Stereocomplexes, Based on Biodegradable Polymers and Bioactive Macromolecules

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SUMMARY: Stereocomplexation between two enantiomeric polymers such as D-PLA and L-PLA have been described in the literature. No reports up till now have been published on the complexation between two non-identical complementary polymers. Stereocomplexes between poly(lactide) enantiomers and a representative peptide, LHRH, was investigated. The complex was shown to be of physical character (NMR, IR, X-ray) without any irriversibility. DSC-scans show an additional signal related to the second (β-) and thermally less stable form of isotactic PLA. A linear correlation was found between the amount of peptide added to D-PLA and the measured enthalpies. An overall decrease in crystallinity was noticed. Less than 5% of the total amount of peptide was found free in solution after complexation with PLA. A controlled release of peptide was measured for about 3 months *in-vitro*. *In-vivo* similar results were obtained as earlier reported for a commercial available controlled release device containing LHRH

Introduction

Among the interactions, known to be involved in complex-formation between two polymer molecules, are Coulomb forces, Van der Waals interactions and hydrogen-bridge formations[1]. Since the early sixties complex formation is known to occur between two polymers of identical chemical composition but of different chirality. These so-called stereocomplexes are thought to exist on mere basis of steric interactions. Besides complexes formed between syndiotactic and isotactic chains as in poly(methylmethacrylates)[2], mere isotactic polymers of opposite chiral configuration were also found to react. Examples are R-

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and S- poly(γ-benzylglutamate)[3], poly(α-methyl-α-ethyl -β-propiolactone)[4], Poly(tert-butylethylene-oxide and -sulfide)[5], Poly(2,3-dimethyl tartaric acid)[6] and α-methyl-benzylmethacrylate[2]. In 1986 several patents have been issued by Du Pont de Nemours & Co. on stereocomplexes of L and D polylactic acid (PLA), its formation and use, for instance in the preparation of controlled release devices or the fabrication of biodegradable implants[7]. Extensive research on the conditions of its preparation and its physical characteristics was done by Ikada, Tsuji and coworkers. Differential Scanning Calorimetry (DSC) shows a shift of the melting temperature of about 50 degrees from Tm=180°C for D- or L-PLA, Mw=100.000, to Tm=230°C for PLA-stereocomplex of similar molecular weight[8]. Clear shifts in signals were detected both in X-ray and in Solid State MAS ^{13}C-NMR spectra[8].

Two types of helices are reported to exist for isotactic PLA: 10_3-helixes: α-form or 3_1-helixes: β-form. Based on computer calculations and Atomic Force Microscopy 3_1-helixes have been predicted for the configuration of D- and L-PLA molecules, intertwined forming 'double strand' helices, which form triangle shaped crystals in a hexagonal cell packing[9].

It should be noted that all previous reports on stereocomplexation are focused on the complexation of enantiomers of the same chemical composition. No research is yet reported on the formation of diastereocomplexes composed of two polymeric chains with different chemical composition and opposite chirality.

Since almost all (macro)molecules found in nature, like proteins, DNA and other bioactive compounds are of Levo-chirality (L), stereocomplexation was tried with D-PLA. The constitution of PLA and peptides is similar, with the obvious differences of the amide bonds in comparison to ester bonds. In aprotic organic solvents poly(amino acids) form less hydrogen bridge bonds. Thus, the crystallization and packing is comparable with PLA in organic solvents.

Based on these findings, this article describes the formation and use of the new type of physical complex between enatioselective poly(lactic acid) (D- and L-PLA) and peptides or proteins, naturally exhibiting the L-configuration. Besides complexation of peptides with only D-PLA also complexation of peptides with D-PLA and L-PLA together has been studied. For the study of stereocomplex-formation with peptides, LHRH, Somatostatin-analogue Vaprotide and poly-alanin have been used as model-peptides. Effects of changes in reaction conditions such as solvent-systems, temperature, concentration of polymer or peptide and reaction time on the complex formation were studied and the complexes were studied for their physical and chemical characteristics.

Experimental methods

Synthesis

PLA and polyalanine were synthesized according to known procedures[7,10]. In brief: Isotactic or atactic polylactic acid was obtained by ringopening polymerization of enantiomeric or racemic lactide, using stannous octoate as catalyst and decanol as initiator. Polyalanine was synthesized by ringopening polymerization of alanin-N- carboxyanhydride. This monomer was obtained by reacting alanin with 0.3 equivalent triphosgene in dry THF.

Complexation of D- and L-PLA has been studied extensively in literature[8]. For its preparation different methods have been reported. Using acetonitrile and stirring at 60 ^{0}C yields the stereo complex in about three days. In an ampoule (2 ml), equipped with a micro-stirrer, D-PLA and peptide or other (bio)polymer of L- chirality (generally 10% w/w) were solved in acetonitrile. In some cases L-PLA (in equivalent amount to D-PLA) was added as well. The solution was warmed at 60 ^{0}C and stirred for three days or longer. Complex usually precipitated out and could be isolated either by centrifugation or filtration. In cases of low molecular D-PLA with 2 till 10% w/w peptide, often no precipitate was formed within three days. L-PLA (100k) was added in order to accelerate the precipitation process. D- + L-PLA complexes were also synthesized at room temperature by adding dichloromethane to the acetonitrile mixture in order to increase the solubility of PLA. Mixing the D- and L-compounds leads to complex formation (cloudiness) which resolves partly by heating (fan). Reacting at 60 ^{0}C for several hours leads to an insoluble precipitation. Although PLA is soluble in various other solvents (chloroform, dichloromethane, THF, dioxane, DMF, DMSO) no precipitate was formed with LHRH other than acetonitrile.

In order to achieve stereocomplexes of PLA containing polyalanine, polyalanine was solved in dichloroacetic acid (DCA) and diluted with acetonitrile. Both isotactic PLA and polyalanine form a gelatinous mass after several hours in this solvent mixture. Overnight the gelatinous mixture of PLA and polyalanine becomes thicker. In 100% DCA, no gelation was observed, but no precipitation was obtained.

Characterization

In order to characterize the various complexes different analytical methods have been used. D-PLA complexes with Vapreotide and LHRH solve in chloroform (2% and 10% complexes taken) but samples put in buffer showed no increase in LHRH release compared to the basic release of the complex put directly in buffer. 20%-LHRH in D-PLA solved for a

good part in D-chloroform except for little remnants (unbound LHRH) ^{1}H NMR showed no additional peaks that could be annotated to LHRH or any deviation in the signals that belong to regular PLA

Decomplexation of the D+L complex containing Leuprolide into polymer and peptide was accomplished by resuspending the complex in acetonitrill, containing 5% w/w (related to the amount of complex) Span80. Almost 100% recovery of the peptide was obtained.

Among the various techniques to analyze the stereocomplexes CD-ORD has been used as a method to ascertain helical formations. Samples were solved in hexafluoro-2-propanol (50 ul) and 3 ml of chloroform was added. D- and L-PLA gave opposite curves, whereas in PLA-complex no or little curve was noticed. Indicating the contra-effects of either PLA. Complex of D-PLA and peptide showed some differences in spectrum. It is difficult, however, to correlate changes in the CD-ORD spectrum to interactions or mere blending.

Since the complexes and the synthesized polyamines do not solve in chloroform or water GPC has not been performed on the PLA-complexes. GPC is performed on the various samples of isotactic and atactic PLA.

Using Solid State 13C MAS NMR, the problem of ample solubility in regular organic solvents was circumvented and we were able to obtain similar spectra for the D- + L-PLA complex as earlier reported[8]. Differences between isotactic PLA and PLA-complex were also found in IR-spectra and X-ray analyses. Complexing peptide with D-PLA, however, resulted in spectra showing only signals from isotactic PLA without further changes. Even at 20% loading of the polymer with LHRH no signals of peptide were noticed. These data indicate that no (irreversible) chemical reaction is involved in the complexation of D-PLA and LHRH since no change in the PLA-spectrum is detected. Similar results were found with FT-IR, Solid State ^{13}C-NMR and X-ray analysis.

Differences between PLA and complexed PLA with peptides were found by Differential Scanning Calorimetry analysis. Table 1 summarizes the measured data for the various complexes and single polymers.

Isotactic PLA melts around 175 ^{o}C, dependent upon its molecular weight, forming a sharp signal on a DSC scan. Characteristic to the PLA complex is the shift in melt temperature of about 55 degrees to 230 ^{o}C.

D-PLA complex with any L-polypeptide, in contrast, shows two melting points: at around 175 ^{o}C and one about 10 degrees less.

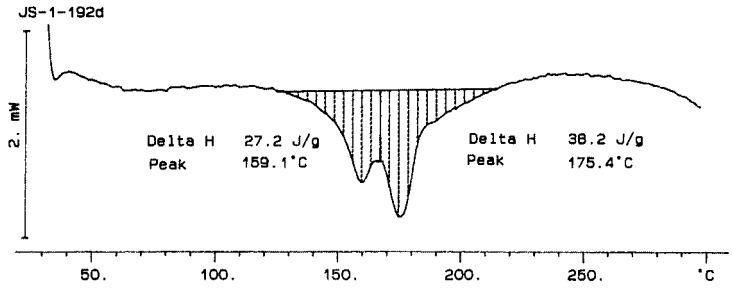


Fig. 1. DSC-scan of stereocomplex

Table 1. DSC-results

DSC	°C	°C
D-PLA, 100.000	181	
L-PLA, 30.00	175	
PLA-complex	209[1)]	231
LHRH	163 [2)]	250
poly-L-Ala [3)]	160	
D-PLA + LHRH	171	179
D-PLA + L-PLA + LHRH	208	231
$D\text{-}PLA_{250k}$ + poly-L-Ala	159	175
$D\text{-}PLA_{250k}$ + L-PLA		227
+ Poly-L-Ala		

[1)] small signal; [2)] very broad signal; [3)] Poly-L-Ala was dissolved in dichloroacetic acid and precipitated in acetonitril..

A similar phenomenon was noticed by Hoogsteen et al. when spinning fibers from a solution of L-PLA in hot chloroform/toluene (near θ-conditions)[11]. It was suggested that the lower melting point was to be attributed to the β-helical form, since it was found less stable than the α-form. From this finding it was hypothesized that in order to form a stereocomplex, PLA has to undergo a conformational change from the α-form to the β-form. Where under standard conditions the α-helix is preferred thermodynamically, in this case formation of complex with a polymer of opposite chirallity stabilizes the β-conformation. This effect was found for complexes of D-PLA with LHRH as well as poly-L-alanin. Additionally the enthalpies of both signals were found to be related to the amount of peptide (percentage of loading) of the D-PLA complex. As can be seen, the total change of enthalpy decreased as D-PLA was complexed with more peptide. This could be caused by a general loss of crystallinity or, indeed, an increase in concentration of the less stable β-conformation.

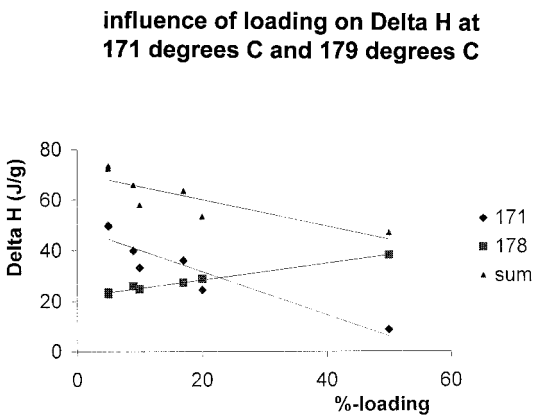


Fig. 2. Relation in enthalpies

D + L PLA complexes with and without a polypeptide such as LHRH-analogues show an additional depression in the DSC-scan around 205 °C, about 25 degrees below the reported meltingpoint for the LPA-complex. No relation, however, is found between enthalpies and amount of added peptide. Poly-Ala itself has not been found to form complexes under similar conditions. Perhaps because of difficulties in solving the polymer.

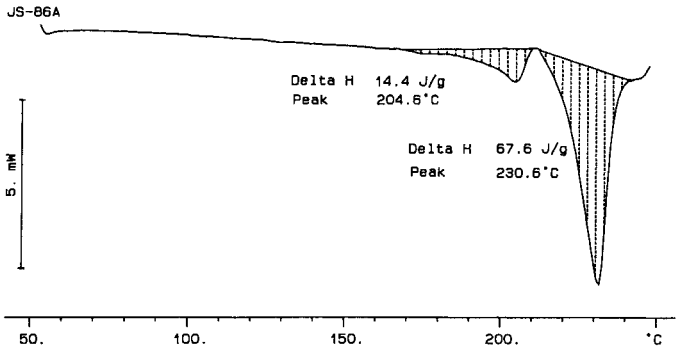
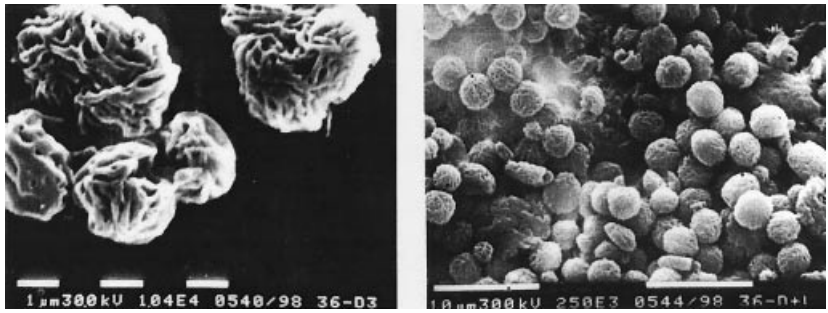


Fig. 3. DSC-scan of PLA-complex containing LHRH

Scanning Electron Microscopy

Scanning Electron Microscopy showed formation of spheres in a range of about 0.3 to 3 μm, both for PLA-complex as well as complexes of D-PLA with peptides. In case of complexes with Vapreotide these particles had a fibrous structure comparable to the one reported for D + L- PLA-complex[12].



D-PLA + Vaprotide

LHRH in D- + L-PLA

Fig. 4. SEM of stereocomplexes containing Vaprotide or LHRH bioactive agents

LHRH (dekapeptide as well as leuprolide) and poly-L-Alanin complexed with PLA, forming both fibrous particles as well as particles with irregular shapes and sizes. Compared to regular microspheres a tremendous increase in surface is observed.

AFM-experiments

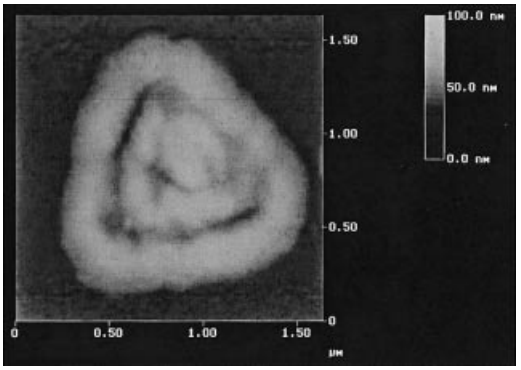


Fig. 5. AFM-image of D- +L-PLA stereocomplex

Triangle shaped crystals were found for D- + L-PLA complexes[13]. A drop was taken from 0.1% polymer solution in ACN and casted on mica. Similar results were reported earlier[13]. Height is about 20 nm. The sides of the triangle are approximately 1.7 micron. AFM-measurements on particles formed in acetonitrile appeared to be highly difficult because of their rough surfaces. Particles were found to diminish in size after being scanned over and over again up until a 'kernel' was found which did not diminish further. Adding a drop of solving agent (hexafluoro-2-propanol) on the mica led to the formation of a polymer film without any characteristics. Dipping in mica into a reaction-mixture of 0.1 % polymer (D- and L-PLA) in ACN at several time intervals led to the conclusion that spherical particles were already formed at early stage in the reaction.

Release

Release of peptide was observed after incubation in buffer pH=7.4 at 37 °C. Release of both LHRH and Vaprotide from complexes have been measured by HPLC.

Complexes for example, containing 5% w/w peptide and D-PLA and L-PLA in ratio 1:1 (PLA between 50 and 100k) released 15% till 45% of the total peptide amount in about 40 days. In some cases release could be measured up until 90 days: 5% loading in 10k DPLA and (after 5 days) L-PLA 60k: release 2% till 45 %. This release was found reproducible.

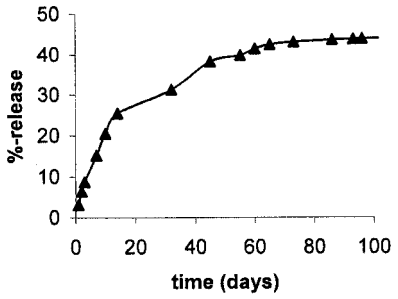


Fig. 6. Release LHRH from D- + L-PLA- complex, 5.3% loading, diameter of microspheres ca 5 μm

No burst-release was noticed within the first 24 hours of the release. Adding PEG_{400} (1:1 or 1:2 in ratio with PLA) resulted in the controlled release of LHRH (dekapeptide) which was twice as much compared to complexes without PEG_{400}. In Vaprotide containing complexes release was still measured up to 30 days until about 35% for complex containing 20% Vapreotide.

In vivo experiment was conducted on male Wistar rats with stereocomplexes containing Luprolide, an LHRH-derivative. Suspensions of stereocomplexes containing 5% (A) and 10% LHRH (B) were prepared and injected i.p. Blood was taken weekly over a period of five weeks post injectum from the tail-artery. The results of the in-vivo release of LHRH are identical to published findings of the release from microspheres (Lupron^{TM}) (Ogawa, 1989)[14].

Conclusion

For the first time dia-stereocomplexes have been described consisting out of biodegradable poly(lactic acid) and polypeptides. As was shown by various spectroscopic techniques no chemical reactions were involved and the complex was found to be reversible. By means of HPLC the amount of peptide complexed with PLA was estimated to be almost 100 %. It was released in a controlled manner both *in-vitro* and *in-vivo*. By means of DSC differences were found in energy uptake between isotactic PLA and complexes of peptide with D-PLA. Based upon earlier reports the various signals were related to the α- and the β- form of the helix conformations of PLA.

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