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Research paper

Acylation of peptides by lactic acid solutions

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Abstract

To simplify the search for effective mechanisms to suppress peptide acylation inside drug delivery devices made of poly(D,L-lactic acid) (PLA) and poly(lactic-co-glycolic acid), we were looking for a suitable model system that would allow screening of strategies for peptide stabilization. With their low pH and the presence of lactic acid oligomers, diluted lactic acid solutions promised to be a suitable test system that mimics the microclimate in degrading PLA devices. We created solutions of 1–50% (w/w) lactic acid by dilution of concentrated lactic acid. Using high performance liquid chromatography (HPLC) and high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) analysis, oligomer hydrolysis was monitored during the equilibration process of the diluted solutions. Their final oligomer content was determined by titration and by calculations based on HPLC data. HPLC-MS analysis of human atrial natriuretic peptide (ANP) stability in different lactic acid solutions at 37°C for 4 weeks demonstrated that ANP underwent acylation even in diluted solutions containing only 0.05% (w/w) lactic acid oligomers. Purity analysis of lactic acid solutions allowed us to compare the conditions in the solution test-system to the microclimate that prevails inside degrading PLA microspheres.

Keywords: Lactic acid; Oligomers; Atrial natriuretic peptide; Stability; Acylation

1. Introduction

A major challenge in the development of biodegradable controlled release devices is a satisfactory stabilization of the incorporated drugs [1]. The acylation of peptide drugs inside degrading poly(D,L-lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) delivery devices [2] is one of the obstacles to be overcome for the successful delivery of bioactive molecules. Several strategies have been proposed for preventing the acylation reaction [2]. To avoid the acidic microclimate that is usually present inside degrading PLA and PLGA [3,4] is one of these strategies, either by enhancing the drainage of polymer degradation products using hydrophilic additives, such as poly(ethylene glycol) or by neutralizing the acidic pH with basic additives, such as magnesium hydroxide. Irrespective of the acylation reaction, both mechanisms had earlier been shown to enhance the stability of proteins incorporated into biodegradable delivery devices [5,6]. To simplify the search for appropriate counter measures to peptide acylation, we were looking for a suitable test system. An easy-to-use model system would be to provide the low pH and the presence of oligomers which is probably the key to the acylation reaction inside degrading PLA [2].

Aqueous lactic acid solution seemed to be a promising candidate for a test system. Of crucial importance to the phenomenon of acylation reactions is the fact that lactic acid (Fig. 1a) is subjected to intermolecular esterification even in dilute solutions, yielding the dimer lactoyllactic acid and also higher oligomers [7]. These activated esters are probably the major source of peptide acylation inside degrading microspheres [2]. Concentrated lactic acid of a concentration of approx. 90% (w/w) contains an absolute amount of approx. 30% (w/w) oligomers [7]. After diluting concentrated lactic acid, the oligomer content decreases comparatively slowly (over several weeks at room temperature) until a dynamic equilibrium of free and esterified lactic acid is reached [7]. Because of the negative inductive effect of the α-hydroxy group, lactic acid has a pK_a value of 3.86, which is significantly lower than that of propionic acid ($pK_a = 4.88$), so that the pH of even diluted lactic acid solutions (1–50% (w/w)) ranges from pH 1.0 to 2.3 (Table 1), thus reflecting the pH that has been measured inside degrading polymer matrices [4].

Having chosen lactic acid solutions as a potential model system, the goals of the present investigations were: (1) to assess the equilibration process and determine the final content of monomers and oligomers in diluted lactic acid

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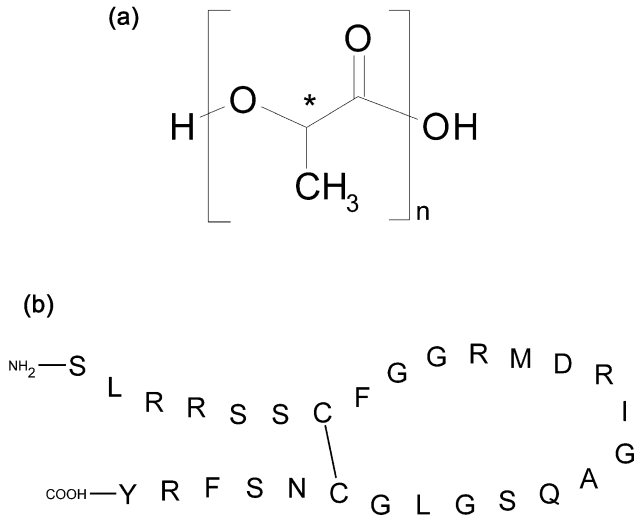

Fig. 1. (a) Structure of lactic acid ($n = 1$) and poly(D,L-lactic acid) ($n \gg 1$). (b) Amino acid sequence of human atrial natriuretic peptide.

solutions; (2) to develop a suitable method for enhanced equilibration of diluted lactic acid solutions within a few days; and (3) to investigate the impact of different oligomer concentrations in solution on peptide acylation. Therefore, several dilutions of concentrated lactic acid were prepared and oligomer hydrolysis was monitored by HPLC analysis during storage at either room temperature or at 90°C for enhanced equilibration. The final composition was determined by HPLC analysis as well as by titration. To investigate the impact of lactic acid oligomers on peptide stability, human atrial natriuretic peptide (ANP, Fig. 1b) was stored in lactic acid solutions of different concentrations, and the appearance of peptide acylation products was monitored by HPLC-MS analysis. In addition, purity analysis of lactic acid solutions with differential scanning calorimetry (DSC) was carried out to compare the conditions in the solution test-system with the microclimate inside degrading PLA microspheres where ANP had earlier been shown to be acylated [2].

2. Materials and methods

2.1. Materials

Concentrated lactic acid, analytical grade ('approx. 90%') was purchased from Merck (Darmstadt, Germany). The exact lactic acid content was determined by titration as described below. Water used throughout the investigations was double-distilled and filtered through a cellulose nitrate filter with pores of 0.2 μm diameter (Sartorius, Göttingen, Germany). Prior to use, the water was retained at boiling point for 5 min to adjust the pH to 7 by removing dissolved carbon dioxide. Acetonitrile was of HPLC grade, all other reagents were of analytical grade or higher purity.

Table 1
pH and absolute oligomer content of lactic acid solutions (from ref. [7]).

Total lactic acid (% w/w)	PH	Oligomers (% w/w)
89	0.0	33.1
50	1.0	3.2
20	1.5	0.5
10	1.8	0.4
5	2.0	0.1
1	2.3	0.0

2.2. Preparation of lactic acid solutions

Diluted lactic acid solutions were obtained by weighing (microbalance AT460 Delta Range from Mettler-Toledo, Gieβen, Germany) the necessary amounts of concentrated lactic acid and of water into 50 ml screw cap vials that were tightly closed. For an adjustment of the oligomer content according to Table 1, the solutions were either stored at room temperature (20 ± 2°C) for 2 months, or at 90 ± 2°C in an incubator (BvW from Memmert, Schwabach, Germany) for accelerated equilibration over 30 h. Samples of each solution were drawn at regular time intervals and were immediately analyzed for relative amounts of lactic acid monomers and oligomers using the HPLC method described below. Additionally, after equilibration, the free acidity and total lactic acid content of all solutions were determined by titration.

2.3. Determination of total and free lactic acid content by titration

The amount of total and free lactic acid in the equilibrated solutions was determined by a two-step titration [8]. An amount of lactic acid solution equivalent to approx. 10 mmol total lactic acid was weighed exactly into an Erlenmeyer flask. In a first step, free lactic acid was titrated against 1 M-sodium hydroxide solution using phenolphthalein as an indicator (V_1). An excess of sodium hydroxide solution was added (V_2) and lactic acid oligomers were hydrolyzed for 60 min. In a second step, the amount of 1 M-sodium hydroxide solution necessary for oligomer hydrolysis was determined by back titration with 1 M-hydrochloric acid (V_3). Three samples of each solution were analyzed. Table 2 illustrates how the amount of total and free lactic acid was determined from titration data. Additionally, the amount of lactic acid monomers and oligo-

Table 2
Determination of the composition of a sample of concentrated lactic acid[a]

	1 M-NaOH (ml)	Lactic acid (w/w, %)
Titration 1 (free acidity)	a = 8.25	71.7
Titration 2 (esters)	b = 1.96	17.0
Total lactic acid	a + b = 10.21	88.7
Monomers	a − b = 6.30	54.7
Oligomers (lactoyllactic acid)	2·b = 3.90	33.9

[a] Sample weight: 1.037 g (M_r (lactic acid) = 90.1; a = V_1; b = $V_2 - V_3$).

mers was calculated under the assumption that all oligomers were dimeric lactoyllactic acid. An example for the calculation of the composition of concentrated lactic acid is given in Table 2.

2.4. HPLC and HPLC-MS analysis of lactic acid solutions

Oligomer hydrolysis in diluted lactic acid solutions was monitored using a HPLC system with a degasser (from Knauer, Berlin, Germany), LC-10AT pump, FCV-10AT$_{VP}$ gradient mixer, SIL-10AD$_{VP}$ autosampler, CTO-6A column oven, SPD-10AV ultraviolet (UV)-detector, and SCL-10A$_{VP}$ controller (all from Shimadzu, Duisburg, Germany). A total of 100 μl of each sample were separated at 40°C on a C4-reversed phase column (Supelcosil LC304, 4.6 × 250 mm) with a C4-precolumn (Supelcosil LC304, 4.6 × 50 mm) from Supelco (Deisenhofen, Germany). The mobile phase consisted of 20% acetonitrile in water (+0.1% trifluoroacetic acid (TFA)) and was set to a flow rate of 1.0 ml/min. Chromatograms were recorded for 10 min by UV detection at 210 nm. The ClassVP 5.0.1 software (Shimadzu, Duisburg, Germany) was used for HPLC data acquisition and analysis to finally calculate the relative peak areas of lactic acid monomers and oligomers. To calculate absolute amounts of monomers and oligomers from HPLC data, the total lactic acid content was divided by the relative peak areas of the monomers and oligomers, respectively.

For identification of oligomers appearing in the chromatograms of lactic acid solutions, the HPLC method described above was transferred to an HPLC system (Series 1100 degasser, binary pump, autosampler, column oven and diode array detector, all from Hewlett-Packard, Waldbronn, Germany) coupled with an electrospray ionization/triple quadrupol mass spectrometer (ES-MS) (TSQ 7000, from ThermoQuest, San José, CA, USA) with API2-source (capillary temperature 350°C, electrospray voltage 4.5 kV). As the mass spectra were obtained in negative-ion mode, addition of TFA to the mobile phase was not necessary for HPLC-MS analysis. Data was acquired and analyzed using the XCalibur software provided with the system (ThermoQuest, San José, CA, USA). For identification of lactic acid oligomers, the total ion current (TIC) of the mass spectrometer was monitored and the appearing signals were further characterized by their corresponding mass spectra.

2.5. Investigation of ANP stability in lactic acid solutions

Human atrial natriuretic peptide (ANP, 28 amino acids, 3080.5 Da, Fig. 1b) was used for the investigation of peptide stability in lactic acid solutions. ANP was dissolved in 1, 5, 10 and 50% lactic acid solutions, yielding a concentration of 20 μg ANP/ml. The solutions were stored in sealed HPLC vials in an incubator at 37°C for 4 weeks. After preparation of the solutions (which took approx. 30 min) and on days 7, 14 and 28 of storage at 37°C, samples of each solution were frozen in liquid nitrogen and stored at −80°C (Herafreeze HFU 586 basic from Kendro, Hanau, Germany) prior to analysis. After all samples had been drawn, the solutions were thawed and analyzed in triplicate using the HPLC-MS system described above. HPLC separation of ANP and its acylation products was carried out on a C18-reversed-phase column and precolumn, applying a mobile phase gradient of 20–40% acetonitrile in water (+0.1% TFA) over 15 min as described earlier [2]. For identification of ANP and its acylation products, the TIC of the ES-MS was monitored for their corresponding mass spectra. Next, for semiquantitative analysis of peptide acylation, triply charged ions of native ANP (m/z 1027.8) and of the mono-acylated reaction product (ANP-LA, m/z 1051.8) were monitored in single-ion mode. The resulting peak areas of the single-ion chromatograms of triply charged ANP and ANP-LA were determined using the LCQuan tool of the XCalibur software package. The relative peak areas of ANP and ANP-LA were expressed as arithmetic mean ± standard deviation of triplicate sample analysis.

2.6. DSC purity analysis of lactic acid solutions

DSC purity analysis is based on the melting point depression caused by small molar amounts of impurities (<2 mol.%) in crystalline samples [9,10], e.g. by lactic acid oligomers and monomers in dilute aqueous solutions. The method had previously been successfully used for assessing the osmotic pressure inside degrading PLA microspheres [3]. Lactic acid solutions with concentrations of up to 2.0 mol.% (9.3% (w/w)) were analyzed on a DSC 2920 equipped with a refrigerated cooling system and an autosampler (TA Instruments, Alzenau, Germany). Using a Sartorius 4401 microbalance (Sartorius, Göttingen, Germany), 1.5–2 μl of each solution were precisely weighed into hermetic aluminum sample pans (TA Instruments, Alzenau, Germany). The samples were sealed using a sample encapsulation press from TA Instruments. An empty, sealed pan served as reference. DSC measurements were carried out using a temperature program optimized for purity analysis. Sample and reference were cooled to −30°C, kept isothermal for 5 min and heated to 10°C with a heating rate of 2°C/min. Purity values of the solutions were obtained using the purity analysis program of the Universal Analysis software package (TA Instruments, Alzenau, Germany) provided with the instrument. Additionally, the osmotic pressure of lactic acid solutions was determined using a semi-micro osmometer (Knauer, Berlin, Germany) which was calibrated with double-distilled water and sodium chloride solutions of known osmotic pressure.

3. Results

3.1. Oligomer hydrolysis in diluted lactic acid solutions

By titration, the total content of concentrated lactic acid

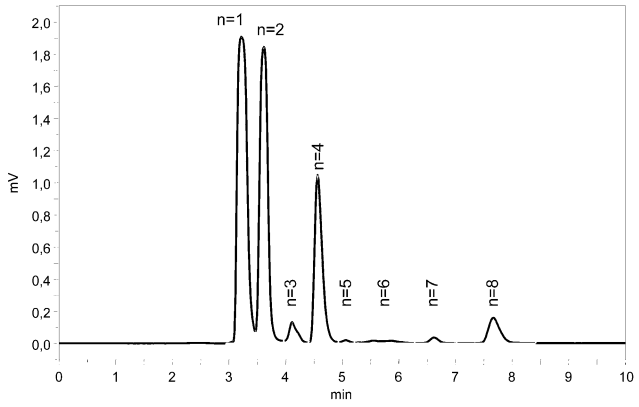

Fig. 2. HPLC chromatogram of lactic acid-20% (w/w), day 3 of the equilibration study (UV detection, 210 nm) ('*n*' represents the number of lactic acid repeating units).

was determined to be 88.7% as shown in Table 2. This value was used as a reference for the preparation of diluted lactic acid solutions with a concentration of 1–50% (w/w) total lactic acid. To monitor the hydrolysis of oligomers in the diluted solutions, samples drawn throughout the equilibration study were analyzed by reversed-phase HPLC, yielding chromatograms with up to six peaks (Fig. 2) which obviously represented the monomer ($n = 1$, retention time 3.2 min) and several oligomers ($n > 1$, retention times 3.7–7.7 min). By HPLC-MS analysis in ES-MS negative-ion mode, the mass spectrum corresponding to each peak was obtained as shown in Fig. 3. In each of the mass spectra, several peaks are present, representing the molecular ion $(M\text{-}H)^-$ and several cluster ions such as, for example, $(2M\text{-}H)^-$, allowing for mass calculation and identification of the oligomers. HPLC-MS analysis revealed that oligomers consisting of two to eight lactic acid units were eluted one after another in reversed-phase HPLC.

After identification of lactic acid monomer and oligomers in the HPLC chromatogram, the corresponding peak areas were determined. The hydrolysis of lactic acid oligomers at room temperature as well as at 90°C was monitored by calculation of relative peak areas of lactic acid monomer and oligomers. As in Fig. 2, free lactic acid ($n = 1$) and lactoyllactic acid ($n = 2$) remained the most prominent peaks throughout the equilibration. The graphs for the equilibration of lactic acid-1% (w/w) and 50% (w/w) at room temperature are displayed in Fig. 4. In this figure, the relative peak areas of lactic acid, lactoyllactic acid and the higher oligomers are shown. One can see that the relative amount of lactic acid monomers increased continuously, whereas the amount of higher oligomers ($n > 2$) decreased until the equilibrium was reached. The hydrolysis of dimers, in contrast, yielded a sigmoid curve which can be explained by the initial formation of dimers resulting from the hydrolysis of higher oligomers. Initially, the relative content of lactic acid monomers was higher in the solutions that were more diluted (58% in lactic acid-1 versus 40% in lactic acid-50%). However, the equilibrium was reached more quickly in solutions of higher total lactic acid concentration. Lactic acid-50% (w/w) reached the equilibrium after approx. 30

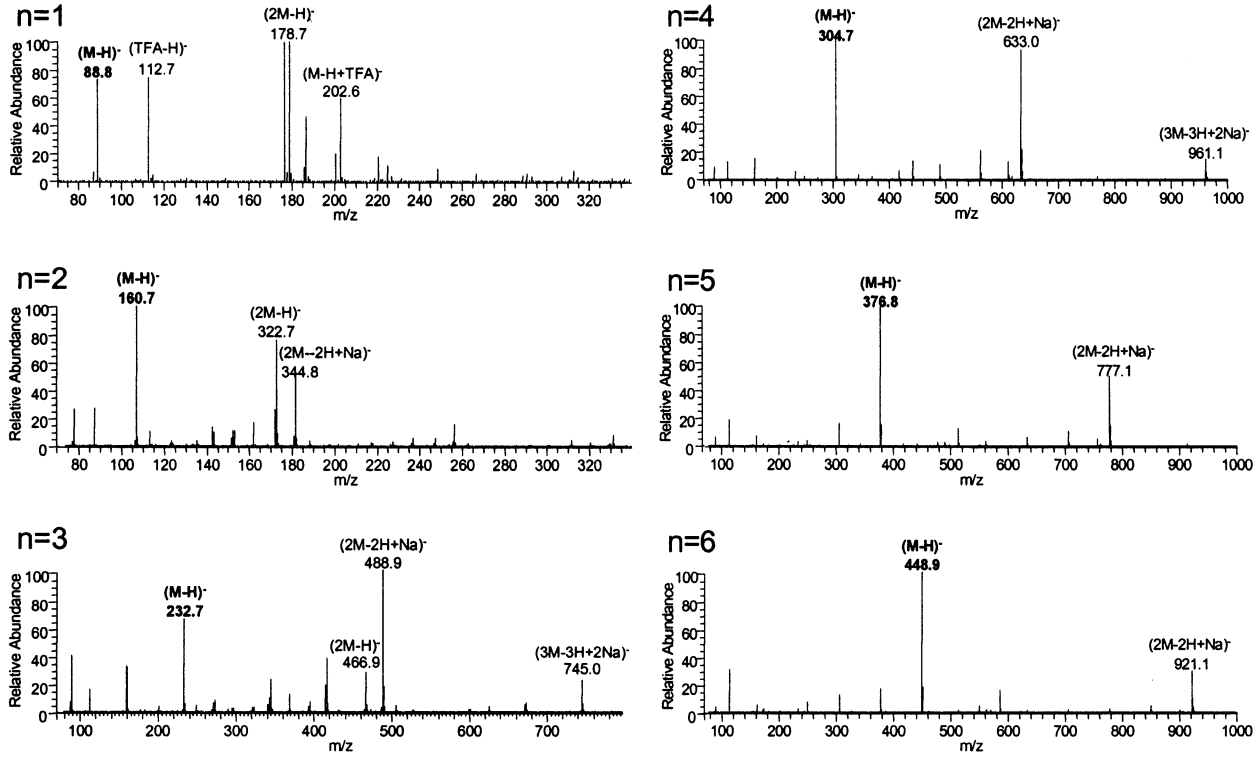

Fig. 3. Negative-ion mass spectra of lactic acid monomer ($n = 1$) and oligomers ($n > 1$).

days of incubation at room temperature, whereas lactic acid-1% (w/w) was equilibrated after approx. 50 days. A higher temperature enhanced oligomer hydrolysis as expected (Fig. 4c). The equilibrium of all lactic acid solutions was reached within approx. 30 h of incubation at 90°C.

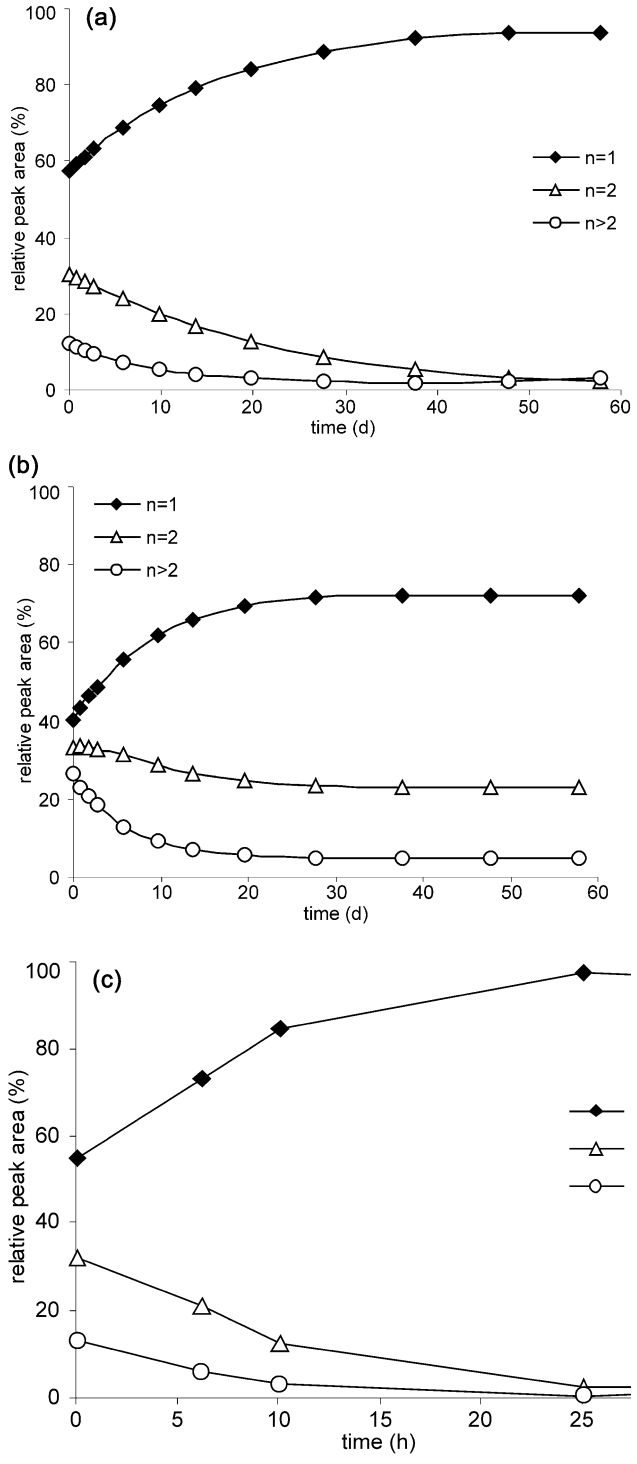

Fig. 4. Hydrolysis of: (a) lactic acid-1% (w/w); of (b) lactic acid-50% at room temperature; and of (c) lactic acid-10% (w/w) at 90°C (relative amounts of mono-, di- and higher oligomers as determined by HPLC analysis, '*n*' represents the number of lactic acid repeating units).

3.2. Composition of lactic acid solutions

After equilibration, the amount of lactic acid monomers ($n = 1$) and all oligomers ($n > 1$) in the solutions was determined by two-step titration and by calculation from HPLC data. In Fig. 5, the values obtained from both methods are shown, demonstrating the increase of oligomer content with increasing total lactic acid content. The higher amount of monomers calculated from titration data compared to calculation from HPLC data resulted from differences in the analytical methods. When titrating free lactic acid with sodium hydroxide solution, a certain amount of the oligomers was already cleaved during this first titration step, yielding a higher volume of sodium hydroxide solution than was really needed for neutralization of free acid, thus leading to higher amounts of lactic acid monomers. In contrast, when calculating relative peak areas of oligomers and monomers from HPLC data, differences in the specific absorption of oligomers and monomers were not considered. A higher specific absorption of the oligomers as compared to lactic acid monomers would, for example, have increased the relative peak area obtained for the oligomers.

3.3. Stability of ANP in lactic acid solutions

ANP was found to be acylated in all of the diluted lactic acid solutions. In Fig. 6, the relative amount of mono-acylated ANP (ANP-LA) is shown as determined by HPLC-MS analysis. In all solutions, an initial amount of approx. 2% ANP-LA was found, as the preparation of the solutions took approx. 30 min prior to freezing in liquid nitrogen, during which the acylation reaction already started. After 4 weeks of incubation, the relative amount of ANP-LA increased to 27% in lactic acid-50% (w/w), whereas in lactic acid solutions of lower concentration, the relative amount of ANP-LA increased more slowly. However, in lactic acid-1% (w/w), containing only 0.05% (w/w) oligomers, acylation was not completely suppressed. Within 4 weeks, the relative amount of ANP-LA initially formed remained at 2.5 ± 0.2%.

3.4. Purity values and osmotic pressure of lactic acid solutions

In an earlier investigation on the microclimate inside degrading PLA microspheres, the lowest sample purity measured was 99.1% [3]. For a comparison of the conditions in the solution test system with the microclimate inside degrading microspheres, DSC purity analysis was also performed with lactic acid solutions. As the van't Hoff-equation, on which purity determination is based, should only be applied for samples of at least 98 mol.% purity [10], lactic acid solutions of molar concentrations ranging from 0.4 to 2 mol.% (= 2.1–9.3% (w/w)) were investigated (Table 3). In Fig. 7, the purity values calculated from DSC analysis are compared to the theoretical values of sample

Total lactic acid (w/w)	Oligomers (w/w) titration	HPLC
89 %	33.1	45.4
50 %	3.97	14.0
20 %	0.46	2.20
10 %	0.15	0.70
5 %	0.07	0.65
1 %	0.05	0.06

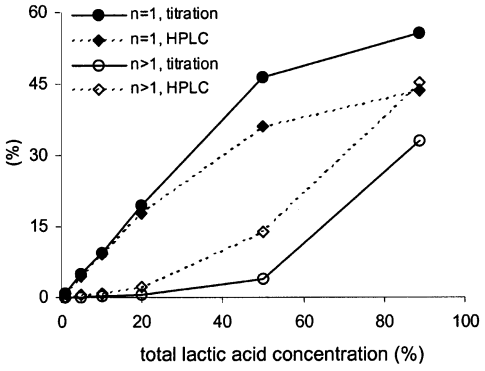

Fig. 5. Absolute amount of lactic acid monomers ($n = 1$) and oligomers ($n > 1$) in solutions of different total lactic acid concentration as determined by titration and by HPLC analysis.

purity. Sample purity determined by DSC analysis was higher than expected from the total amount of lactic acid. This resulted from the presence of oligomers in the solutions, subsequently yielding a lower amount of osmotically active molecules. The negative dissociation grade calculated from purity values and osmotic pressure of the solutions, increased with increasing lactic acid concentration (Table 3), thus demonstrating the presence of an increasing amount of oligomers. According to these results, the above-mentioned purity value measured inside degrading PLA microspheres would therefore be reflected by the purity of a solution of approx. 5% (w/w) lactic acid.

4. Discussion

We chose lactic acid solutions as a model system for the investigation of parameters influencing peptide acylation as the solutions provide the low pH and the presence of oligomers that are characteristic for the microclimate inside degrading PLA. For the successful use of lactic acid solutions as a testing system, their composition of free and esterified lactic acid had to be determined. When preparing lactic acid solutions by diluting concentrated lactic acid, oligomer hydrolysis may require several weeks until an equilibrium is reached and the final composition of the solutions can be analyzed. Whereas at room temperature, the equilibration process of diluted lactic acid solutions took almost 2 months, it could be significantly accelerated at a higher temperature. Equilibration at 90°C was completed within 30 h, a feasible time-range for the preparation of lactic acid solutions as a testing system.

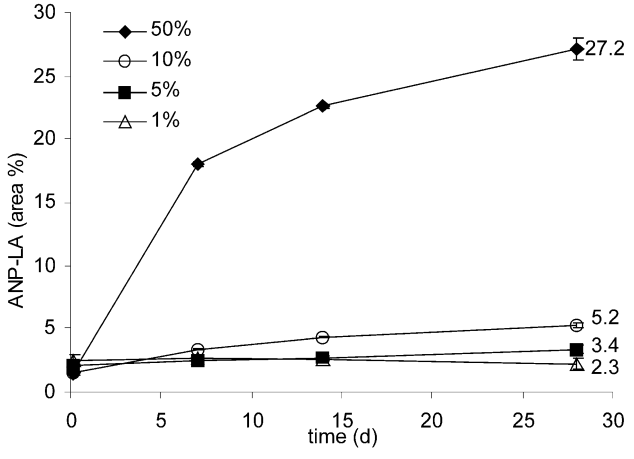

Fig. 6. Relative amount of ANP-LA in lactic acid solutions of different concentrations (1–50% (w/w)) under storage at 37°C. The values shown in the diagram represent the relative amount of ANP-LA after 28 days of incubation.

The composition of lactic acid solutions cited from the literature [7] (Table 1) had been determined by titration, assuming that only lactic acid and its dimers were present in solution. HPLC analysis demonstrated that a significant amount of higher oligomers consisting of up to eight lactic acid units was also present in solutions of higher lactic acid concentration. The results from titration and HPLC analysis yielded differences in the absolute oligomer content of the solutions. However, it became clear that even lactic acid-1% contained a small amount of oligomers. In future studies, the investigation of variations of different parameters, such as ionic strength or pH of the solutions, may lead to changes in oligomer content of the solutions. Assessing the oligomer content by HPLC and titration will allow for a comparison with the standard solutions described in the present study to evaluate the effect of the changed parameters on the composition of the solutions.

The investigations on ANP stability in lactic acid solutions demonstrated on the one hand, that with reduced oligomer content, peptide acylation was also reduced. On the other hand however, even at low oligomer concentrations, the acylation reaction was not suppressed entirely. It may thus be impossible to prevent peptide acylation in degrading PLA devices by simply enhancing the drainage of degradation products, e.g. by enhanced water uptake due to the incorporation of hydrophilic additives. When looking at the lowest purity value measured in degrading PLA microspheres [3], one can see that it corresponds to the purity of a solution of approx. 5% (w/w) total lactic acid concentration

Table 3
Osmotic pressure, purity values and dissociation grade of lactic acid solutions

Concentration		Osmotic pressure (mosmol/kg)		Sample purity (%)		Dissociation grade
Mol.%	% (w/w)	Theoretical	Determined	Theoretical	Determined	
0.00	0.00	0	0	100.0	100.0	–
0.43	2.11	234	205	99.6	99.7	−0.14
0.84	4.07	452	392	99.2	99.4	−0.17
1.23	5.89	653	580	98.8	99.0	−0.17
1.66	7.80	866	765	98.3	98.8	−0.19
2.01	9.31	1033	930	98.0	98.6	−0.18

(Table 3). In a 5% (w/w) lactic acid solution, an amount of 3.4% ANP-LA was found after 4 weeks of incubation at 37°C using the present model system. In PLA microspheres, approx. 55% ANP-LA had been found after 4 weeks of polymer degradation in an earlier study [2]. This may be explained by an inhomogeneous distribution of the oligomers in the degrading polymer matrix, leading to higher local concentrations of oligomers than reflected by the purity value. Additionally, in lactic acid solutions there is a relatively high amount of monomers present which contribute to the osmotic pressure of the solution, but are not reactive for peptide acylation. In degrading microspheres by contrast, there may be a much higher relative amount of oligomers present which are the primary products of polymer degradation and which may react with the incorporated peptides. Furthermore, inside the polymer matrix, incorporated peptides and oligomers stemming from polymer degradation are closer to each other than in diluted solutions, thus further increasing the probability of a reaction.

Although the prediction of absolute amounts of acylated peptides in miocrospheres was not possible, lactic acid solutions as a model system may provide valuable information on the efficacy of counter measures to peptide acylation.

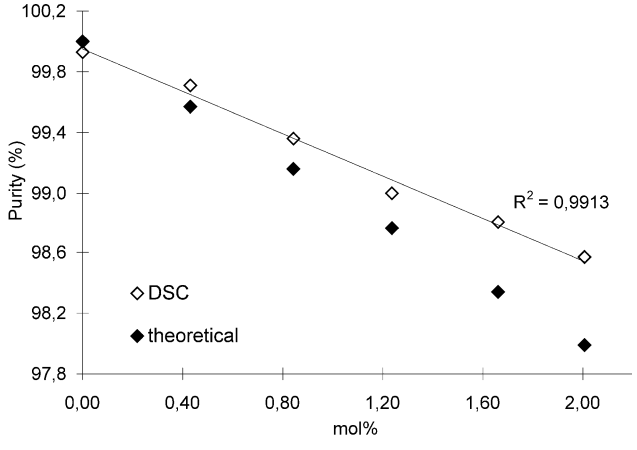

Fig. 7. Purity of lactic acid solutions (theoretical values and values determined by DSC purity analysis).

5. Conclusions

For investigations on peptide stability, lactic acid solutions are a suitable model system which can be prepared from concentrated lactic acid within a reasonable time. Lactic acid solutions allow for the variation of several parameters relevant to peptide acylation, such as oligomer concentration and pH, and will thus be helpful in the search for effective counter measures to peptide acylation in biodegradable delivery devices.

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