Peptide Acylation by Poly(α-Hydroxy Esters)

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Purpose. Poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) microspheres were investigated concerning the possible acylation of incorporated peptides.
Methods. Atrial natriuretic peptide (ANP) and salmon calcitonin (sCT) were encapsulated into PLA and PLGA microspheres. Peptide integrity was monitored by HPLC-MS analysis during microsphere degradation for four weeks. sCT fragmentation with endoproteinase Glu-C was used for identifying modified amino acids. Peptide stability in lactic acid solutions was investigated to elucidate possible mechanisms for preventing peptide acylation.
Results. Both peptides were acylated by lactic and glycolic acid units inside degrading microspheres in a time-dependent manner. After 21 days, 60% ANP and 7% sCT inside PLA microspheres were acylated. Fragmentation of sCT with endoproteinase Glu-C revealed that besides the N-terminal amine group, lysine, tyrosine or serine are further possible targets to acylation. Stability studies of the peptides in lactic acid solutions suggest that oligomers are the major acylation source and that lower oligomer concentration and higher pH substantially decreased the reaction velocity.
Conclusions. The use of PLA and PLGA for drug delivery needs substantially more circumspection. As, according to FDA standards, the potential hazards of peptide acylation products need to be assessed, our findings may have significant implications for products already on the market. Techniques to minimize the acylation reaction are suggested.

KEY WORDS: biodegradable polymer; poly(lactic acid); poly(lactic-co-glycolic acid); peptide stability; acylation.

INTRODUCTION

The current progress of genomics and proteomics gives new hope to find better treatments for diseases that range from Alzheimer's to cancer (1). The strategy of identifying new targets involved in a specific disease's pathobiochemistry will hopefully lead to the identification of numerous therapeutically relevant protein and peptide drugs in the near future. On their way from the lab bench to the bedside it seems a small and insignificant step to deliver such new substances to target cells and tissues. However, over the last three decades, much effort was taken to develop sophisticated drug delivery strategies specifically for this purpose (2). Their intention is to overcome the intrinsic problems of protein and peptide drugs that evolve from the short half-life in a biological environment.

Biodegradable polymer microparticles and implants that release proteins and peptides for days and weeks (3,4) and concomitantly protect the drug reservoir from enzymatic degradation, are among the most advanced concepts of protein and peptide delivery (5–7). Although this appears to be a straightforward, well established, and ready to use approach, protein and peptide delivery systems made of biodegradable polymers remain rare. Good examples are growth factors, which are precious drugs for regenerative medicine and tissue engineering applications (8,9). Numerous substances are still awaiting processing to controlled release devices to take full advantage of their biological potential (8–10). This progress may seem surprisingly slow as there are a number of polymers with excellent safety records and broad variability with respect to drug release control. Poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) (Fig. 1a) are such materials and, according to contemporary literature, extremely popular substances for the manufacturing of protein and peptide release systems (11,12).

However, although PLA and PLGA are the only FDA-approved biodegradable polymers in use for the manufacturing of commercially available protein and peptide depot systems in humans (11,13,14); a glance at the literature reveals that numerous problems evolve from their specific properties. The presence of moisture inside PLA and PLGA microspheres during storage and the accumulation of acidic polymer degradation products in degrading polymers are the main reasons for the instability of peptides and proteins and, subsequently, an obstacle to their controlled release from PLA and PLGA microspheres and implants (15). The accumulation of degradation products increases osmotic pressure and lowers pH inside degrading polymers, which has so far been discussed as being the major factor of protein and peptide instability (16,17). In contrast, chemical reactions between polymers and proteins, such as acylation reactions, remain undiscussed in any systematic stability study with PLA or PLGA, although acylation has been shown to play a major role with degrading polyanhydrides (18). As the acylation of peptides and proteins could have severe detrimental effects, such as a loss of activity (19,20), a change of receptor specificity (21,22) or of immunogenicity (20,22), it has to be carefully considered when developing controlled release systems for peptides and proteins. Our goal, therefore, was to scrutinize the possibility of an acylation reaction by investigating the chemical integrity of salmon calcitonin (sCT) and human atrial natriuretic peptide (ANP), two model substances (Fig. 1b), inside degrading PLA and PLGA microspheres.

MATERIALS AND METHODS

Materials

Poly(D,L-lactic acid) with equal amounts of D- and L-lactic acid units (PLA, Mw 17,000, Resomer R202) and poly-(lactic-co-glycolic acid) (PLGA, Mw 17,000, Resomer RG502) with a lactide/glycolide ratio of 50/50, both end-capped with ethanol, were obtained from Boehringer Ingelheim (Ingelheim, Germany). sCT (32 amino acids, Mw 3431.5) and ANP (30 amino acids, Mw 3080.5) were obtained from Novartis (Basel, Switzerland) and Suntory (Tokyo, Japan), respectively. Endoproteinase Glu-C for enzymatic pep-

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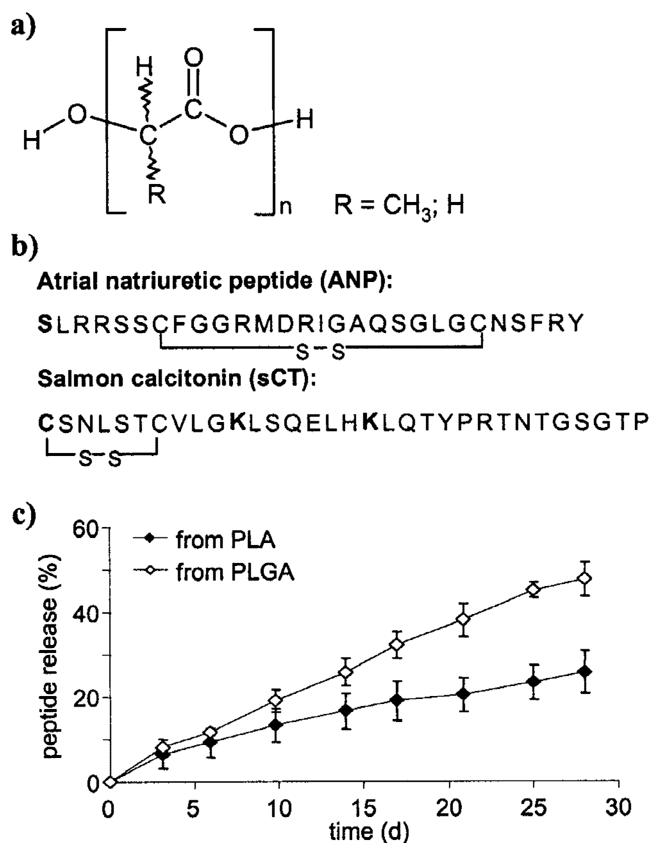

Fig. 1. (a) Chemical structure of poly(D,L-lactic acid) (PLA, R = CH_3) and poly(lactic-co-glycolic acid) (PLGA, R = H or CH_3). (b) Amino acid sequences of ANP and sCT (bold letters indicate amino acids carrying a free primary amine group). c) Profiles of ANP release from PLA (♦) and PLGA (◇) microspheres.

tide cleavage was purchased from Roche Molecular Biochemicals (Mannheim, Germany). All reagents were used in analytical grade or higher. Water used for the investigations was double-distilled and filtered through a 0.2 μm cellulose nitrate filter (Sartorius, Göttingen, Germany) before use.

Microsphere Degradation Studies

Microspheres with an average size of approx. 50 μm were manufactured using a double emulsion/solvent evaporation technique as described by Brunner *et al.* (16). The microspheres were frozen in liquid nitrogen and freeze-dried in a desiccator, which was cooled with dry ice and evacuated using a RV5 two-stage pump (Edwards, Crawley, Sussex, UK). When the pressure was as low as 10^{-2} mbar after approx. 12–18 h of drying, the dry ice was removed and the drying continued until the desiccator had reached room temperature at a pressure of 10^{-2} mbar. The microspheres were then stored under vacuum in a desiccator until use.

For the investigation of peptide release and stability, 80 mg microspheres were weighed into polypropylene tubes (Corning Costar, Bodenheim, Germany) and incubated in 2 ml isotonic phosphate buffer pH 7.4 containing 0.02% sodium azide. The samples were eroded under gentle shaking (frequency 30 min^{-1}) at 37 °C in a shaking water bath (model 1086 from GFL, Burgwedel, Germany). The buffer was changed at regular intervals and microsphere samples were taken after 7, 14, 21, and 28 days of degradation. After freeze-drying of the microspheres, all samples were stored at −80 °C until further analysis.

For the analysis of peptide stability inside polymer particles, the following extraction method was used which had been validated to yield an extraction efficiency of 100%. Microspheres (20 mg) were weighed into a 1.5 ml micro test tube (Eppendorf, Hamburg, Germany), dissolved in 600 μl of dichloromethane, and 600 μl of an acetonitrile/water/trifluoroacetic acid-mixture (15/85/0.1) were added. After mixing with 2,200 rpm on a Reax Control (Heidolph, Schwabach, Germany), the dispersion was allowed to settle at room temperature for 10 min before the dichloromethane phase was finally separated from the mixture by centrifugation at 200 g for 3 min (GS-15R, Beckman, Palo Alto, CA, USA). The upper fraction containing the extracted peptide was used for further analysis as described below.

Enzymatic Peptide Cleavage

sCT was digested enzymatically by adding Glu-C, an endoproteinase that cleaves peptides on the carboxyl side of glutamate. sCT extracted from microspheres after 28 days of degradation and native sCT, which served as a control, were submitted to the following protocol: 1 mg native sCT was dissolved in 950 μl ammonium carbonate buffer (25 mM, pH 7.8) containing urea (1 mM) and methyl amine (20 mM). In the case of sCT extracted from microspheres, 500 μl of the extracted peptide solution were diluted with 450 μl of the same buffer. In both cases, 50 μl bidistilled water containing 50 μg Glu-C were added. The reaction mixture was kept at room temperature for 30 min. 100 μl of the reaction mixture were diluted with 900 μl water and analyzed by HPLC under UV detection at 274 nm. Cleavage products were identified by HPLC-MS.

Peptide Stability Studies in Lactic Acid Solutions

Lactic acid solutions with defined oligomer content were obtained via dilution of concentrated lactic acid [89% (w/w)] and subsequent accelerated equilibration at 90 °C for 24 h to reach a stable amount of lactic acid oligomers (23). To obtain lactic acid solutions of pH 5, the pH was adjusted by adding sodium hydroxide prior to equilibration. For the peptide stability studies, ANP or sCT were dissolved in different lactic acid solutions [1%, 5%, 10%, and 50% (w/w)] with a peptide concentration of 20 μg/ml. The solutions were incubated at 37 °C and samples were collected at days, 0, 7, 14, and 28.

HPLC and HPLC-MS Analysis

Peptide samples were investigated by high-performance liquid chromatography (HPLC) analysis, using an HPLC system with a degasser (from Knauer, Berlin, Germany), LC-10AT pump, FCV-10AT_{VP} gradient mixer, SIL-10AD_{VP} autosampler, CTO-6A oven, SPD-10AV UV-detector, RF-551 fluorescence detector, and SCL-10A_{VP} controller (all from Shimadzu, Duisburg, Germany). For ANP analysis, a linear gradient of 20–40% acetonitrile in water [+0.1% trifluoroacetic acid (TFA)] for 20 min, and for sCT analysis a linear gradient of 30–45% acetonitrile in water (+0.1% TFA) for 20 min served as mobile phase at a flow rate of 1.0 ml/min. 100 μl of the samples were separated at 40 °C, using a combina-

tion of a C18-reversed phase precolumn (LC318, 4.6 × 50 mm) and analytical column (LC318, 4.6 × 250 mm) from Supelco (Deisenhofen, Germany). Chromatograms were recorded at 274 nm (UV detection) and at 274 nm/308 nm (excitation/emission), using the fluorescence detector. For the analysis of peptide cleavage products, a C4-reversed phase precolumn (LC304, 4.6 × 50 mm) and column (LC304, 4.6 × 250 mm, from Supelco, Deisenhofen, Germany) were used with the system settings described above. The mobile phase gradient was a step-gradient of 4–90% acetonitrile in water (+0.1% TFA) in five isocratic steps over 15 min.

For HPLC-mass spectrometry (HPLC-MS) analysis, the analytical methods were transferred to a Hewlett-Packard HPLC system with Series 1100 degasser, binary pump, autosampler, column oven, and diode array detector (all from Hewlett-Packard, Waldbronn, Germany), coupled with a TSQ7000 electrospray-mass spectrometer (ThermoQuest, San José, CA, USA) with API2-source (capillary temperature: 350 °C, spray voltage: 4.5 kV). The XCALIBUR® software package (ThermoQuest, San José, CA, USA) was used for data acquisition and analysis. Peptides were detected in the total ion chromatograms of the mass spectrometer. They were characterized concerning their molecular mass by analysis of their individual mass spectra. For semi-quantitative measurements, the triply-charged ions of the peptides and their acylation products were monitored in single-ion mode and the peak areas of the corresponding single-ion chromatograms were compared using the LCQUAN® tool of the XCALIBUR® software package.

RESULTS

Peptide Release from PLA and PLGA Microspheres

The release of ANP from PLA and PLGA microspheres followed almost zero order kinetics (Fig. 1c). After 4 weeks, approx. 25% of the incorporated peptide had been released from the PLA microspheres while approx. 50% had been released from PLGA microspheres. In contrast, less than 4% of sCT were released from PLA and PLGA microspheres (data not shown). Pronounced adsorption of sCT to PLGA microspheres has been described by Calis *et al.* (24) which may be one reason for the limited release of sCT from the microspheres. However, with the majority of the peptides still inside the microspheres, the system was ideal for the investigation of the peptide integrity under polymer degradation. During the monitored time-period of 4 weeks, polymer degradation of PLA and PLGA microspheres was still in the accumulation phase, i.e., degradation products were formed but not yet released from the matrix (25,26).

Peptide Stability inside Degrading PLA and PLGA Microspheres

To obtain information on the chemical structure of non-released peptides, ANP and sCT were extracted from microsphere samples for up to 28 days of degradation. ANP and sCT extracted from the microspheres before degradation served as a control. For these samples only one signal was detected in the total ion chromatogram of the mass spectrometer (TIC) at the retention time of native ANP (9.1 min, Fig. 2a) and native sCT (data not shown), respectively. In the corresponding mass spectra, several multiply-charged ions, $(M+2H)^{2+}$, $(M+3H)^{3+}$, and $(M+4H)^{4+}$, were detected (Fig. 2b). Additionally, cluster ions of multiply-charged peptides with trifluoroacetic acid from the mobile phase were detected. From the m/z values of the peptides, their average molecular masses were calculated to be 3080.5 amu in the case of ANP and 3431.5 amu in the case of sCT, indicating that the peptides remained unchanged in terms of their chemical integrity (Table I).

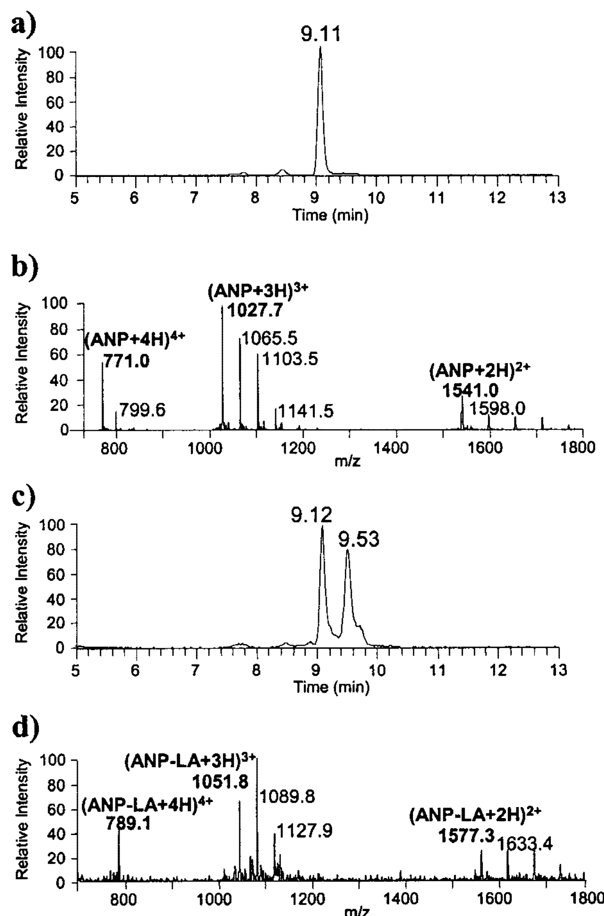

Fig. 2. (a) HPLC-MS total ion chromatogram (TIC) and (b) electrospray mass spectrum (retention time 9.1 min) of native ANP extracted from PLA microspheres after manufacturing, (c) TIC and d) electrospray mass spectrum (retention time 9.5 min) of ANP acylated by one lactic acid unit (ANP-LA) extracted from PLA microspheres after 21 days of degradation.

However, after the microspheres had been incubated in phosphate buffer at 37 °C, these results became subject to dramatic changes. In the case of ANP extracted from PLA microspheres after 3 weeks of degradation, an additional peak appeared in the TIC at a retention time of 9.5 min (Fig. 2c). From the multiply-charged ions in the mass spectrum at this retention time (Fig. 2d), a molecular mass of 3152.5 amu was calculated that would exactly match the sum of the molecular mass of the peptide chain (3080.5 amu) and one lactic acid molecule (72 amu). In the case of ANP extracted from PLGA, similar changes were observed. Besides the identification of ANP acylated by lactic acid units, triply-charged ions at m/z 1046.8 and 1066.1 were found at a retention time of 9.5 min, stemming from ANP carrying one (ANP-GA, Mw 3138.5 amu) or two molecules of glycolic acid (ANP-2GA, Mw 3196.5 amu) (Table I).

Table I. Molecular Masses and Mass Per Charge Ratios (m/z) of Multiply Charged Ions ($(M+nH)^{n+}$) of the Model Peptides and Their Acylation Products

Peptide	Molecular mass (amu)	m/z (amu)		
		$(M+2H)^{2+}$	$(M+3H)^{3+}$	$(M+4H)^{4+}$
ANP	3080.5	1541.3	1027.8	771.1
ANP-LA[a] (+72 amu)	3152.5	1577.3	1051.8	789.1
ANP-GA[a] (+58 amu)	3138.5	1570.3	1047.2	785.6
ANP-2GA[a] (+116 amu)	3196.5	1599.3	1066.5	800.1
sCT	3431.5	1716.8	1144.8	858.9
sCT-LA[a] (+72 amu)	3503.5	1752.8	1168.8	876.9
sCT-GA[a] (+58 amu)	3489.5	1745.8	1164.2	873.4
sCT-2GA[a] (+116 amu)	3547.5	1774.8	1183.5	887.9

[a] -LA, -GA and -2GA indicate the acylation of the peptide by one lactic acid unit or by one or two glycolic acid units, respectively.

Similar results were obtained for sCT. Peptide derivatives extracted from degraded PLA microspheres with a molecular mass of 3503.5 amu indicated an attachment of lactic acid units to sCT. From PLGA microspheres, additionally, sCT derivatives were extracted with an increased molecular mass that correlates with the molecular mass of one or two glycolic acid units (Mw 3489.5 amu and 3547.5 amu, Table I).

Peptide Stability during Microsphere Degradation

Based on these results, we suspected that the peptides might have been acylated during polymer degradation. To prove that the observed changes were related to polymer degradation and to a chemical reaction we investigated the kinetics of the process. Therefore, peptides were extracted from the microspheres after different times of degradation and investigated by HPLC-MS analysis. In Figs. 3a and 3b, the relative ANP and ANP-LA content of PLA and PLGA microspheres is shown, demonstrating that the ANP-LA content increases continuously along with the degradation time. The conversion in PLA microspheres seemed to be faster (Fig. 3a) than in PLGA microspheres (Fig. 3b). However, the additional attachment of glycolic acid units in PLGA microspheres was not considered for this analysis and would further increase the amount of altered peptide. In Figs. 3c and 3d, the relative sCT-LA content in PLA (Fig. 3c) and PLGA microspheres (Fig. 3d) is displayed. The reaction product content changed again continuously with time, but was substantially slower than with ANP under these conditions. These results were a first hint that the peptides were indeed undergoing a chemical reaction leading to the covalent attachment of monomers.

Based on the assumption that the molar extinction coefficients of ANP-LA and sCT-LA at 274 nm do not differ substantially from those of the parent peptides, the amount of the acylated peptides can be calculated from the peak areas of the UV chromatograms at 274 nm. Like this, after 21 days of microsphere degradation, 60% ANP and 7% sCT inside PLA microspheres were found to be acylated. However, if the tyrosine side chain was an acylation target, the basic assumption of unchanged specific extinction may not be valid due to the contribution of tyrosine to UV absorption at 274 nm.

Identification of Reaction Sites in the Peptide Chain

To identify amino acids that are potential binding partners for an acylation reaction, sCT was cleaved enzymatically. By incubation with Glu-C we selectively cleaved the peptide chain between amino acids 15 and 16. Two fragments of Mw 1579.5 amu [sCT(1–15)] and 1870.0 amu [sCT(16–32)] were detected by HPLC-MS after cleavage of native sCT (Fig. 4a/b). Glu-C-treated sCT extracted from PLA microspheres after 28 days of degradation contained peptide derivatives of these fragments, each carrying one lactic acid unit (Fig. 4c). Enzymatic cleavage of sCT extracted from PLGA microspheres after the same degradation time yielded additional fragments acylated mainly with glycolic acid units. From these results, we concluded that besides the N-terminal amine group, lysine, tyrosine, and serine could be targets for the acylation by polymer units.

Identification of Acylation Sources

To differentiate between the two potential sources of acylation, namely low molecular mass degradation products such as monomers and oligomers, and the polymer surface, we investigated the stability of peptides in lactic acid solutions. Concentrated lactic acid [approx. 90% (w/w)] contains approx. 36% oligomers, such as dimers and higher oligomers (23). Even diluted lactic acid [1% (w/w)] still contains approx. 0.01% oligomers (23). ANP and sCT were, therefore, incubated at 37 °C in lactic acid solutions of different concentrations [1–50% (w/w)] at pH 2 and pH 5. Samples were regularly analyzed for acylation products over 4 weeks using HPLC-MS analysis. Acylation products of both peptides with lactic acid units were detected in all samples. Figure 5a shows the relative amount of sCT-LA which increased with increasing lactic acid concentration. At pH 5, the amount of acylated sCT was significantly reduced compared to the solutions at pH 2 (Fig. 5b). Similar results were obtained for ANP (data not shown).

DISCUSSION

The analysis of ANP and sCT extracted from PLA and PLGA microspheres after 3 weeks of degradation revealed that some peptide molecules had apparently undergone structural changes. Besides the presence of unchanged peptides, HPLC-MS analysis revealed the existence of derivatives with a molecular mass increased by 72 amu, 58 amu, and 116 amu. These molecular mass changes were indicative of an acylation of nucleophiles inside the peptide chain by lactic and glycolic acid units. Non-covalent interactions would have led to other

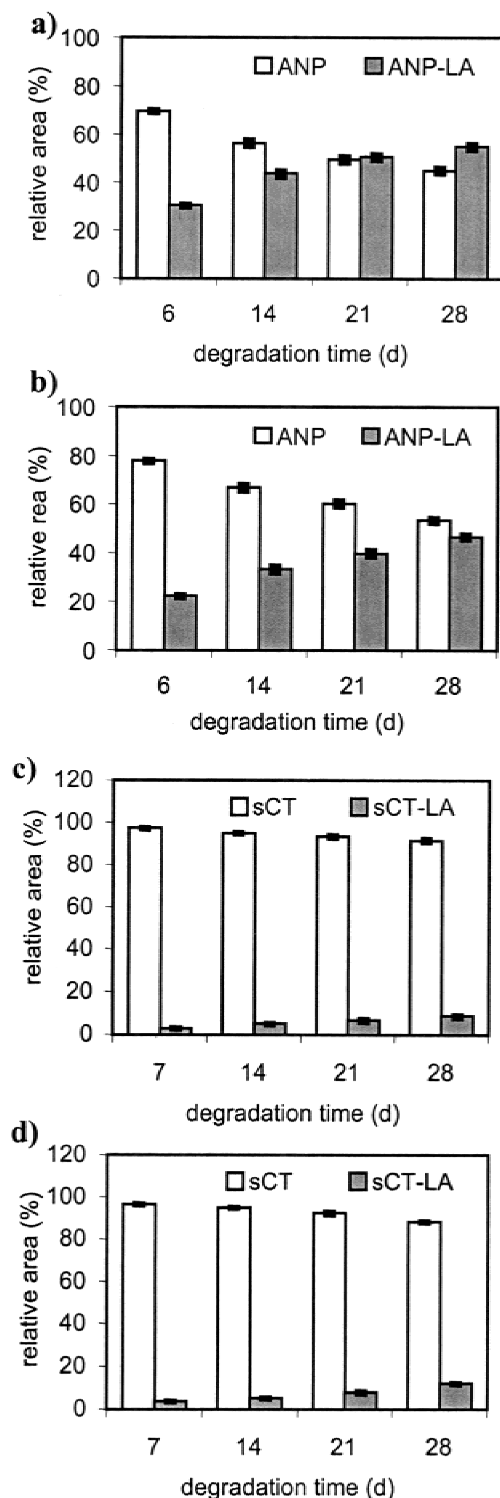

Fig. 3. ANP and ANP-LA extracted from (a) PLA and (b) PLGA microspheres, and sCT and sCT-LA, extracted from (c) PLA and (d) PLGA microspheres after 6 or 7, 14, 21, and 28 days of microsphere degradation in phosphate buffer pH 7.4.

mass differences, e.g., to a mass increase by 90 amu and 76 amu in case of ionic interactions between a peptide and lactic and glycolic acid, respectively. To support our hypothesis, we investigated the kinetics of this process. We observed that the content of acylation products increased continuously with time compared to the content of native peptide (Fig. 3), which indicates that the changes were the result of a chemical reaction related to polymer degradation. The breakdown of the polymer backbone, or at least the presence of water seems, thereby, to be a prerequisite for the reaction as no acylated peptides were found in undegraded microspheres after three months of storage in a desiccator (Fig. 2a/b).

As a next step we identified the reaction sites in the peptide chain. Amino acids that are a target for an acylation need to carry nucleophilic functional groups, such as primary amines (18). If unmodified, the N-terminal amine group of a peptide could act as a nucleophile for the acylation reaction. However, experiments with Glu-C suggested that other nucleophiles in the peptide chain were also acylated. Thus, the cleavage experiments with the endoproteinase revealed that both sCT fragments were acylated (Fig. 4c). Therefore, the amine group of lysine or the hydroxy groups of tyrosine or serine can be considered as additional acylation targets.

After we had identified amine and hydroxy groups as potential reaction partners in the peptide chain, we tried to identify the acylation source. There were two options: the surface of the degrading polymer itself with the acylation taking place at the interface between insoluble polymer and peptide-rich hydrated areas, or oligomers that stem from polymer degradation and are known to accumulate inside degrading microspheres (27,16). To investigate the role of oligomers, we tested the stability of peptides in aqueous lactic acid solutions. The results showed that the peptides were acylated in a concentration-dependent manner (Fig. 5), from which we were able to conclude that the degradation products alone could be the source of acylation. The impact of polymer surfaces on the process, however, remains unclear and will be the subject of future investigations. Furthermore, the results suggest that a fast release of degradation products from PLA or PLGA drug delivery systems would be highly desirable. First empirical observations with poly(ethylene glycol) as a porogen seem to confirm positive effects on protein stability (28). Another approach that was discussed for the stabilization of proteins in PLGA devices is the induction of a higher pH in the degrading polymer (17). The reduced amount of acylated peptides that was found in lactic acid solutions at pH 5 confirms that raising the pH inside eroding PLA or PLGA by adding bases might be a useful approach to prevent peptide acylation.

pH also appears to play a major role in the mechanism of the acylation reaction. Recently, a degradation mechanism for lactic acid oligomers at acidic pH was proposed, involving the acylation of a nucleophile by the lactate end-group at the hydroxy end of the PLA chain (29). Usually, the reaction involves water as a reaction partner and results in polymer hydrolysis. During the reaction with peptides, nucleophiles in the peptide chain may become involved in this reaction as shown in Fig. 6. The different extent to which ANP and sCT were acylated in our experiments illustrates the importance of the peptide structure. That proteins undergo the same modifications when exposed to PLA or PLGA is supported by the fact that they usually contain even more binding sites that are frequently used for reactions with other nucleophiles, such as amine reactive dyes for labeling purposes (30).

The implications of our findings for the manufacturing of drug delivery systems for proteins and peptides using biodegradable polymers such as PLA and PLGA are of paramount importance. First of all, the acylation of peptides and proteins

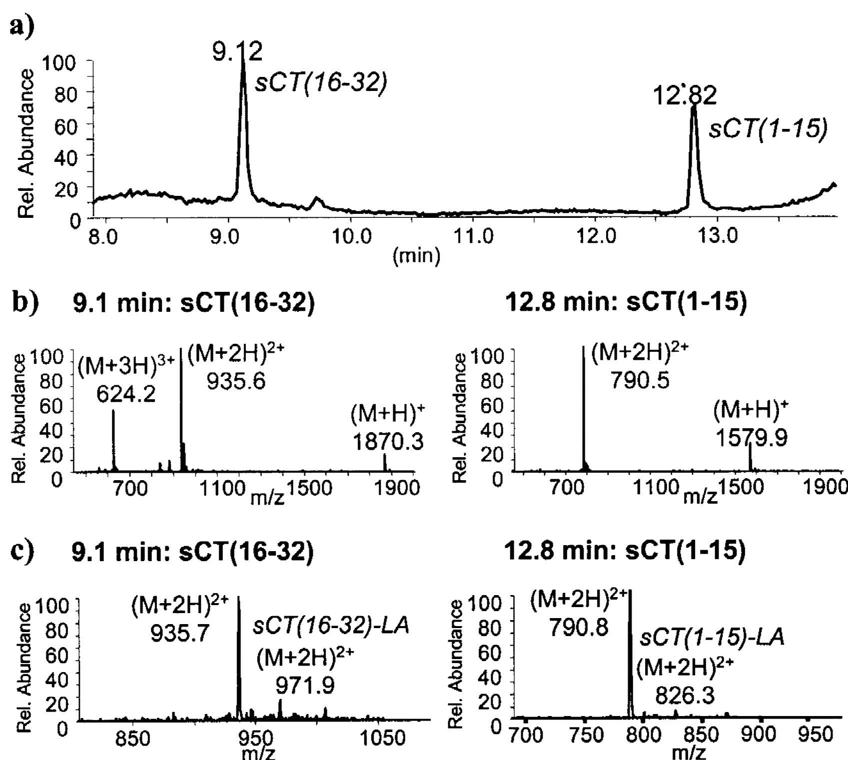

Fig. 4. (a) TIC of the two sCT fragments obtained by enzymatic cleavage of native sCT with Glu-C. (b) Electrospray mass spectra of fragments of native sCT at retention times 9.1 min (sCT(16-32)) and 12.8 min (sCT(1-15)). (c) Electrospray mass spectra of fragments of sCT extracted from microspheres after 28 days of degradation at retention times 9.1 min (additional ion representing sCT(16-32)-LA) and 12.8 min (additional ion representing sCT(1-15)-LA).

poses a severe threat to their stability inside PLA and PLGA and can affect important properties such as biological activity, immunogenicity, and toxicity (19–22). This is, for example, of utmost importance to all those in the field that currently use these polymers for their safety record to deliver growth factors in tissue engineering applications. More problems evolve from the fact that the amount of acylated peptides we found can be considered an impurity that has to be investigated for toxicity and adverse pharmacological effects. [ICH (07/1996) Guideline for industry: Q5C: Quality of biotechnological products: stability testing of biotechnological/biological products. ICH (11/1997) Guidance for industry: Q3B: Impurities in new drug products]. As the mechanism that we revealed had been unknown up until this point, there are several PLA- and PLGA-based delivery systems for proteins and peptides on the market that have possibly not undergone such testing. First investigations of commercial products such as for goserelin release from PLGA indicate that the peptide was also subject to acylation by lactic and glycolic acid units (data not shown). The fact that PLA and PLGA are the only approved degradable polymers in use for the manufacture of parenteral protein and peptide release systems for humans, will render the formulation of drug delivery devices on the basis of degradable polymers even more complicated in the future.

Despite these problems, a number of counter measures appear to be promising. Increased pH or an enhancement of monomer release seem, at a first glance, to be suitable parameters to slow down and hopefully suppress the acylation

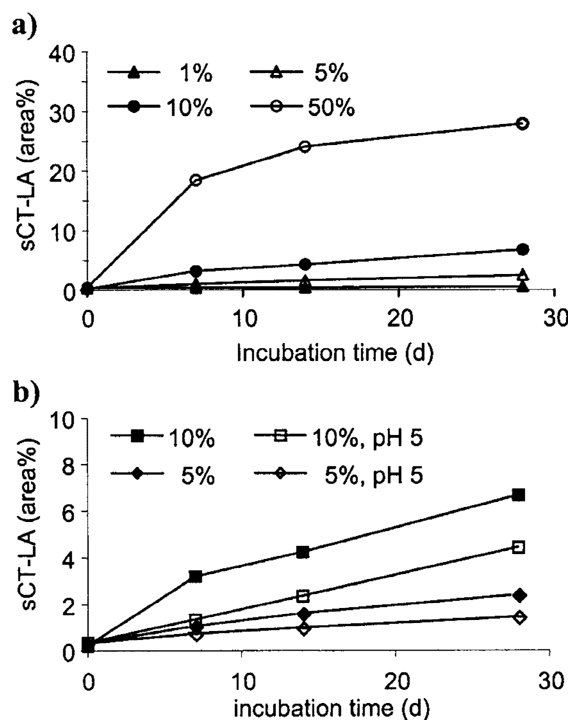

Fig. 5. Relative amounts of acylated sCT after incubation in lactic acid solutions: (a) as a function of lactic acid concentration, (b) as a function of lactic acid concentration and pH.

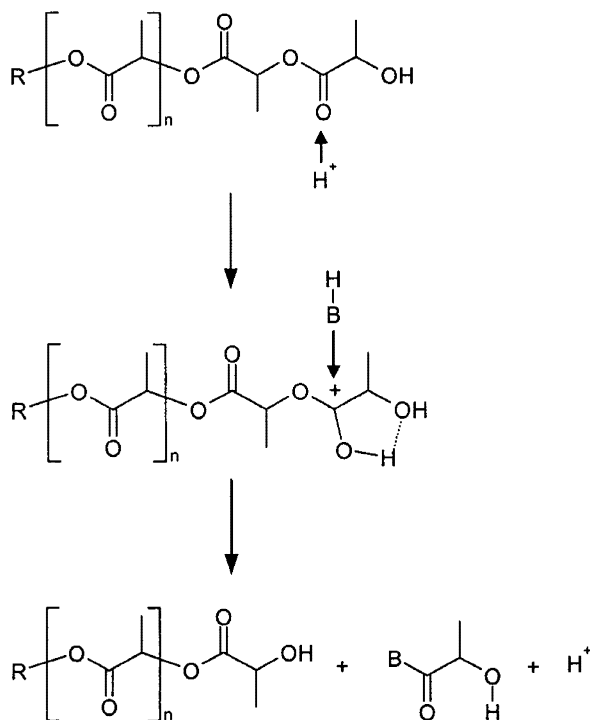

Fig. 6. Proposed reaction mechanism of acid catalyzed polymer degradation involving the acylation of a target. "BH" represents a nucleophile or water.

process. However, substantially more studies will be needed to evaluate the usefulness of these and other approaches.

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REFERENCES

1. D. Bailey, E. Zanders, and P. Dean. The end of the beginning for genomic medicine. *Nature Biotechnology* **19**:207–209 (2001).
2. R. Langer. New methods of drug delivery. *Science* **249**:1527–1533 (1990).
3. H. Okada. One- and three-month release injectable microspheres of the LH-RH superagonist leuprorelin acetate. *Adv. Drug Deliv. Rev.* **28**:43–70 (1997).
4. P. J. Camarata, R. Suryanarayanan, D. A. Turner, R. G. Parker, and T. J. Ebner. Sustained release of nerve growth factor from biodegradable polymer microspheres. *Neurosurgery* **30**:313–319 (1992).
5. S. D. Putney and P. A. Burke. Improving protein therapeutics with sustained-release formulations. *Nat. Biotechnol.* **16**:153–157 (1998).
6. S. P. Schwendeman, M. Cardamone, M. R. Brandon, A. Klibanov, and R. Langer. Stability of proteins and their delivery from biodegradable polymer microspheres. In S. Cohen and H. Bernstein (eds.), *Microparticulate Systems for the Delivery of Proteins and Vaccines,* Marcel Dekker, New York, 1996 pp. 1–49.
7. J. L. Cleland, A. Mac, B. Boyd, J. Yang, E. T. Duenas, D. Yeung, D. Brooks, C. Hsu, H. Chu, V. Mukku, and A. J. Jones. The stability of recombinant human growth hormone in poly(lactic-co-glycolic acid) (PLGA) microspheres. *Pharm. Res.* **14**:420–425 (1997).
8. J. E. Babensee, L. V. McIntire, and A. G. Mikos. Growth factor delivery for tissue engineering. *Pharm. Res.* **17**:497–504 (2000).
9. K. Y. Lee, M. C. Peters, K. W. Anderson, and D. J. Mooney. Controlled growth factor release from synthetic extracellular matrices. *Nature* **408**:998–1000 (2001).
10. W. M. Saltzman. Growth factor delivery in Tissue Engineering. *MRS Bulletin* **21**:62–65 (1996).
11. R. T. Bartus, M. A. Tracy, D. F. Emerich, and S. E. Zale. Sustained delivery of proteins for novel therapeutic drugs. *Science* **281**:1161–1162 (1998).
12. W. R. Gombotz and D. K. Pettit. Biodegradable polymers for protein and peptide drug delivery. *Bioconjug. Chem.* **6**:332–351 (1995).
13. S. Cohen and H. Bernstein. *Microparticulate Systems for the Delivery of Proteins and Vaccines,* Marcel Dekker, New York, 1996.
14. S. P. Baldwin and W. M. Saltzman. Materials for protein delivery in tissue engineering. *Adv. Drug Deliv. Rev.* **33**:71–86 (1998).
15. M. van de Weert, W. E. Hennink, and W. Jiskoot. Protein stability in poly(lactic-co-glycolic acid) microparticles. *Pharm. Res.* **17**:1159–1167 (2000).
16. A. Brunner, K. Mäder, and A. Göpferich. pH and osmotic pressure inside biodegradable microspheres during erosion. *Pharm. Res.* **16**:847–853 (1999).
17. G. Zhu, S. R. Mallery, and S. P. Schwendeman. Stabilization of proteins encapsulated in injectable poly(lactide-co-glycolide). *Nature Biotechnology* **18**:52–57 (2000).
18. A. J. Domb, L. Turovsky, and R. Nudelman. Chemical interactions between drugs containing reactive amines with hydrolyzable insoluble biopolymers in aqueous solutions. *Pharm. Res.* **11**: 865–868 (1994).
19. S. A. Kidwai, A. A. Ansari, and A. Salahuddin. Effect of succinylation (3-carboxypropionylation) on the conformation and immunological activity of ovalbumin. *Biochem. J.* **155**:171–180 (1976).
20. M. A. Qasim and A. Salahuddin. Changes in conformation and immunological activity of ovalbumin during its modification with different acid anhydrides. *Biochim. Biophys. Acta* **536**:50–63 (1978).
21. S. Murase, N. Yumoto, M. G. Petukhov, and S. Yoshikawa. Acylation of the alpha-amino group in neuropeptide Y(12–36) increases binding affinity for the Y2 receptor. *J. Biochem. Tokyo* **119**:37–41 (1996).
22. S. P. Schwendeman, H. R. Costantino, R. K. Gupta, M. Tobio, A. C. Chang, M. J. Alonso, G. R. Siber, and R. Langer. Strategies for stabilising tetanus toxoid towards the development of a single-dose tetanus vaccine. *Dev. Biol. Stand.* **87**:293–306 (1996).
23. C. Holten, A. Müller, and D. Rehbinder. *Lactic acid: properties and chemistry of lactic acid and derivatives,* Verlag Chemie, Weinheim, 1971.
24. S. Calis, R. Jeyanthi, T. Tsai, R. C. Mehta, and P. P. DeLuca. Adsorption of salmon calcitonin to PLGA microspheres. *Pharm. Res.* **12**:1072–1076 (1995).
25. A. Göpferich. Polymer bulk erosion. *Macromolecules* **39**:2598–2604 (1997).
26. F. von Burkersroda, L. Schedl, and A. Göpferich. Why degradable polymers undergo surface erosion or bulk erosion. *Biomaterials submitted* (2001).
27. A. Göpferich. Polymer bulk erosion. *Macromolecules* **39**:2598–2604 (1997).
28. W. Jiang and S. P. Schwendeman. Stabilization and controlled release of bovine serum albumin encapsulated in poly(D,L-lactide) and poly(ethylene glycol) microsphere blends. *Pharm. Res.* **18**:878–885 (2001).
29. S. J. De Jong, E. R. Arias, D. T. S. Rijkers, C. F. Van Nostrum, J. J. Kettenes-Van den Bosch, and W. E. Hennink. New insights into the hydrolytic degradation of poly(lactic acid): participation of the alcohol terminus. *Polymer* **42**:2795–2802 (2001).
30. R. P. Haugland. *Handbook of fluorescent probes and research chemicals,* Molecular Probes Inc., Eugene, Oregon, USA, 1996.