pH and Osmotic Pressure Inside Biodegradable Microspheres During Erosion[1]

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Purpose. To measure changes in pH as well as osmotic pressure in aqueous pores and cavities inside biodegradable microspheres made from polymers such as poly(D,L-lactic acid) (PLA) and poly(D,L-lactic acid -*co*- glycolic acid) (PLGA).

Methods. The internal osmotic pressure inside eroding PLA microspheres was analyzed with differential scanning calorimetry (DSC) in a temperature range of 10 to −25°C. The osmotic pressure was calculated from the melting peaks of the aqueous phase using purity analysis. For pH determination, PLGA microspheres were loaded with a pH-sensitive spin probe which allowed the determination of pH by electron paramagnetic resonance (EPR).

Results. The osmotic pressure in PLA microspheres increased to 600 mOsm within four days and decreased to 400 mOsm after two weeks. The pH in PLGA microspheres in this study was ≤4.7. Basic drugs such as gentamicin free base or buffering additives led to a pH increase. In no case, however, did the internal pH exceed a value of 6 within 13 hours.

Conclusions. DSC and EPR are useful techniques to characterize the chemical microenvironment inside eroding microspheres. This data in combination with detailed information on peptide and protein stability could allow in the future to predict the stability of such compounds within degradable polymers.

KEY WORDS: biodegradable polymers; pH; osmotic pressure; protein stability; peptide stability.

INTRODUCTION

During the last twenty years microspheres made of biodegradable polymers such as PLA or PLGA have become an attractive dosage form for the delivery of peptide and protein drugs (1–6). They improve the bioavailability of peptides and proteins by protecting them against inactivation in vivo and concomitantly replace multiple injections or continuous infusions which are necessary due to the short half lives of these substances. Despite tremendous research efforts, however, there are only a few protein or peptide loaded microsphere preparations commercially available so far. We hypothesize that insufficient stability of peptides and proteins inside eroding microspheres is one of the problems that may account for this (7,8).

For example, during the erosion of PLGA polymer matrices, acidic oligomers and monomers lead to a pH-drop (9). Under certain conditions, such as low drug contents, microspheres may tend to be a closed system and, as degradation products, can not freely diffuse out of the microsphere matrix thus, a shift towards an acidic microclimate inside the eroding microspheres can be assumed (10). We expected the accumulation of degradation products inside microspheres would not only lead to a decrease of the pH inside aqueous pores and cavities, but also to a rise in osmotic pressure. Both changes may affect the stability of peptides and proteins (11). The values for pH and osmotic pressure are valuable parameters in predicting the stability of drugs intended for encapsulation into microspheres. Unfortunately, our knowledge of the conditions that prevail inside degrading particles is limited by difficulties in the analytical assessment of these parameters. Therefore, the goal of our work was to develop and apply methods that allow the assessment of the chemical environment inside eroding microspheres.

The development of pH-sensitive nitroxides has permitted the non-invasive pH-measurement by electron paramagnetic resonance (EPR) (12). EPR has been used to study pH-changes in biodegradable PLGA-implants in vivo (9) and to detect pH-gradients inside eroding polyanhydrides in vitro (13). The possibility of the assessment of the microacidity inside degrading microparticles by EPR has been demonstrated recently (14). However, little data have been published on the influence of basic or buffering additives on the pH inside degrading microparticles (15). Therefore, one goal of the present study was to provide experimental data about additive-induced changes of the microenvironment. To our knowledge, the measurement of osmotic pressure inside degrading microparticles has yet to be considered. To measure the osmotic pressure, a special technique was developed using differential scanning calorimetry (DSC) and evaluation of DSC data based on the van't Hoff equation (17). The knowledge of pH and osmotic pressure values inside eroding microspheres may become one of the key parameters in the design of biodegradable microspheres and will avoid cost-intensive development of polymer drug delivery systems.

MATERIALS AND METHODS

Materials

Poly (D,L-lactic acid) (PLA Mw 2,000; Resomer® R104) and poly (D,L-lactic acid-co-glycolic acid) (PLGA Mw 14,000; Resomer® RG502 and PLGA Mw 8,000, Resomer® RG502H) were obtained from Boehringer Ingelheim (Ingelheim, Germany). 98% hydrolyzed poly(vinyl alcohol) (Mw 13,000–23,000) from Aldrich (Milwaukee, Wisconsin, USA) was used as a surfactant. The hydrophilic spin probe 4-amino-2,2,5,5-tetramethyl-3-imidazoline-1-yloxy (AT) was obtained from the Institute of Organic Chemistry of the Russian Academy of Sciences in Novosibirsk (Russia). All other chemicals were purchased in analytical grade from Sigma (St. Louis, Missouri, USA).

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Methods

Preparation of Microspheres for Measurements of Osmotic Pressure

Microspheres were prepared by using a modified solvent evaporation technique. 300 mg polymer was dissolved in 1 ml methylene chloride. 200 µl double distilled water was emulsified therein under sonication for 90 seconds with a Branson Sonifier B-12 (Branson, Carouge-Genève, Switzerland). After adding 2 ml of 1% aqueous poly(vinyl alcohol) solution under vortex mixing, a multiple emulsion formed spontaneously and was poured into a 0.3% poly(vinyl alcohol) solution. The organic solvent was evaporated for 2 hours. The resulting microspheres were collected by centrifugation at 1500 rpm (centrifuge Centrikon T-42C, Kontron, Neufahrn), washed with distilled water and freeze dried.

DSC-Analysis

The microspheres were incubated in 0.1 M phosphate buffer pH 7.4 for 14 days at 37°C. The buffer was replaced daily to maintain sink conditions with respect to the release of degradation products. At regular times three samples of 2–3 mg microspheres were collected and placed directly at the bottom of an aluminum DSC-pan. The water on the surface was removed with Kim-wipes.® Afterwards, the pans were tightly sealed (encapsulating Press P/N5141 from Polymer Laboratories, Walldorf, Germany), weighed and frozen at −25°C until analysis. DSC thermograms were taken on a PL-DSC (Module ETIA, CCI-III, V5.00, Polymer Laboratories, Walldorf, Germany), with a programmable gas interface and cryogenic cooling system. Each sample was stored in a desicator at room temperature for 30 minutes and then analyzed in triplicate with a temperature scan from 10°C to −25°C and back to 10°C using a heating rate of 5 C/min. The obtained raw data was corrected for the time lag due to heat transfer to the sample using the purity analysis software provided by the DSC manufacturer. To correlate the obtained purity values with osmotic pressure, sodium chloride solutions of various molarity were analyzed using the same DSC protocol. The resulting relation between purity and molarity was used as calibration curve for microsphere data.

Preparation of Microspheres for pH Measurements

50 µmol of the hydrophilic spin probe AT were incorporated per g PLGA microspheres using the solvent evaporation technique described above. Acetic acid anhydride was used as solvent for the organic phase. Different batches of microspheres were prepared: a) with no further additives in the inner water phase, b) with addition of 1.5 mg gentamicin free base, and c) with addition of 1.87 M sodium acetate buffer pH9. Amines such as gentamicin are thereby most likely protected against reaction with acetic anhydride (16) by the protonation through acetic acid which is created during acetic anhydride hydrolysis. Sodium acetate was added in a concentration high enough to neutralize all acetic acid from acetic anhydride hydrolysis calculated on the assumption of total (100%) hydrolysis. The batch was used as a control to make sure measured pH values were not influenced by traces of acetic acid.

pH-Measurements in Microspheres with EPR

pH-calibration curves for AT in different buffer systems (Sörensen-phosphate buffer, Sörensen citrate buffer, McIlvaine buffer) were obtained at 2.1 GHz (S-band), 9.4 GHz (X-band), and 1.1 GHz (L-band). The different microsphere batches were examined in the dry state and after incubation in buffer for eight hours. EPR spectra were taken at regular time intervals. Microspheres were measured at 2.1 GHz in 2 ml Eppendorf centrifuge tubes, which served as incubation vessels. To make sure that EPR signals stem from the inside of microspheres and not the surrounding buffer medium containing released AT, the particles were centrifuged and resuspended repeatedly in fresh buffer. The supernatant was examined for EPR signals, and the measurements on microspheres were started after no more EPR signals could be obtained from the supernatant. This made sure that the pH values measured stemmed from the inside of microspheres only. As the EPR signal from the microspheres did not allow for spatial resolution, all pH values are average values which may be subject to local variations. To account for the release of spin probe during an erosion experiment, the measurements were performed at spin probe concentrations at which the pH is independent from the signal intensity. EPR spectra were taken using a MT 1 EPR spectrometer with a surface coil (Magnettech GmbH, Berlin, Germany), which allowed the measurement to be performed directly in the incubation tubes.

RESULTS

The osmotic pressure during microsphere erosion was determined indirectly by measuring melting point depressions of the free water inside microspheres (18). The direct measurement of the melting point depression would require a high sensitivity and reproducibility, which is difficult to achieve (19). To avoid this problem, melting point depressions were determined by evaluation of DSC melting peaks with a technique called purity analysis. For quantitative evaluations a detailed peak shape analysis is necessary, which can be performed by special software packages.

The algorithms of these software packages are based on the thermodynamics of reactions (17): Under equilibrium conditions and during a reversible process (such as melting), the work done is the maximum work possible. The change in free energy is:

$$\Delta G = G_p - G_e = \Delta G_0 + RT\ln\frac{a_p}{a_e} \qquad (1)$$

where a_e and a_p represent the arbitrary activities of educts and products, and ΔG_0 is the standard free energy. Considering ΔG equals zero for a system in equilibrium, such as during melting equation 1 simplifies to equation 2 in which a_1^L and a_1^S express the activities of the melting substance 1 in the liquid and the solid phases:

$$\Delta G_0 = -RT\ln\frac{a_1^L}{a_1^S} \qquad (2)$$

By differentiation of equation 2 with respect to temperature at constant pressure p, one obtains:

$$\left(\frac{\partial \ln\left(\frac{a_1^L}{a_1^S}\right)}{\partial T}\right)_p = -\frac{1}{R}\left(\frac{\partial\left(\frac{\Delta G_0}{T}\right)}{\partial T}\right)_p \quad (3)$$

At this stage the Gibbs-Helmholtz equation, which correlates the change in free energy with the reaction enthalpy ΔH_0, can be introduced:

$$\left(\frac{\partial\left(\frac{\Delta G_0}{T}\right)}{\partial T}\right)_p = \frac{-\Delta H_0}{T^2} \quad (4)$$

By substitution of equation 4 into equation 3 the van't Hoff equation is obtained:

$$\left(\frac{\partial \ln\left(\frac{a_1^L}{a_1^S}\right)}{\partial T}\right)_p = \frac{\Delta H_0}{RT^2} \quad (5)$$

Assuming the molar heat of fusion ΔH_0 is constant over the temperature range of interest, integration of equation 5 yields:

$$\ln\left(\frac{a_1^L}{a_1^S}\right) = \frac{\Delta H_0}{R} \cdot \frac{T_0 - T_m}{T^2} \quad (6)$$

T_m is the sample temperature after equilibration and T_0 represents the melting point of the pure component and $T_0 - T_m$ is the melting point depression. Rearranging equation 6 gives:

$$T_m = T_0 - \frac{RT^2}{\Delta H_0} \ln\left(\frac{a_1^L}{a_1^S}\right) \quad (7)$$

The following assumptions allow further simplification of equation 7:

1. The system contains only one impurity, with small values of activity a_2. It follows that, $\ln a_1^L = \ln(1 - a_2^L)$, and if $a_2^L << 1$, $\ln(1 - a_2^L) \approx a_2^L$.
2. The activity of the impurity in the solid phase equals zero, leading to $\ln a_1^S = \ln(1 - a_2^S) \approx 0$,
3. In the case of dilute solutions activities can be replaced by concentrations, $a_2^L \approx x_2^L$
4. $T_0 \approx T_m = T$;
5. Above the eutectic temperature, $x_2^L = x_2 \frac{1}{f}$, where f represents the molten fraction at the corresponding sample temperature.

With these assumptions, equation 7 simplifies to:

$$T_m = T_0 - \frac{RT_0^2 x_2}{\Delta H_0} \cdot \frac{1}{F} \quad (8)$$

$T_0 - T_m$ represents the melting point depression, which is a function of the molar gas constant, the melting point of the pure solvent T_0, the melting point of the solution T_m, the mole fraction of impurity x_2, and the heat of fusion ΔH_0. Assuming that the impurity is 1 mol% or less the melting point depression moves in a narrow range of some tenth degree Kelvin. Equation 8 suggests that plotting T_m versus 1/F gives a straight line whereby x_2 can be calculated from the slope. Due to thermal lags within the DSC system, however, the data obtained by DSC measurements are concave curves and do not necessarily fit the straight line. These curves can be linearized graphically or by automated software packages (Fig. 1).

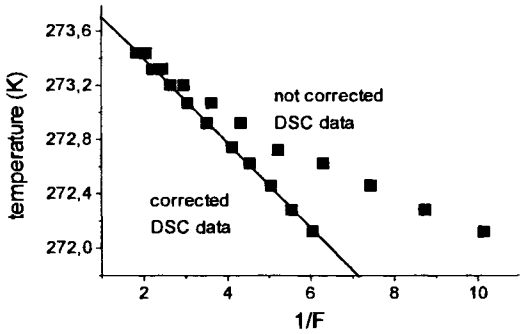

Fig. 1. Scheme for graphical linearization of 1/F plot.

To prove purity values and osmotic pressure are related, we investigated sodium chloride solutions of different molarity. The purity of sodium chloride solutions is a linear function of the molar concentration as shown in Fig. 2. The values of molarity can be transformed into osmotic pressures by multiplication with the kryoscopic constant of water.

In Fig. 3 typical thermograms obtained from microspheres prior to erosion as well as after one day and after five days of erosion can be seen. The melting peak broadened during erosion and a shift to earlier times was noticed. When the obtained thermograms were analyzed for purity prior to erosion a purity of approximately 99.9% was measured (Fig. 4) This value corresponds to the osmotic pressure of the encapsulated medium. Purity reaches a minimum of 99.1% after four days, corresponding to an osmotic pressure of at least 600 milliosmol. After 15 days of degradation purity values increased to a level that equals about 400 milliosmol. It can be concluded from these experiments, that during the first week of erosion a significant decrease of sample purity occurs, which is related to a significant increase of osmotic pressure resulting from the polymer degradation products.

To determine the effect of these changes on pH, microspheres were investigated by EPR. AT was chosen as a spin probe for EPR experiments because of its pK_a value and its hydrophilic properties. pH calibration curves of AT at 2.1 GHz

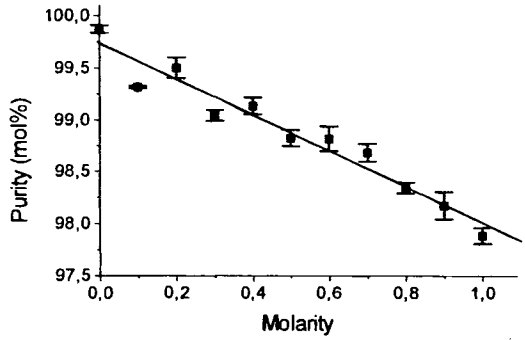

Fig. 2. Calibration curve for DSC purity analysis obtained with sodium chloride solutions of different molarity.

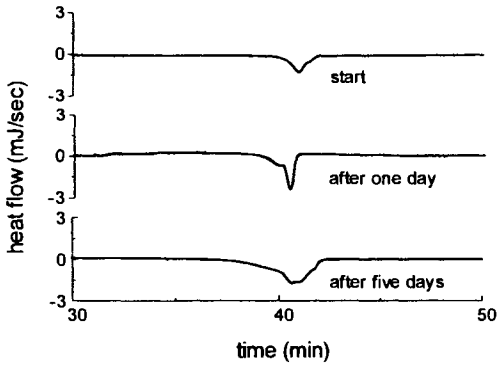

Fig. 3. A typical set of thermograms showing the broadening of DSC melting peaks of eroding PLA microspheres.

were in good agreement with results obtained at 9.4 and 1.1 GHz and a pK_a of 6.1 could be confirmed (Fig. 5), (9,12,13). The critical point with microspheres was to make sure EPR signals stemmed from the inside of the microsphere matrix only and were not contaminated with signals related to released spin probes in the erosion buffer. There are three different possibilities to avoid this problem, namely, (i) the addition of paramagnetic ions, which leads to line broadening of the released nitroxide molecules, (ii) the addition of ascorbic acid, which reduces the released nitroxides to EPR-silent hydroxylamines, and (iii) repeated exchange of the buffer solution. Methods (i) and (ii) change the ionic strength and the osmotic pressure of the release medium. Therefore, we decided to exchange the buffer several times. Before taking EPR spectra microspheres were centrifuged and resuspended in fresh buffer. This procedure was repeated until no further spin probes could be detected in the supernatant.

Different PLGA microspheres loaded with the spin probe AT were prepared. In the first series of experiments, microspheres made of the more hydrophobic, end-capped PLGA 14,000 were investigated. EPR-spectra taken of non-eroded microspheres consisted of the characteristic line spectrum of highly immobilized nitroxide molecules. In contrast to previous experiments with implants of 2 mm thickness, no change in the line shape could be found after exposing the microspheres to phosphate buffer. The EPR signal intensity was reduced during erosion. These results suggest water solubilized nitroxide molecules diffuse rapidly out of the microsphere. It can be estimated, the amount of water solubilized nitroxide molecules does not exceed 1% of the total amount of the incorporated

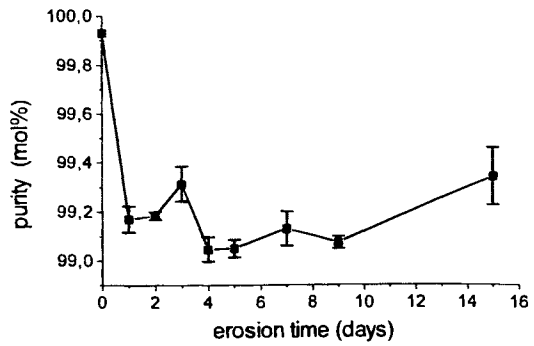

Fig. 4. Changes in sample purity during erosion of PLA microspheres.

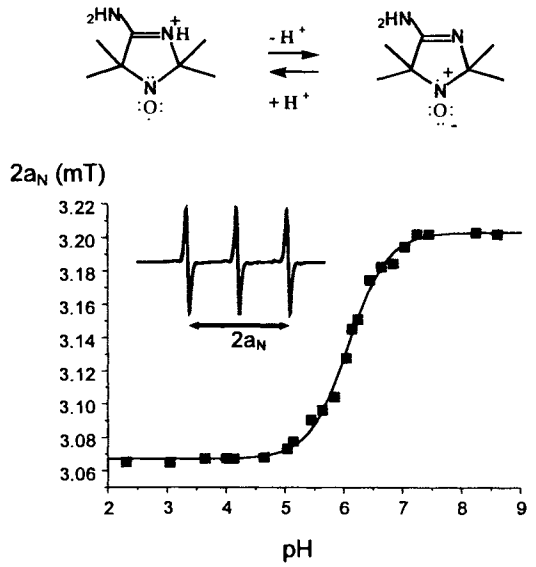

Fig. 5. pH-dependence of hyperfine coupling constant $2a_N$ of pH-sensitive spin probe AT.

spin probe. This lack of spectral contributions of water solubilized AT molecules prevented pH-measurements in these microparticles.

A second series of experiments was performed with the more hydrophilic, non-end-capped PLGA 8,000. To study the influence of buffer additives and incorporated basic drugs on the microacidity inside the microspheres, gentamicin free base or sodium acetate was incorporated together with AT. The EPR-spectra taken from microspheres prior to erosion were not affected by these additives (Fig. 6). The spectra showed AT is immobilized inside the microsphere matrix. After incubation in phosphate buffer pH 7.4, changes in EPR-spectra were detected (Fig. 7). The total EPR spectrum was characterized by the highly mobile spin probe which confirms AT dissolved in water. In contrast to the more hydrophobic polymers, water penetrated much faster inside the microspheres made of non-end-capped PLGA. The isotropic hyperfine coupling constant $2a_N$ (Fig. 5) was used to calculate pH-values inside eroding microspheres (13). Microspheres containing no further additives gave a pH value of $\leq$ 4.7 (Fig. 8). This acidic pH results from the free carboxylic acid groups of the polymer, its degradation products, and traces of acetic acid from the hydrolysis of acetic anhydride during preparation. Addition of a basic drug such as gentamicin free base increased pH-values up to 5.7; the addition of sodium

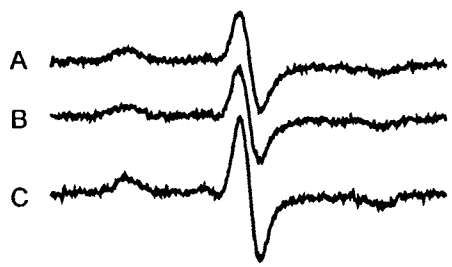

Fig. 6. 2.1 GHz EPR spectra of PLGA microspheres loaded with AT in the dry state; scan range: 10 mT, B_0 = 77 mT. Besides AT microspheres contained (A) no further additives, (B) genta icin base and (C) sodium acetate buffer.

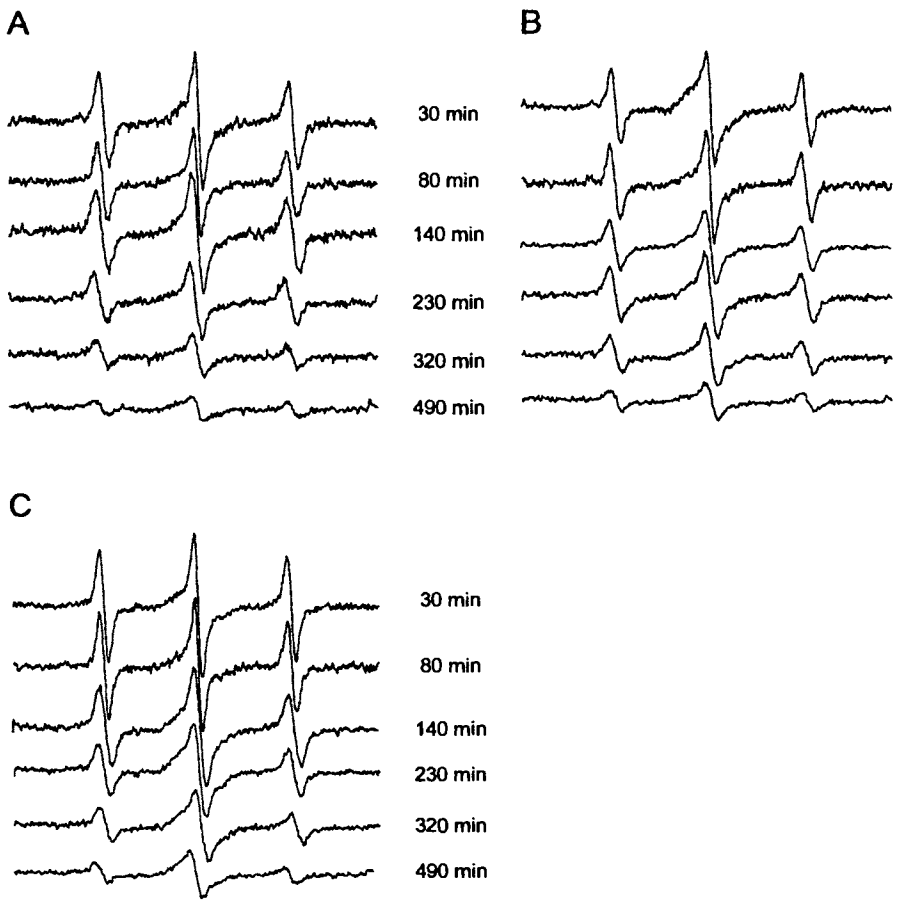

Fig. 7. 2.1 GHz EPR spectra of PLGA microspheres loaded with AT after buffer exposition (phosphate buffer pH 7.4); Scan range: 6 mT, B_0 = 77 mT. Microspheres were prepared with a W/O/W emulsion technique and acetic anhydride as organic solvent. Besides AT microspheres contained (A) no further additives, (B) gentamicin base and (C) sodium acetate buffer.

acetate resulted in a pH of 5.9. Released spin probes after the first centrifugation showed the spin molecules were not protonated in the buffer medium and gave a corresponding pH-value ≥7.4.

DISCUSSION

DSC in combination with purity analysis proved to be an efficient tool to probe the microenvironment inside eroding microspheres. The osmotic pressure increase inside PLA microspheres during the first days of erosion shows polymer degradation products are not readily released out of the polymer matrix. The chosen low molecular weight PLA was within the critical average molecular weight range determined by Park to yield a fraction of water soluble oligomers (20). The accumulation of the polymer degradation fragments inside the microsphere matrix led to an increase in osmotic pressure. With progressing erosion, the microspheres erode and the diffusion of oligomers and monomers can proceed more easily, which is expressed in decreasing osmotic pressure during the second week of erosion.

EPR was useful to determine pH-values experimentally inside biodegradable microspheres. The procedure of continuous centrifugation and buffer replacement made sure signals were only detected from the inside of microspheres. Incorporation of buffer salts or basic drugs changed the acidic microclimate in the microsphere matrix. Measuring pH inside microspheres with EPR is a non-invasive and continuous method, which can also be extended to in vivo experiments. In addition, EPR also may provide insight toward the elucidation of release mechanisms of incorporated low molecular drugs. With the more hydrophobic end-capped PLGA14,000 the amount of detected mobile spin probes was insufficient to obtain a clear signal after exposure to the erosion medium. Mobilization of the encapsulated spin probe by penetrating water molecules is very slow, and diffusion inside the microsphere matrix appears to be less important than in devices of larger geometry. The more hydrophilic non-end-capped polymers showed a high amount of mobilized spin probe in buffer. Here diffusion appears to play an important role in the release mechanism.

Internal pH inside eroding microspheres has been discussed controversly so far. A number of early papers on the issue describe a connection between peptide or protein instability within PLGA microspheres and internal pH values generated during degradation (21,22,23). Human growth hormone, for example, showed the same degradation profiles after encapsulation as in solution at pH 7.4. This gave rise to the assumption, that the internal pH of the degrading microspheres had to be similar to the one of the bulk solution (24). This point of view was supported by NMR studies on the microclimate of eroding PLGA microspheres (25). Our results show a different behavior

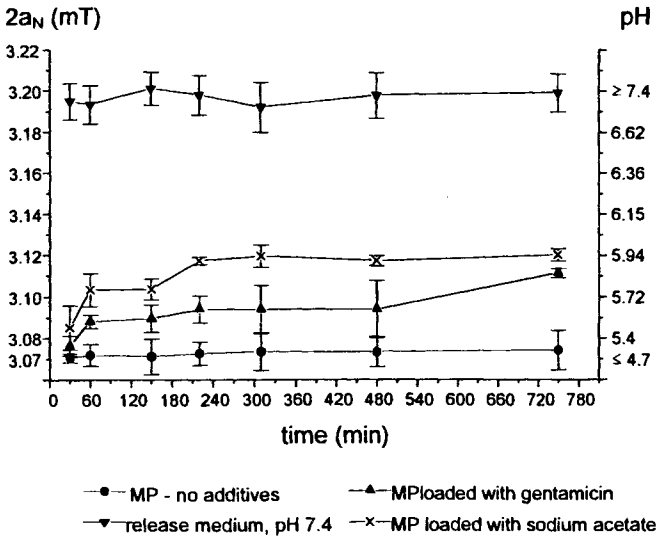

Fig. 8. Experimental results of pH measurements in microspheres made of Resomer RG502H. The hyperfine coupling constant $2a_N$ of spin probe AT allows to determine pH-values in the environment of the spin probe.

of eroding PLGA microspheres and we believe EPR is a more suitable technique for the assessment of internal microsphere changes. Our results are in accordance with the more recent findings of an acidic microclimate determined by 4 independent methods (26,27).

The careful characterization of the chemical microclimate inside microspheres is an extremely valuable tool for a better understanding of microsphere drug delivery systems and for the rational design of such dosage forms. If detailed data on different polymers becomes available in the future this may help to choose the best polymer-drug combination that guarantees high peptide and protein stability, and optimized drug release behavior.

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