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Research paper

Hetero-stereocomplexes of D-poly(lactic acid) and the LHRH analogue leuprolide. Application in controlled release

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Abstract

Reversible hetero-stereoselective complexes were obtained by mixing acetonitrile solutions of enantiomeric D-poly(lactic acid) (D-PLA) and leuprolide, an L-configured nonapeptide LHRH analogue. The complex spontaneously aggregated and precipitated in high yields (95%) from acetonitrile solutions, forming uniform, porous microparticles with a mean unweighed particle size of 1.7 μm. The complexation of L-configured peptide occurred only with D-PLA, and not with L-PLA or racemic D,L-PLA. Various factors affecting the release pattern of leuprolide from the hetero-stereocomplexes were investigated. Complexes with D-PLA of low molecular weight (<10,000 Da) displayed lower release rates of leuprolide than high molecular weight D-PLA (>50,000 Da). Changing the leuprolide: D-PLA ratio from 1:50 to 1:10 (w/w) in the stereocomplex, resulted in a faster release of leuprolide. Similarly, the release rate of leuprolide was twice as fast when adding poly(ethylene glycol) to the acetonitrile complexation solution. Leuprolide was released from most of the formulations in a first order pattern, with only a small burst release during the first 24 h. Addition of water to the complexation solution significantly increased the initial release of the peptide. Low testosterone levels for over 25 days were observed in an in vivo release study of leuprolide from a hetero-stereocomplex formulation, monitoring testosterone levels in the blood of rats after sub cutaneous injection.

Keywords: Leuprolide; LHRH release; Stereocomplex; Poly(lactide); Peptide release

1. Introduction

Stereoselective polymers of identical chemical composition but of opposite enantiomeric configuration form stable homo-stereocomplexes [1–11]. Lacking possibilities for electrostatic interactions or hydrogen bridging, the main force favoring this complex was shown to rely on stereospecific Van der Waals interactions [12]. Stereocomplexation was demonstrated in various pairs of opposite polymeric enantiomers, including D- and L-PLA [9,13,14].

Characteristically, the PLA-stereocomplex was insoluble in common solvents and displayed a melt transition peak at 230 °C, 50° higher than D- or L-PLA enantiomers ($T_m = 180$ °C). Alterations noticed in Wide Angle X-ray, FT-IR and Solid State NMR spectra confirmed the formation of the stereocomplex and provided information about the PLA-stereocomplex crystal structure [14–18]. A conformational change of PLA was suggested to occur during the stereocomplex formation, in which the 10_3 α-helix, as reported for isotactic PLA, turns into a 3_1 helix. PLA-stereocomplex particles displayed a longer biodegradation period under physiological conditions, compared to the polymeric enantiomers [12,19].

Most of the research on polymer stereocomplexes has been focused on homo-stereocomplexes between opposite enantiomers with identical chemical compositions. We have discovered a novel reversible stereoselective complex, formed between L-peptides and D-configured poly(lactic acid) (D-PLA) [20,21]. Based on the resemblance between L-PLA and a naturally L-configured peptide, as both polymer backbones differ only slightly in torsion angle values [22], the peptide backbone was suggested to adopt an α-helix conformation and to interact with D-PLA as illustrated in Scheme 1.

Besides the obvious possibility of hydrogen bond formation, mainly stereoselective Van Der Waals

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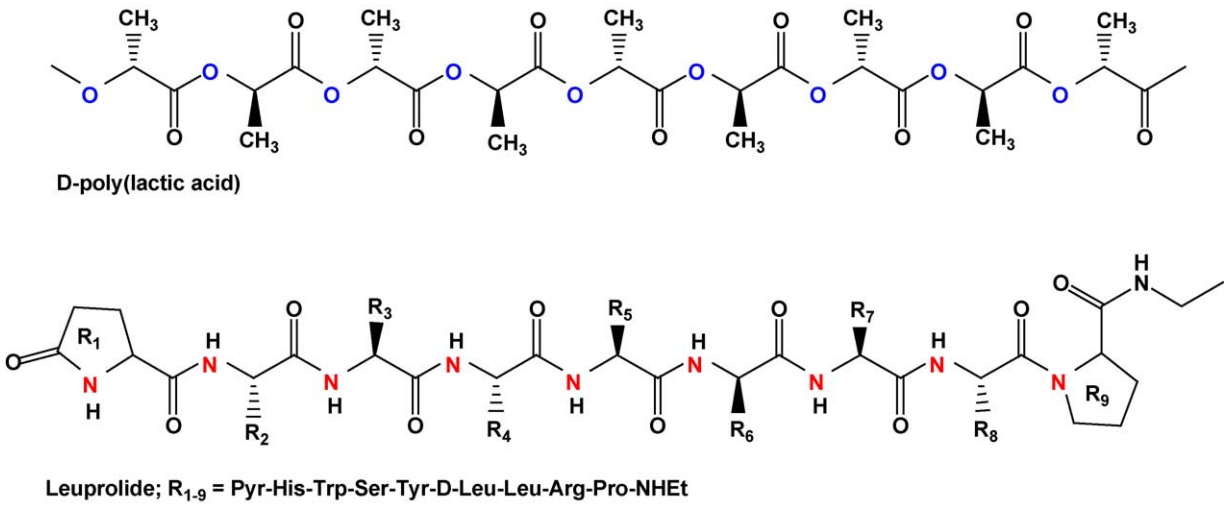

Scheme 1.

interactions were suggested to induce the stereoselectivity of the complex formation [20].

Leuprolide release from PLA microparticles has been studied both in vitro and in vivo [23,24]. The main problems encountered in the controlled release of peptides from biodegradable polymer matrices are: the fast initial release of the bioactive agent from the polymer matrix, in particular during the first 24 h (burst release) and the loss in bioactivity. Okada already achieved significant improvement to lower this burst effect with the development of 1 and 3-month release injectable microspheres [25]. Although little is known yet about the molecular interactions between various polymer matrices and the peptidic moieties, this knowledge may form the key in the production of more efficient release devices [26,27]. The advantages of the concept of stereocomplexation are: (a) molecular interaction that depends on the polymer backbone and the D-PLA, resulting in a controlled release that is only little dependent on diffusion; (b) no need for microsphere preparation process, since the particles are spontaneously generated, without need for surfactants or other additives. This process may be useful for the preparation of various peptide and protein release devices, including insulin, growth hormones and others.

This manuscript describes the stereocomplex formation between leuprolide and D-PLA under various conditions with a focus on the potential use of the complex for controlled delivery of peptides.

2. Materials

Chemicals and solvents were purchased from Sigma-Aldrich Israel or from Mallinckrodt-J.T. Baker B.V. Deventer, Holland. D- and L-Lactides were obtained from Purac BV, Gorinchem, Holland. Stannous(II)bis-2-ethylhexanoate ($Sn(Oct)_2$), was obtained from Sigma-Aldrich, Israel. Leuprolide acetate was purchased from Novetide Ltd, Israel.

Thermal analysis was determined on a Mettler TA 4000-DSC differential scanning calorimeter (DSC), calibrated with zinc and indium standards, at a heating rate of 10 °C/min. Molecular weights were estimated using a gel permeation chromatography (GPC) system consisting of a Spectra Physics (Darmstadt, Germany) P1000 pump and equipped with a refractive index detector ERC-7510, ERMA Inc., a Rheodyne (Coatati, CA) injection valve with a 20 μl loop, and a Spectra Physics Data Jet integrator connected to a computer. Samples were eluted with $CHCl_3$ through a linear Styrogel column (Waters, 10 μm pore size) at a flow rate of 1 ml/min. The molecular weights were determined relative to polystyrene standards (Polyscience, Warrington, PA) with a molecular weight range of 400–400,000. Sonication was performed in an ultra-sonic bath Transsonic T 460, Elma, Germany. Particle size was determined by a Coulter N4 SD Sub-Micron Particle Size Analyzer, Coulter Electronics, USA. Scanning electron microscopy (SEM) was performed on a SEM 505, Philips, Holland. Gold coating: polaron E 5100-2, (Holywell) Polaron Equipment Ltd, England. NMR studies were performed on a Varian, 300 MHz, using samples dissolved in deuterated chloroform, DMSO or acetonitrile. HPLC analysis was performed on a HP 1100 (Hewlett-Packard, USA), column: RP C-18 5 × 250 mm equipped with guard column C-8 LichroCard, Merck, Germany. Testosterone levels were determined by a competitive immunoassay using an automated chemiluminescence system ACS 180 (Chiron Diagnostics Corp., USA).

2.1. Synthesis of poly(lactic acid)

Stereoselective and racemic PLA were synthesized by ring opening polymerization of lactide using stannous 2-ethylhexanoate ($Sn(Oct)_2$) as catalyst and different

alcohols as co-catalysts [13]. In a typical polymerization reaction, lactide (10 g, 70 mmol) together with octanol (90 mg, 0.7 mmol), were dissolved in dry toluene (300 ml) and refluxed over a Dean-Stark apparatus for 2 h. At reflux, $Sn(Oct)_2$ (280 mg, 0.7 mmol) was added. After stirring at 135 °C for 3 h toluene was distilled off. In the synthesis of higher molecular weight polymers (>50 kDa) no alcoholic co-catalyst was added. After initial polymerization with $Sn(Oct)_2$ for 3 h, toluene was distilled off. The crude material (M_w between 20 and 30 kDa) was further polymerized in bulk overnight at 135 °C. D-PLA of a molecular weight exceeding 100 kDa was prepared by melting D-lactide (10 g, 70 mmol) with $Sn(Oct)_2$ (60 mg, 0.15 mmol) under anhydrous conditions in a sealed ampoule. The solution was heated at 135 °C for 4 h. For the synthesis of low molecular weight PLA ($M_w = 3$ kDa) co-catalyst (benzyl alcohol, 3 g, 28 mmol) was added to lactide (20 g, 140 mmol) at a 1:5 ratio in 300 ml toluene and dried by refluxing over a Dean-Stark apparatus for 3 h. At 135 °C $Sn(Oct)_2$ (280 mg, 0.7 mmol) was added and the mixture was stirred for 2 h. The polymers were dissolved in a small amount of chloroform and precipitated in an ether/petroleum ether mixture or in isopropanol. NMR: $\delta = 5.15$ (1H, q, CH); $\delta = 1.60$ (3H, d, CH_3). At $\delta = 1.0–1.4$ (m, alkyl) peaks of alkyl are detected in those polymers synthesized with decanol as co-catalyst.

2.2. Formation of hetero-stereocomplexes of leuprolide and D-PLA

Stereocomplexes of poly(D-lactic acid) (D-PLA) with leuprolide were prepared by mixing D-PLA (19 mg) and leuprolide (1 mg, 5% (w/w)) in acetonitrile (1 ml) in glass ampoules containing a micro stirrer. After sealing the ampoule, the mixture was stirred at 60 °C for 3 days. The reaction mixture became turbid and formed a white precipitate, which was isolated by filtration or centrifugation. The precipitate was dried overnight in vacuum over P_2O_5.

Alternatively, after stirring for 2 days the still clear solution in acetonitrile was sprayed into a beaker (500 ml), containing a mixture of isopropanol (100 ml) and liquid nitrogen (300 ml). The mixture was left to thaw at −4 °C, overnight. The precipitate was collected by centrifugation of the isopropanol and dried in vacuum over P_2O_5.

The obtained precipitates were characterized by differential scanning calorimetry (DSC) and SEM. Peptide content of the precipitate and remaining supernatant were determined by HPLC. SEM micrographs were taken from various complexes under high vacuum using 30 kV accelerating voltage. Particles were deposited on a carbon film followed by gold coating, performed in vacuum for 1 minute at 20 mA.

2.2.1. NMR analyses

Complexes containing D-PLA (120 kDa) and leuprolide up to 20% (w/w) were analyzed by ^{13}C MAS Solid State NMR and compared to control samples. For control samples D-PLA as obtained from synthesis (see above) and D-PLA (120 kDa) after precipitation from a 1% (w/w) cooled acetonitrile solution were taken. Samples varying from 40 to 80 mg were put in a probe and, where necessary, additional weight pieces were added. After tuning and shimming the spectra were obtained while the probe was spun at 8000 Hz.

liquid state NMR was conducted on a Varian in deuterated DMSO Typically 10 mg was dissolved in 0.40 ml. NOESY experiments were done. Alternatively, NMR spectometry was performed during complex formation. D-PLA (120 or 10 kDa, 10 mg) was dissolved in a mixture of deuterated acetonitrile and D_2O (9:1, 0.5 ml) at 50 °C. Leuprolide (2.5 mg) was added and the solution was transferred to an NMR tube. Regular ^1H NMR spectra were obtained roughly 15 min after mixing. NOESY experiment (4 h) was started and performed also at 50 °C.

2.2.2. Particle size analysis

Stereocomplex particles (10 mg) were resuspended in acetonitrile (3 ml) and diluted if necessary in order to get a particle concentration of approximately 10^5 per ml. The particle size was measured at 25 °C taking the unweighed volume mean diameters.

2.3. In vitro release of leuprolide

Hetero-stereocomplex particles (20 mg) were suspended in 0.1 M phosphate buffer pH 7.4 (1 ml) in disposable syringes, equipped with a filter and stopper, and put on an orbital shaker at 100 rpm at 37 °C. At specific time intervals, the buffer was removed from the syringe via the filter. Fresh buffer was returned into the syringe via the same filter in order to release stuck particles. Phosphoric acid (50 μl) was added to the samples and leuprolide concentrations were determined using Reversed Phase HPLC at a flow of 1 ml/min of 30% acetonitrile/70% 0.01 M TEAP-buffer pH = 3 and UV- detection at 278 nm. The release data was fit with a random intercept model fit with generalized least squares, using STATA-8 software. The data was fitted against the following functions: linear, square root and natural logarithm.

2.4. In vivo studies

The in vivo activity of the leuprolide/D-PLA hetero-stereocomplex was determined in male Wistar rats weighing 250 g ($n = 5$). The hetero-stereocomplex formulation of D-PLA 120 kDa (270 mg) and leuprolide (15 mg, 5%, w/w) and the drug-free control formulation were prepared as described above. Yield was 271 mg (95%) and the mean particle size was 1.7 μm.

Complex formation was verified by DSC. The complex was resuspended in a 5% glucose solution, vortexed thoroughly, sonicated in a sonication bath, and lyophilized.

Prior to injection, the formulations were resuspended in deionized water and injected sub cutaneously into the rats.

The injected amount of formulation contained a total of 1.25 mg leuprolide per kg rat to be released in a sustained manner. Blood was collected from the tail-artery once in every week over a period of 5 weeks post injection. The samples were centrifuged for 10 min at 5000 rpm and serum was isolated. Testosterone levels were determined by a competitive immunoassay. Serum (15 μl) and steroid releasing agent (50 μl, 0.1 μg/ml) were mixed in order to release testosterone from endogenous binding proteins in the serum. In a competitive binding reaction, this was added to a fixed amount of acridinium ester-labeled testosterone (50 μl, ~3.2 ng/ml) and incubated with 300 μl polyclonal rabbit anti-testosterone antibodies (33 ng/ml) for 5 min. The rabbit anti-testosterone antibody was bound to a mouse anti-rabbit antibody, which was coupled to paramagnetic particles in a solid phase. The amount of chemical luminescence was inversely related to the amount of testosterone present in the sample.

3. Results and discussion

3.1. Polymer synthesis

The synthesized polymers were soluble in dichloromethane, chloroform, dimethyl sulfoxide (DMSO) and in acetonitrile (>50 °C in case of PLA with molecular weights exceeding 20 kDa). All polymers were characterized by DSC and GPC (Table 1).

3.2. Complex formation

A solution of D-PLA and leuprolide in acetonitrile was stirred at 60 °C for 3 days forming a precipitate. This phenomenon did not occur when using L-PLA or racemic D,L-PLA instead, or using a solution of D-PLA in acetonitrile without leuprolide, since these solutions remained clear. See also findings, published elsewhere [20,28].

The morphology of the precipitated particles of the hetero-stereocomplex was studied by SEM. Uniform spherical particles were formed. Notable is the porous nature of the particles, creating a large surface area (Fig. 1), compared to common PLA microspheres having a compact and smooth surface area. Using a particle size analyzer the size of the particles was analyzed by suspending the precipitate in acetonitrile. Particle size was measured under standard conditions, using unweighed volume mean diameters. The particle size was between 1.0 and 3.3 μm with a mean particle size of 1.7μm.

The complexation of D-PLA with leuprolide in different solvents, including dichloromethane, dimethylsulfoxide (DMSO), dimethylformamide (DMF) or tetrahydrofuran (THF), did not form a precipitate other than in acetonitrile. Leuprolide was found to remain stable in acetonitrile solution at 65 °C for 5 days.

A decrease in free leuprolide concentration was observed during the complex formation and precipitation. Less than 5% of peptide was found in the acetonitrile supernatant after isolation of the precipitate by centrifugation, when reacting leuprolide with D-PLA at a 1:10 (w/w) ratio. A linear correlation was found between the ratio of leuprolide: polymer, that was put into the reaction, and the free leuprolide concentration in acetonitrile solution after isolation of the precipitate (Fig. 2).

D-PLA of low molecular weights (10 kDa or less) precipitated with leuprolide in lower yields, 20–40% of the peptide and polymer entry. D-PLA of high molecular weight (>50 kDa) precipitated almost completely (>95%) from the solution during complexation.

3.3. Characterization of hetero-stereocomplex particles

In spectra obtained by Solid State ^{13}C MAS NMR at 500 MHz no differences in shifts of the signals were observed compared to the spectrum of the PLA except for a small narrowing of the peaks that could indicate an increase

Table 1
Properties of the lactide polymers used in this study

Polymer	Co-catalyst	Mol. ratio LA:Cat	M_w[a] (Da)	Poly dispersity index	Melt transition temperature[b] (°C)
D-PLA	Benzyl alcohol	5 : 1	2800	1.05	120
	Octanol	50 : 1	10,000	1.2	158
	Octanol	100 : 1	17,200	2.5	169
	Decanol	1000 : 1	122,000	2.2	179
	–	–	250,000	2.0	190
L-PLA	Benzyl alcohol	5 : 1	2800	1.05	120
	–	–	120,000	1.4	178
DL-PLA	Octanol	100 : 1	43,000	1.53	No transition

[a] M_w-weight average molecular weight, determined by GPC.
[b] Measured by differential scanning calorimetry; polymers were prepared from reaction of D- or L-lactide, alcohol as co-catalyst, and stannous octoate in dry toluene at 135 °C for 3 h. Polymers of high molecular weight were obtained by further polymerization in bulk overnight. Polymers were purified by precipitation in a 1:1 (v/v) ether/petroleum ether mixture. All polymers were soluble in acetonitrile at 50 °C.

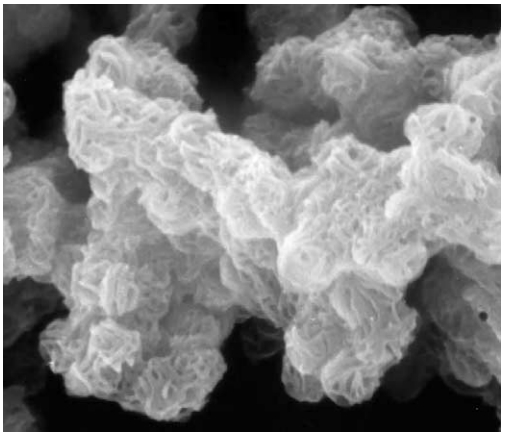

Fig. 1. Scanning electron microscopy. Image of a leuprolide/D-PLA hetero-stereocomplex, obtained by spontaneous precipitation from a solution of D-PLA (120 kDa) and leuprolide (2%, w/w) in acetonitrile at 60 °C.

in crystallinity. This was also observed for D-PLA, precipitated from acetonitrile. In NOESY experiments (both in deuterated DMSO and in acetonitrile) cross peaks were found for protein and polymer intramolecular protons. Intermolecular dependencies, however, were not observed. This is in contrast to the homo-stereocomplex formation between D-PLA and L-PLA, in which shifts were observed that were attributed to the stereo-interactions between the polymers [17]. This result may be explained by the small amount of peptide that interacted with D-PLA (up to 10%, w/w). More studies are suggested to verify evidence for interaction by NMR.

Changes were seen in the thermal behavior of the hetero-stereocomplexes as was investigated by differential scanning calorimetry (DSC). Fig. 3 shows the thermal behavior, obtained for the different formulations of the stereoselective

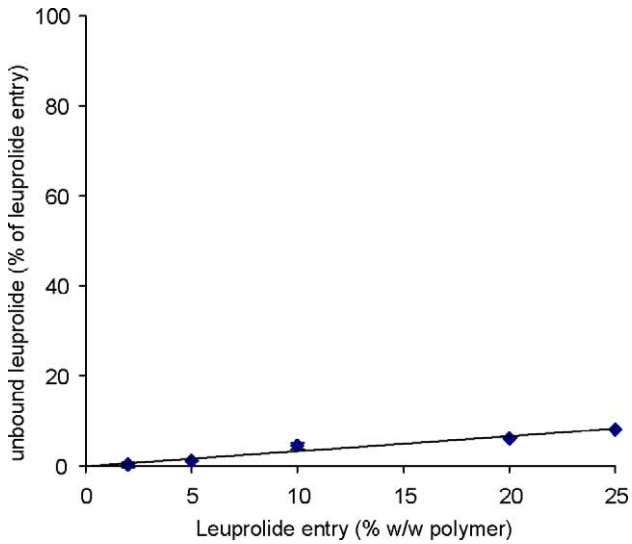

Fig. 2. Correlation between unbound leuprolide and leuprolide entry. Leuprolide (1 mg) was reacted with increasing amounts of D-PLA (100 kDa) in acetonitrile (2 ml). After stirring at 60 °C the spontaneously precipitated complexes were isolated and unbound leuprolide in the acetonitrile reaction solution was determined by HPLC.

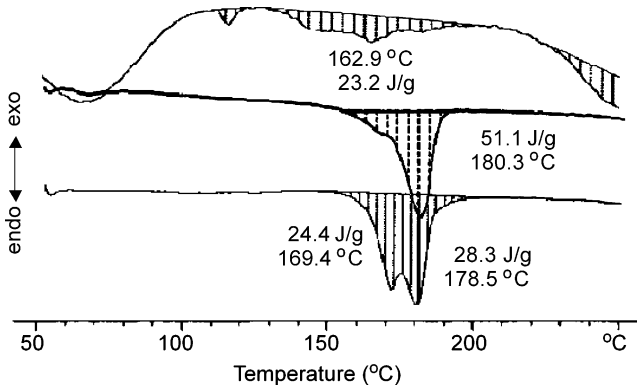

Fig. 3. DSC thermoscans. (A) Leuprolide; (B) D-PLA 120 kDa; (C) hetero-stereocomplex of leuprolide and D-PLA 120 kDa, obtained by precipitation after mixing acetonitrile solutions of D-PLA and leuprolide (10%, w/w) at 60 °C and stirring for 3 days. DSC was determined on a 5 mg dry powder of the complex at a heat rate of 10 °C/min.

complex. This behavior is discussed elsewhere in more detail [28]

As opposed to racemic PLA lacking a defined transition point, enantiomeric D- or L-PLA melted around 178 °C, depending upon its molecular weight. The D-PLA/leuprolide hetero-stereocomplex shows two transition temperatures, the regular melt transition at 178 °C and a new transition at 169 °C. A similar phenomenon in the DSC thermal behavior of L-PLA was reported when spinning fibers of L-PLA from a hot chloroform/toluene solution (close to θ-conditions) [29].

3.4. Release studies

As a result of the reversible nature of the stereoselective complex of L-peptide and D-PLA, release of the peptide component was observed in a controlled manner in a release medium at physiological conditions (0.1 M phosphate buffer at 37 °C).

Close to 100% recovery of leuprolide from the various precipitates was achieved by resuspending obtained precipitates in acetonitrile containing Span 80 (5%, w/w) that was stirred overnight at 60 °C. This indicates that the complexation can be reversed by surfactants that disrupt the interaction between the peptide and the polymer.

3.4.1. Influence of leuprolide content and the molecular weight of D-PLA

Different formulations have been prepared, varying the molecular weight of D-PLA, and the relative amount of leuprolide (Fig. 4A and B). Generally, characteristic of the release of leuprolide from hetero-stereocomplexes is the lack of a fast release of the peptide during the first 24 h (burst-effect) and a close to first-order type of the leuprolide release profile.

High molecular weight D-PLA complexes (M_w >50 kDa) with leuprolide 5% (w/w), released 40% of the leuprolide content over a period of 25 days. Using random intercept

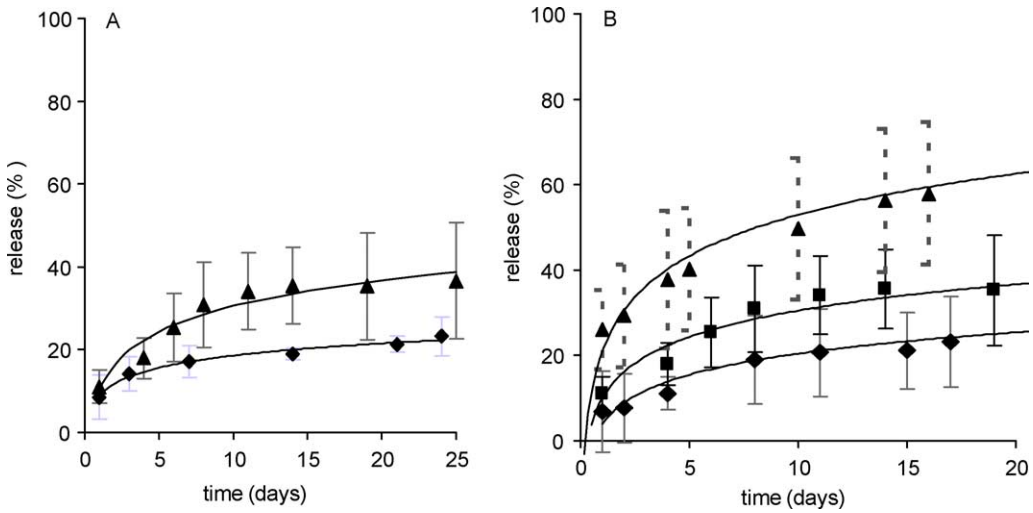

Fig. 4. Release of leuprolide from hetero-stereocomplexes with D-PLA. Influence of molecular weight and leuprolide concentration. Complexes of leuprolide and D-PLA precipitated from solution in acetonitrile while stirring at 60 °C for 3 days. (A) Influence of D-PLA molecular weight on the release rate (leuprolide 5%, w/w). ◆ (black diamond) low molecular weight D-PLA (< 10 kDa) or ▲ (black triangle) high molecular weight D-PLA (> 50 kDa). (B) Influence of the leuprolide concentration (w/w polymer) in the hetero-stereocomplex on the release profile (D-PLA > 50 kDa). ◆ (diamond) 2% (w/w), ■ 5% (w/w) and ▲ (triangle) 10% (w/w). The release of leuprolide was conducted in 0.1 M phosphate buffer solution pH 7.4, at 37 °C. Leuprolide concentration in the releasing medium was determined by HPLC.

model fit with generalized least squares, the best fit for the release profile was found to be logarithmic. Low molecular weight polymer complexes (M_w < 10 kDa) released only a little over 20% of the peptide content during the same time period. Also in this case the best fit was found to be logarithmic. In similar experiments, the influence of the leuprolide: D-PLA ratio on the release pattern was investigated (Fig. 4B). Changing from 1:50 to 1:10 (w/w) ratio, a faster release of the complexed peptide was observed, from 20 to 60% of the leuprolide content at mean release rates of 0.19 to 1.7 μg/day/mg complex, respectively. In contrast to the release profile of complexes with 5% (w/w) leuprolide, curve fiting of the release profiles of complexes with 2 and 10% leuprolide contents showed a slight preference to a square root function. However, the differences between the various curve fittings were very small due to the high standard deviations.

The leuprolide is released based on the detachment of the stereoselective complex as a result of its reversible nature. After 25 days the release slows down significantly. Up to 50% of the remaining leuprolide was recovered from the particles after the 30-day release period by suspending in an acetonitrile solution of spam-80 (5%, w/w) at 60 °C overnight. It is suggested that the biodegradation of the PLA causes the complex further to disengage causing an additional release of leuprolide. However, the degradation process is far slower [30].

3.4.2. Spray-freeze in liquid nitrogen

Hetero-stereocomplex formation was shown to be initiated immediately upon mixing L-configured peptide with D-PLA (10 or 120 kDa) [20]. However, hetero-stereocomplex particles spontaneously precipitated only after stirring at 60 °C for 3 days. The isolation of hetero-stereocomplex particles, formed in the first stage of the complexation reaction, was accomplished by quenching the reaction mixture in liquid nitrogen. The isolated particles consisted of the stereocomplexes, as was verified by DSC. In general, the release rates were higher than those from complex particles, generated by precipitation at 60 °C (Fig. 5). The effect of the molecular weight of D-PLA on the release pattern of leuprolide from the complexes obtained from quenching in liquid nitrogen, was similar to precipitates formed in acetonitrile at 60 °C. High molecular

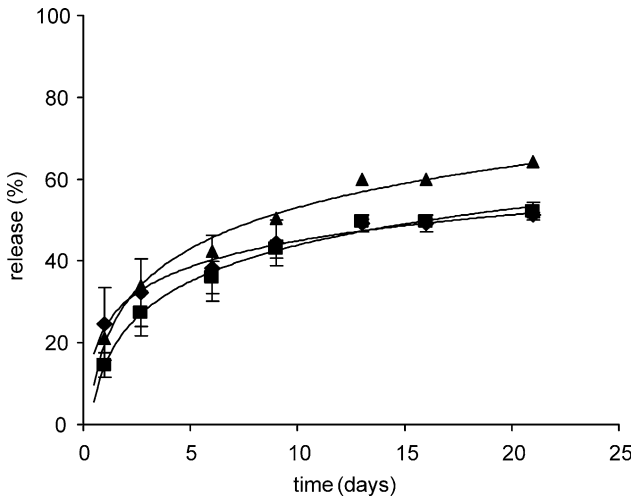

Fig. 5. Release of leuprolide from D-PLA complex prepared by spray freezing in liquid nitrogen. Leuprolide (2 mg, 10%, w/w) was reacted with D-PLA (38 mg) in acetonitrile (1 ml) at 60 °C. After stirring for 1 day, the still clear reaction mixture was sprayed into liquid nitrogen. The influence of the molecular weight of D-PLA (◆ (diamond) 3 kDa, ■ (square)10 kDa and ▲ (triangle) 50 kDa) on the release profile was determined. Release studies were conducted as described in Fig. 4.

weight D-PLA (100 kDa) complexes released 60% leuprolide over a period of three weeks at a mean release rate of 0.73 μg/day/mg complex, between day 6 and 21, whereas low molecular weight D-PLA (10 and 3 kDa) released 50% over a similar time period at a mean rate of 0.54 μg/day/mg complex. The release curves were best fitted with a logarithmic function.

3.4.3. Effect of additives (water, PEG_{400})

The effects of additional water or PEG_{400} to the complexation reaction mixture on the release profile of leuprolide is shown in Fig. 6. Adding water to the acetonitrile solution (0.5–2.5%, v/v) along with D-PLA and leuprolide led to a microparticulate system that showed a significant burst release during the first 24 h (45% of the total leuprolide content) followed by a constant release of an additional 35% of the drug content for a period of 30 days.

The addition of poly(ethylene glycol) (PEG_{400}) (5%, v/v) to the reaction mixture, the release rate of leuprolide from the obtained particles was doubled compared to the average release from stereoselective complexes without the presence of PEG_{400} (80% of the leuprolide content was released over a period of 30 days Addition of PEG_{400}, however, did not induce an appreciable burst effect in the release of leuprolide during the first 24 h (Fig. 6). A slight increase of free leuprolide was measured in the acetonitrile solution after isolation of the precipitates formed in the presence of PEG_{400} (3% of leuprolide, compared to 1.2% without addition of PEG_{400}). The various release profiles could be best fitted with a logarithmic curve.

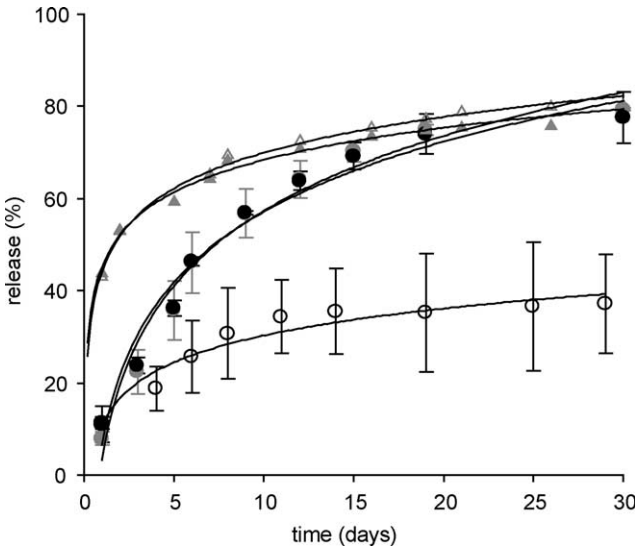

Fig. 6. Release of leuprolide from D-PLA complex, effect of addition of water or PEG_{400} to the reaction mixture. Leuprolide/D-PLA hetero-stereocomplexes were obtained from acetonitrile solution. Addition of water: △ (open triangle, 10 μl, 0.5%, v/v) or ▲ (gray closed triangle, 50 μl, 2.5%, v/v) to the reaction mixture (leuprolide 2%, w/w, D-PLA 50 kDa). Addition of PEG_{400}: ● (gray closed circle, 50 μl, 5%, v/v) or ● (black closed circle, 100 μl, 10%, v/v) to the reaction of leuprolide (5%, w/w) with D-PLA (100 kDa). ○ (open circle) release of leuprolide (5%, w/w) from hetero-stereocomplex with D-PLA (>50 kDa) without additives. Release studies were conducted as described in Fig. 4.

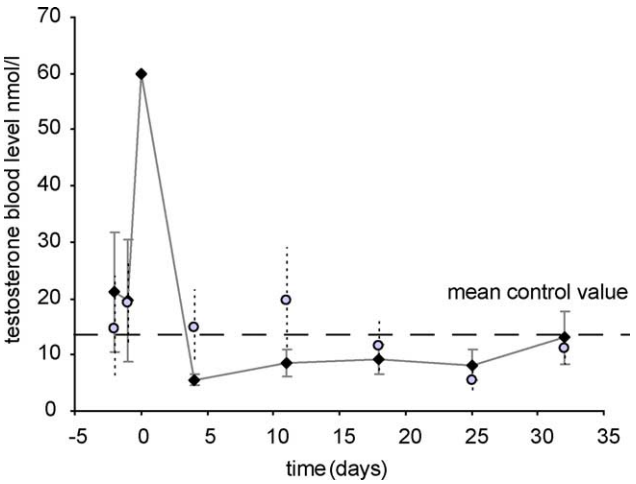

Fig. 7. In vivo activity of leuprolide stereocomplex. ◆ (diamond) leuprolide (5%, w/w) with D-PLA 120 kDa, ○ (circle) control group. Particles were suspended in isotonic glucose solution and injected subcutaneous in male Wistar rats ($n = 5$). Testosterone blood levels were determined by drawing blood from the rat tail artery at specific time intervals. Testosterone concentrations were determined by chemiluminescence, using an automated competitive immunoassay with acridinium ester-labeled testosterone.

Although results that were presented earlier by Okada suggested two exponential curves one following the other in the leuprolide release from microspheres [25], we found that the release of leuprolide from stereocomplexes were best described by a logarithmic function.

3.4.4. In vivo evaluation of leuprolide-D-PLA hetero-stereocomplex

In vivo release of leuprolide was studied by monitoring testosterone blood levels of rats. Leuprolide, a potent LHRH agonist, initially causes a boost in testosterone synthesis. Via a negative feedback, control the testosterone systhesis is blocked. The results of the in vivo release of leuprolide from leuprolide/D-PLA hetero-stereocomplexes, expressed in testosterone level in the blood, were similar to those published by Ogawa et al. [31] (Fig. 7). Low testosterone blood levels were recorded for a period of 25 days. After 4 weeks, the suppressed testosterone concentration was slowly restored to its control value.

4. Summary

Hetero-stereocomplexes consisting of biodegradable poly(D-lactic acid) and the L-configured peptide leuprolide with application in the controlled release of leuprolide, is described. Stereoselective complex formation was demonstrated to occur exclusively with D-configured PLA and not with L-PLA or racemic D,L-PLA. SEM analysis showed uniform, ~1.7 μm in size particles having a fibrous

and porous structure. DSC-thermoscans showed a second peak appeared 10° lower than the melting peak of enantiomeric PLA.

Release of leuprolide was found to be dependent on the molecular weight of D-PLA. Over a period of 25 days, 20% of the total leuprolide content (5%, w/w drug loading) was released from the hetero-stereocomplex with low molecular weight D-PLA compared to 40% from complexes with high molecular weight D-PLA. Increasing the leuprolide content in the complex also increased the amount of released peptide over a period of 20 days. Addition of PEG_{400} or water to the reaction mixture in acetonitrile, yielded hetero-stereocomplexes from which 80% of the leuprolide content was released after 30 days. However, whereas water induced a significant burst release of 45% during the first 24 h, release from complexes prepared in the presence of PEG_{400} remained uniform throughout the release period.

All formulations were of a first-order nature, as statistic analysis showed that the curves were best fitted on a logarithmic fit. Fig. 8 summarizes the release rates of the release during the first 24 h from different hetero-stereocomplex formulations containing 5% (w/w) leuprolide. It can be concluded that the release from low molecular weight D-PLA complexes is slower than that from high molecular weight D-PLA.

Subcutaneous administration of leuprolide stereocomplexes to rats showed a significant decrease on the testosterone blood levels for 3 weeks which indicate continuous release of active leuprolide during this period.

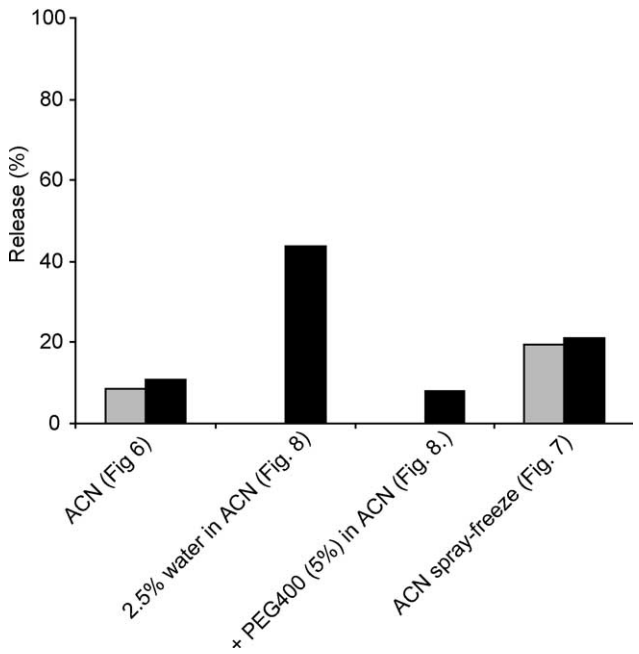

Fig. 8. Burst effects of leuprolide. Summary of release from hetero-stereocomplexes of D-PLA and leuprolide (5%, w/w), described in Figs. 4–6. Release of leuprolide in first 24 h. Black bars: high molecular weight PLA, white bars: low molecular weight PLA; ACN, acetonitrile.

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References

[1] Y. Baba, A. Kagemoto, Heats of dissociation of mixture of poly (*g*-benzyl L- and D-glutamate), Macromolecule 10 (1977) 458–460.
[2] D. Grenier, R.E. Prud'homme, Complex formation between enantiomeric polyesters, J. Polym. Sci. Polym. Phys. 22 (1984) 577–587.
[3] I. Iribarren, C. Aleman, C. Regano, A.M. deIlarduya, J.J. Bou, S. MunozGuerra, Stereocopolyamides derived from 2,3-di-*O*-methyl-D- and -L-tartaric acids and hexamethylenediamine. 2. Influence of the configurational composition on the crystal structure of optically compensated systems, Macromolecules 29 (1996) 8413–8424.
[4] H. Matsubayashi, Y. Chatani, H. Tadokoro, P. Dumas, N. Spassky, P. Sigwalt, Crystal structures of optically active and inactive poly(*tert*-butylethylenesulfide), Macromolecules 10 (1977) 996–1002.
[5] D.W. Lim, T.G. Park, Stereocomplex formation between enantiomeric PLA-PEG-PLA triblock copolymers: characterization and use as protein-delivery microparticulate carriers, J. Appl. Polym. Sci. 75 (2000) 1615–1623.
[6] J. Spevacek, B. Schneider, Aggregation of stereoregular poly(methyl methacrylates), Adv. Colloid Interface Sci. 27 (1987) 81–150.
[7] R. Slivniak, A.J. Domb, Stereocomplexes of enantiomeric lactic acid and sebacic acid ester-anhydride triblock copolymers, Biomacromolecules 3 (2002) 754–760.
[8] K.L. Singfield, G.R. Brown, Optically-active polyethers. 1. Studies of the crystallization in blends of the enantiomers and the stereoblock form of poly(epichlorohydrin), Macromolecules 28 (1995) 1290–1297.
[9] Y. Ikada, K. Jamshidi, H. Tsuji, S.-H. Hyon, Stereocomplex formation between enantiomeric poly(lactides), Macromolecules 20 (1987) 904–906.
[10] A.M. Liquori, G. Anzuino, V.M. Coiro, M. D'Alagni, P. de Santis, M. Savino, Complementary stereospecific interaction between isotactic and syndiotactic polymer molecules, Nature 206 (1965) 358.
[11] J. Slager, A.J. Domb, Biopolymer stereocomplexes, Adv. Drug Deliv. Rev. 55 (2003) 549–583.
[12] D. Brizzolara, H.J. Cantow, K. Diederichs, E. Keller, A.J. Domb, Mechanism of the stereocomplex formation between enantiomeric poly(lactide)s, Macromolecules 29 (1996) 191–197.
[13] M. Spinu, C. Jackson, M.Y. Keating, K.H. Gardner, Material design in poly(lactic acid) systems: block copolymers, star homo- and copolymers, and stereocomplexes, J. Macromol. Sci. Pure Appl. Chem. A33 (1996) 1497–1530.
[14] H. Tsuji, S.H. Hyon, Y. Ikada, Stereocomplex formation between enantiomeric poly(lactic acid)S. 3. Calorimetric studies on blend films cast from dilute-solution, Macromolecules 24 (1991) 5651–5656.
[15] H. Tsuji, S.H. Hyon, Y. Ikada, Stereocomplex formation between enantiomeric poly(lactic acid)S. 4. Differential scanning calorimetric studies on precipitates from mixed-solutions of poly(D-lactic acid) and poly(L-lactic acid), Macromolecules 24 (1991) 5657–5662.
[16] H. Tsuji, F. Horii, S.H. Hyon, Y. Ikada, Stereocomplex formation between enantiomeric poly(lactic acid)S. 2. Stereocomplex formation in concentrated-solutions, Macromolecules 24 (1991) 2719–2724.
[17] H. Tsuji, F. Horii, M. Nakagawa, Y. Ikada, H. Odani, R. Kitamaru, Stereocomplex formation between enantiomeric poly(lactic acids)S. 7. Phase-structure of the stereocomplex crystallized from a dilute acetonitrile solution as studied by high- resolution solid-state C-13 Nmr-spectroscopy, Macromolecules 25 (1992) 4114–4118.

[18] H. Tsuji, Y. Ikada, Stereocomplex formation between enantiomeric poly(lactic acid)s. XI. Mechanical properties and morphology of solution-cast films, Polymer 40 (1999) 6699–6708.
[19] T. Okihara, M. Tsuji, A. Kawaguchi, K. Katayama, H. Tsuji, S.H. Hyon, Y. Ikada, Crystal-structure of stereocomplex of poly(L-lactide) and poly(D-lactide), J. Macromol. Sci. Phys. B30 (1991) 119–140.
[20] J. Slager, Y. Cohen, R. Khalfin, Y. Talmon, A.J. Domb, Peptides form stereoselective complexes with chiral polymers, Macromolecules 36 (2003) 2999–3000.
[21] J. Slager, A.J. Domb, Stereocomplexes based on poly(lactic acid) and insulin: formulation and release studies, Biomaterials 23 (2002) 4389–4396.
[22] P. De Santis, A.J. Kovacs, Molecular conformation of poly(*S*-lactic acid), Biopolymers 6 (1968) 299–306.
[23] B.H. Woo, J.W. Kostanski, S. Gebrekidan, B.A. Dani, B.C. Thanoo, P.P. DeLuca, Preparation,characterization and in vivo evaluation of 120-day poly(D,L-lactide)leuprolide microspheres, J. Control Release 75 (2001) 307–315.
[24] B.H. Woo, K.-H. Na, B.A. Dani, G. Jiang, B.C. Thanoo, P.P. DeLuca, In vitro characterization and in vivo testosterone suppression of 6-month release poly(D,L-lactide leuprolide microspheres, Pharm. Res. 19 (2002) 546–550.
[25] H. Okada, One- and three-month release injectable microspheres of the LH–RH superagonist leuprorelin acetate, Adv. Drug Deliv. Rev. 28 (1997) 43–70.
[26] R. Gref, in: L.M. Sanders, H. Hendren (Eds.), Protein Delivery-Physical Systems, Plenum Press, New York, 1997.
[27] W. Wang, Instability, stabilization and formulation of liquid protein pharmaceuticals, Int. J. Pharm. 185 (1999) 129–188.
[28] J. Slager, A.J. Domb, Hetero-stereocomplexes prepared from D-poly(lactide) and leuprolide. I. Characterization, Biomacromolecules 4 (2003) 1308–1315.
[29] W. Hoogsteen, A.R. Postema, A.J. Pennings, G. Tenbrinke, P. Zugen-maier, Crystal-structure, conformation, and morphology of solution-spun poly(L-lactide) fibers, Macromolecules 23 (1990) 634–642.
[30] F. Tsuji, Autocatalytic hydrolysis of amorphous-made polylactides: effects of L-lactide content, tacticity, and enantiomeric polymer blending, Polymer 43 (2002) 1789–1796.
[31] Y. Ogawa, H. Okada, T. Heya, T. Shimamoto, Controlled release of LHRH agonist, leuprolide acetate, from microcapsules: serum drug level profiles and pharmacological effects in animals, J. Pharm. Pharmacol. 41 (1989) 439–444.