10$) (■), solution of free rhIL-2 in HEPES buffered saline ($n=7$) (□), empty hydrogel ($n=4$) (▲) and HEPES buffered saline ($n=10$) (△).

4. Conclusions

Stereocomplexed PEG–PLA hydrogels were rapidly formed in situ by mixing aqueous solutions of PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$ star block copolymers. These hydrogels degraded under physiological conditions and the single enantiomeric solutions had a low viscosity, thus allowing easy injection. Proteins could be easily loaded into the stereocomplexed hydrogels by mixing protein containing aqueous solutions of PEG–$(PLLA)_8$ and PEG–$(PDLA)_8$ copolymers. The *in vitro* release of the relatively small protein lysozyme (d_h is 4.1 nm) followed first order kinetics, wherein a high cumulative release of approximately 90% was obtained in 10 days. Importantly, the released lysozyme retained its enzymatic activity, emphasizing the protein-friendly hydrogel preparation method. The larger protein IgG (d_h is 10.7 nm) could be released *in vitro* with nearly zero order kinetics for 16 days. The release of the therapeutic protein rhIL-2 followed almost zero order kinetics for 7 days, wherein up to 45% was released. The therapeutic efficacy of rhIL-2 loaded stereocomplexed PEG–PLA hydrogels was demonstrated using mice bearing fast growing, large malignant tumors. The PEG–$(PLLA)_8$/PEG–$(PDLA)_8$/rhIL-2 mixtures could be easily injected intratumorally. Compared to injection with free rhIL-2, the therapeutic effect of the released protein started approximately 1–2 weeks later, indicating that the stereocomplexed PEG–PLA hydrogels act as a slow releasing depot of rhIL-2. Combining a single injection with free rhIL-2 with slow release of rhIL-2 from the stereocomplexed hydrogels may be a

promising alternative for the current standard therapy wherein frequent, painful injections with free rhIL-2 are given.

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