Monitoring Microviscosity and Microacidity of the Albumin Microenvironment Inside Degrading Microparticles from Poly(lactide-co-glycolide) (PLG) or ABA-triblock Polymers Containing Hydrophobic Poly(lactide-co-glycolide) A Blocks and Hydrophilic Poly(ethyleneoxide) B Blocks

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Purpose. The purpose of this study was to monitor the microenvironment of an encapsulated model protein during the release from biodegradable microparticles (MP) made from three different polymers, namely poly(lactide-co-glycolide) (PLG) and ABA-triblock polymers containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethyleneoxide) B blocks with an A:B ratio of 90:10 (ABA10) and 70:30 (ABA30).

Methods. MP loaded with spin labeled albumin were prepared by a w/o/w technique. The particles were characterized by light scattering and electron microscopy. In vitro release of albumin was determined by size exclusion chromatography. Light microscopic experiments were conducted to visualize water penetration in the matrix. The protein microenvironment inside the degrading microparticles was characterized noninvasively by 2 GHz EPR spectroscopy.

Results. Water penetrated rapidly into all MP in the range of few minutes. A burst release was observed for PLG. The release from ABA block-polymers continued for over 14 days despite the rapid solubilization of the protein inside the microparticles. The initial microviscosity of the protein environment inside the ABA particles after exposure to buffer was 2 mm^2/s and increased with time. A gradual decrease of the pH to a value of 3.5 was observed within the MP.

Conclusions. The data indicate that the microviscosity and microacidity inside protein loaded microparticles can be studied nondestructively by EPR spectroscopy. Our results clearly demonstrate that ABA-block polymers are superior to PLG allowing a controlled release of proteins from swollen microspheres.

KEY WORDS: biodegradable polyesters; albumin; EPR; microparticles.

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ABBREVIATIONS: ABA, triblock polymers containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethyleneoxide) B blocks; EPR, electron paramagnetic resonance spectroscopy; HSA, human serum albumin; PEO, polyethyleneoxide; PLG, poly(lactide-co-glycolide); SEC, size exclusion chromatography; SLA, spin labeled albumin.

INTRODUCTION

Peptide and protein based drugs have attracted increased attention during the last years. Their therapeutical utility is limited by their poor peroral bioavailability and their short half lives in biological tissues. Due to these characteristics, multiple daily injections are often required. To overcome compliance problems, biodegradable polymers have been developed to provide a continuous and controlled release from days to months (1). Microparticles and implants have been developed for the parenteral administration of different synthetic GnRH mimetic peptides and the prolactine inhibitor bromocriptine (2). In general, the delivery system acts as a carrier which releases the drug at a predetermined rate, degrading thereby (or a short period after complete release) into nontoxic compounds which are excreted by the body. It has been observed that the matrix can deactivate incorporated compounds, especially proteins, prior to their release (1). Irreversible aggregation of proteins due to hydrophobic interactions between hydrophobic polymers and the hydrophilic protein is one of the main factors contributing to the loss of activity. In addition, an acidic microenvironment inside the degrading matrix can develop with time due to the formation of degradation products. It has been found that the pH inside degrading PLG implants drops *in vivo* to acidic values as low as pH 2 and acid induced protein degradation can be expected to take place under these conditions (3).

A strategy was developed to minimize the hydrophobic interactions by the incorporation of hydrophilic poly(ethyleneoxide) segments into the hydrophobic polyesters. ABA-triblock polymers containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethyleneoxide) B blocks were synthesized in order to achieve a hydrophilic microenvironment for the incorporated protein within the delivery matrix (4). It has been demonstrated that these block polymers contain hydrophobic polyester A blocks and hydrophilic poly(ethyleneoxide) microphases within the polymer matrix (5). They absorb water rapidly after exposure to buffer. Inspite, of this swelling behaviour, incorporated proteins are released with near zero order kinetics over weeks (6,7). The advantages of the block polymers with respect to protein release kinetics and stability became evident in comparison to PLG polymers (8) and were confirmed in the present study. A high amount of albumin was released initially (burst release), followed by a strongly decreased release rate. ABA block polymers also hypothetically minimize the risk of acid induced protein degradation, because the formation of a swollen structure with high water content could lead to a rapid exchange of hydrogen ions with the surrounding matrix (7).

The understanding of the interplay between the polymer matrix, the incorporated protein and the release medium requires an appropriate characterization. One disadvantage of the methods commonly employed (electrophoresis, scanning electron microscopy, gel permeation chromatography, differential scanning calorometry) is the requirement of a destructive sample preparation, which increases the number of samples and may lead to artifacts. Also, information concerning the physical state of the incorporated protein within the polymer matrix cannot be obtained by these methods *in situ*. Due to their nondestructive nature, electron paramagnetic resonance spectroscopy (EPR) does not require isolation steps and is therefore well suited for

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serial measurements (9). Stable nitroxide radicals with different physicochemical characteristics are available and can be incorporated as model drugs (spin probes) or covalently linked to drugs of higher molecular weight (spin labeled drugs). The sensitivity of the EPR spectra of nitroxides to the microviscosity and micropolarity of the surrounding matrix can be used to investigate the mechanisms of drug delivery *in vitro* and *in vivo* (10,11).

Furthermore, using specially designed nitroxides it is also possible to perform pH-measurements (12). The existence of pH-gradients inside degrading polyanhydrides was demonstrated under *in vitro* conditions by spectral-spatial-EPR-Imaging (13) and very acidic (pH 2) microenvironments have been found in PLG implants in mice by *in-vivo-EPR* spectroscopy (3).

The aim of the present study was to monitor noninvasively and continuously the physical state of an incorporated protein inside degrading microparticles by EPR-spectroscopy *in vitro*. Human serum albumin (HSA) was selected as a model protein. The pH-sensitive spin label 4-bromomethyl-2,2,3,5,5-pentamethyl-imidazolidine-1-oxyl was covalently attached to HSA to characterize microviscosity and pH. Three different polymers, namely PLG, ABA 10 and ABA 30, were selected to study the influence of the polymer composition on the characteristics of the protein environment inside the microparticles.

MATERIALS AND METHODS

Spin Labeled HSA

4-bromomethyl-2,2,3,5,5-pentamethyl-imidazolidine-1-oxyl (R-Br) was obtained from Prof. Grigoriev, Inst. of Organic Chemistry, Russian Academy of Sciences, Novosibirsk, Russia. Human serum albumin was a gift from Chiron-Behring, Germany. The covalent labelling of albumin with R-Br was performed according to Khramtsov (14). 100 mg HSA were dissolved in 5 ml NaAc-buffer (pH=5.0, 0.01M). The solution was mixed for three hours and then cooled to 2°C. 5.5 mg R-Br were dissolved in 5 ml buffer (pH 5.0; 0.01 M), cooled to 2°C and added to the HSA-solution. The mixture was stirred for 16 hours at 2°C. Unreacted R-Br was removed by gel filtration (Sephadex G25, Pharmacia, Uppsala, Sweden). Afterwards, the solution was freeze dried.

Poly(D,L-lactide-co-glycolide), lactide:glycolide ratio 50:50, PLG, (RG 502; Mw = 15.8 kDa) was purchased from Boehringer Ingelheim (Germany). Biodegradable linear ABA block copolymers consisting of poly(L-lactide-co-glycolide) A-blocks attached to central poly(ethyleneoxide) B-blocks were synthesized as previously described (4). The ABA block copolymers were composed as follows: ABA10: PLG:PEO ratio 90:10, Mw = 36.4 kDa; ABA30: PLG:PEO ratio 70:30, Mw = 26.7 kDa. Serum albumin (BSA, Sigma, Germany) was used as model drug for the *in vitro* release study. All other materials were of analytical purity.

Preparation of Microparticles

Microspheres were prepared by a modified W/O/W double-emulsion technique. 630 mg of the polymer were dissolved in 2.33 ml of dichloromethane in a 10 ml polypropylene syringe. 400 µl of an aqueous BSA solution (containing 70 mg of BSA, HSA, or spin-labeled HSA) were emulsified in the organic polymer solution using an ultra turrax homogenizer (Janke & Kunkel, Germany) at 20000 rpm for 30 s. This primary W/O emulsion was then rapidly injected into 300 ml of an aqueous PVA solution containing 0.1% (w/v) PVA. Homogenization was carried out using an ultra turrax S 25 N-25 F (Janke & Kunkel, Germany) at 8000 rpm for 30 s. For the removal of dichloromethane the resultant W/O/W emulsion was stirred for 3 h. The microspheres were isolated and washed by centrifugation at 5000 rpm for 10 min. Subsequently, the microspheres were lyophilized (Edwards Freeze Dryer Modulyo, 15 h, −50°C, 3.3 mbar) and stored at + 4° under desiccation.

Scanning Electron Microscopy

The microspheres were sputter-coated with a gold layer at 25 mA in argon atmosphere at 0.3 hPa for 2 min (Edwards/Kniese Sputter Coater S 150). The coating procedure was repeated three times. Surface structure of the microspheres were determined using a scanning electron microscope in vacuo (0.001 mbar) at a voltage of 25 kV (Hitachi S501, Hitachi Denshi, Japan).

Laser Light Scattering

Particle size distribution of the microspheres was analyzed by laser light scattering using a Malvern MastersizerX (Malvern Instruments, UK). The utilized 300 mm lens covered a particle size range of 1.2-600 µm. Each sample was measured in triplicate.

In Vitro BSA Release

About 20 mg of the microspheres were suspended in 2 ml of phosphate buffer pH 7.4 in a 2 ml Eppendorf cup (Eppendorf, Germany) at 37°C. At predetermined time intervals, the buffer was withdrawn and 2 ml of fresh medium were added to the microspheres. The total amount of albumin released was quantified using size exclusion chromatography (autosampler AS 2000a, L-6000 pump, Merck-Hitachi, Merck, Germany, columns: TSK pre column and TSK G3000 SWXL, Tosohaas). Albumin was detected fluorimetrically (excitation: 280 nm, emission: 340 nm, Fluorescence-Spectrometer F-1000, Merck-Hitachi, Merck, Germany). The flow rate was 0.7 ml/min using a mobile phase of phosphate buffer containing 0.3 M NaCl. The peak-areas of albumin were calibrated using standards of known concentrations.

Light Microscopy

Water penetration into the different microsphere batches was visualized using light microscopy (Optiphat-2, Nikon, Japan). Micrographs of the particles were taken before and immediately after addition of phosphate buffer pH 7.4.

EPR Measurements

About 15 mg of albumin loaded microparticles were suspended in 1.5 ml buffer in Eppendorf plastic vials which were incubated in horizontal direction in a water-thermostat (30 moving cycles per minute). The buffer was changed every second

day to assure sink conditions. All studies were performed at least in triplicate.

EPR spectroscopy was performed with a surface coil equipped 2 GHz spectrometer MT1 from Magnettech GmbH, Berlin, Germany, using the following parameters: central field: 78 mT, field scan: 10 mT, modulation amplitude: 0.1 mT.

The microparticles were collected with an Eppendorf centrifuge at 3000 rpm in order to separate the EPR signals from the buffer and the inner sphere of the particles. The supernatant buffer was carefully removed by means of a syringe and characterized by EPR-spectroscopy. The particles were resuspended in buffer and the procedure was repeated until no EPR signals were detectable in the buffer (three times). Thereafter, the microparticles were measured by EPR using the parameters mentioned above. The measurement of the particles could be performed directly in the Eppendorf vial due to the use of a surface coil of appropriate size and no further sample preparation was required.

RESULTS

The EPR spectrum of the spin labeled albumin is sensitive to microviscosity and pH of the protein environment as illustrated in Fig. 1. The high mobility of the spin labeled protein in a low viscous environment leads to a partial averaging of the anisotropic hyperfine coupling of the electron spin with the nitrogen nucleus (nucleus spin I = 1) and all three transitions (I_{+1} - first absorption line; I_0 - central absorption line, and I_{-1} third line) can be recognized (Fig. 1f and g). Higher viscosity of the protein environment results in less averaging of the anisotropy, increased line widths and decreased signal amplitudes, particularly of the first (I_{+1}) and the third (I_{-1})line (Fig. 1a-e). These spectral changes can be used to quantify the viscosity by means of the a/b ratio (Fig. 1B). The hyperfine splitting constant $2a_N$ is sensitive to the pH of the environment (Fig. 1f and g) due to the imidazolidine derived structure of the radical (3,12). Protonation of the nitrogen in position 3 of the imidazolidine ring induces a decreased spin density on the nitrogen atom of the nitroxyl moiety, leading to a decreased hyperfine coupling constant.

Albumin loaded microspheres were prepared by a modified W/O/W double emulsion technique. Poly(lactide-co-glycolide) and two ABA triblock copolymers with different content of PEO were used for microencapsulation.

As demonstrated by laser light scattering experiments, particle sizes and particle size distributions of the different microsphere formulations were determined to be in the same range. Therefore, a direct comparison of the microspheres was possible (Fig. 2).

Figure 3 illustrates that independent of the polymer used spherical particles were obtained. In contrast to PLG, the use of the more hydrophilic ABA block copolymers yielded microspheres with a rough appearance. In the case of ABA30 a number of pores was observed on the surface.

The results of the microscopic investigations demonstrated that water penetrates immediately into the particles, but penetration rate of water at very early times differed as function of the polymer used. Light microscopic micrographs of dry placebo microspheres from PLG and ABA30 only showed dark particles due to total light reflection (Fig. 4). In the case of the particles from ABA30, addition of phosphate

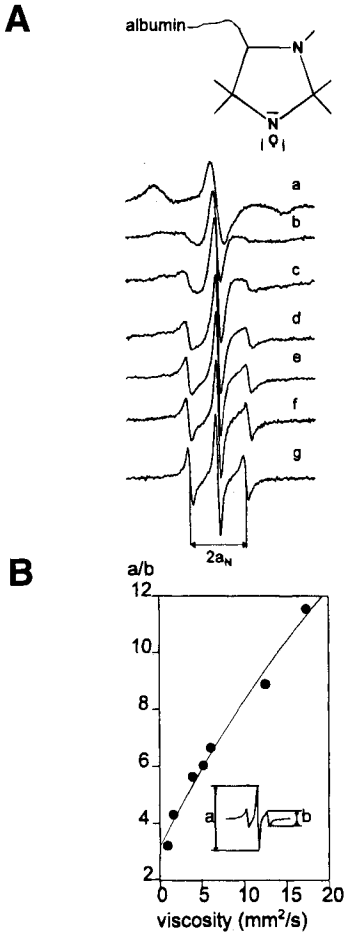

Fig. 1. A: Influence of viscosity and pH on the spectral shape of spin labeled albumin. Higher viscosity results in an increase in the ratio **a/b** between the signal amplitudes of the second and the third peak. The pH affects the constant of hyperfine splitting 2aN. a) dry powder, b) glycerine-water mixture with a viscosity of 17.37 mm^2/s, c) solubilized in a glycerine-water mixture with a viscosity of 12.53 mm^2/s, d) solubilized in a glycerine-water mixture with a viscosity of 3.922 mm^2/s), e) solubilized in a glycerine-water mixture with a viscosity of 1.669 $mm^{2/s}$, f) solubilized in phosphate buffer, pH 7.4 ($2a_N$ = 3.162 mT), g) solubilized in 0.1 M HCl ($2a_N$ = 2.912 mT). B: Relation between the a/b ratio and viscosity of glycerine water mixtures.

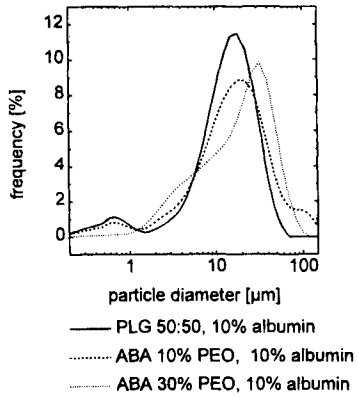

Fig. 2. Particle size distribution of microparticles measured by laser light scattering.

buffer solution pH 7.4 very rapidly led to a water penetration into the polymer matrix. After only a few seconds of contact with the buffer solution, a light border representing the penetrating water front was observed. Two minutes later water already reached the center of the particles. In contrast, water did not penetrate into the matrix of the PLG microspheres in this time period. After circa 10 min of contact time, water also seemed to have reached the inner areas of the more hydrophobic polymer matrix.

In vitro release of albumin from the different polymers was measured over a period of 30 days. As summarized in Fig. 5a, the release profile of BSA from PLG was characterized by an initial burst of more than 60%, followed by a period with negligible drug release. Additionally, the formation of larger microparticulate aggregates was noticed. In contrast, albumin was released from both ABA triblock copolymers in a continuous pattern. Moreover, it was found that albumin was released more rapidly from the polymer with the higher PEO content, ABA30, compared to ABA10. No apparent changes of the appearance, such as larger aggregates or decreased ability for resuspension were observed for the ABA microparticles.

The EPR spectra of PLG microparticles (Fig. 6a) indicate that water penetrates rapidly into the particles, leading to a spectral change from totally immobilized albumin (see Fig. 6a, 0 min and Fig. 1a) to a spectrum which is dominated by mobile albumin solubilized in an environment with a moderate viscosity of about 10 mm^2/s (see top of Fig. 6a, 10 min and Fig. 1d). The EPR signal intensity decreases with time. Additionally, changes of the shape of the EPR-spectra indicate that the albumin which is not released during the first days is localized in a highly viscous environment, comparable to 80:20 glycerine-water mixtures (see Fig. 6a, 145 h and times thereafter and Fig. 1b).

Exposure to buffer leads to rapid solubilization of albumin inside ABA microparticles within a low viscous environment (Fig. 6b and 6c). Despite the rapid solubilization, there were only small changes of the signal intensity during the first days. The microvicosity of the protein environment inside the MP can be quantified by calculating the ratio between the signal amplitude of the second (central) peak I_0 and the third (high field) peak I_{-1} (Fig. 7). The experimental data indicate that the exposure to buffer yields an initial microviscosity of around 2 mm^2/s inside the particles. With time, the signal intensity decreases due to the release of the protein. In addition, changes of the spectral shape were also observed. The a/b ratio increases slowly and continuously with time, indicating an increased viscosity of the albumin environment. The increase was more rapid for ABA30 as compared to ABA10. Finally, the spectral shapes became very similar to those which were recorded from albumin solubilized in a viscous glycerine/water mixture (17.376 mm^2/s) 80:20 (v/v) (Fig. 1b).

The use of the pH-sensitive labeled albumin allowed the assessment of the acidity of the protein environment inside the microparticles. The analysis of the hyperfine coupling constant indicates that the pH of the microparticulate core inside ABA polymers decreases slowly and reaches a pH value of 3.5 after 200 h (Fig. 8). Reliable pH measurements are not possible after 50 h for PLG and after 200 h for

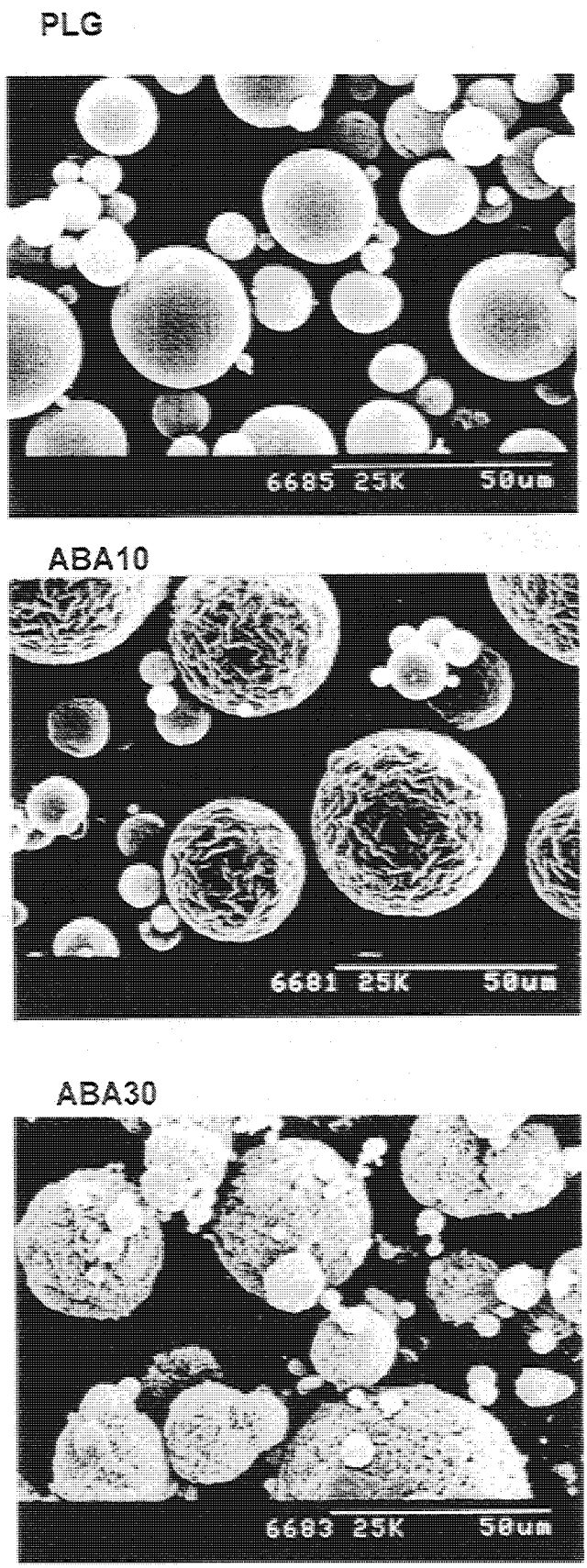

Fig. 3. Scanning electron micrographs of the different microsphere formulations.

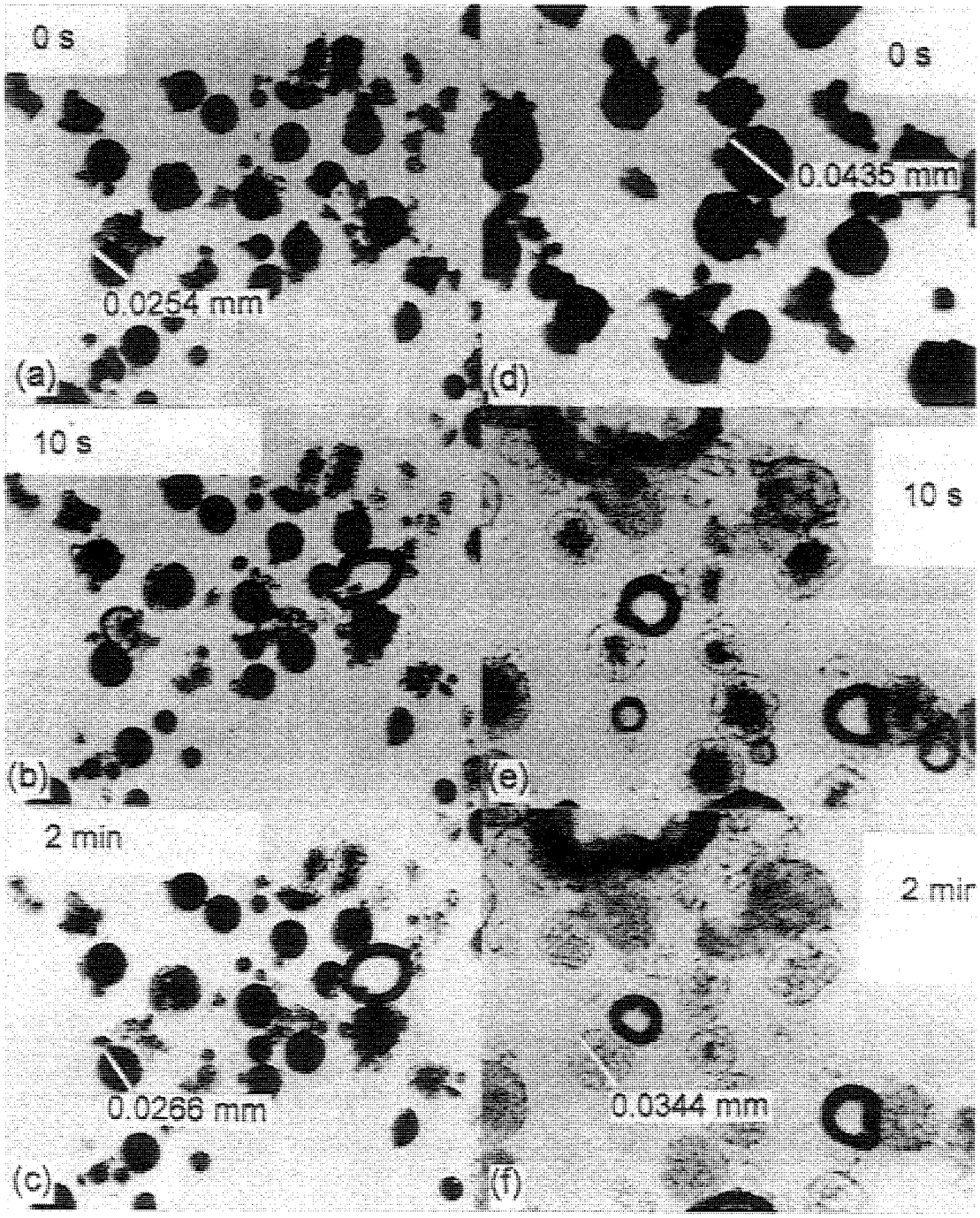

Fig. 4. Light microscopic photographs of microspheres: a) PLG before exposure to phosphate buffer pH 7.4; b) PLG immediately after exposure to buffer; c) PLG 2 min after exposure to buffer; d) ABA30: before exposure buffer; e) ABA30 immediately after exposure to buffer; f) ABA30 2 min after exposure to buffer.

ABA microparticles due to the changes of the spectral shape caused by the increase in microviscosity and the decreasing signal to noise ratio.

DISCUSSION

Albumin loaded microparticles were made from PLG and two different ABA triblock copolymers. Monitoring of the physical state of albumin inside biodegradable microparticles was possible by the incorporation of a pH-sensitive labeled albumin. As demonstrated by SEC, an incomplete burst release of over 65% of the total albumin loading, followed by a period of no peptide delivery, was observed for the PLG microspheres. Moreover, the EPR signal intensity of the PLG microspheres decreased rapidly during the first hours. Water solubilized albumin was detectable inside the microparticles for the first two

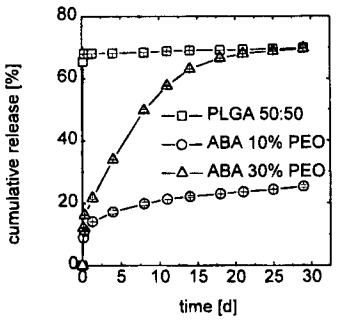

Fig. 5. *In vitro* release of albumin from the differently composed polyesters.

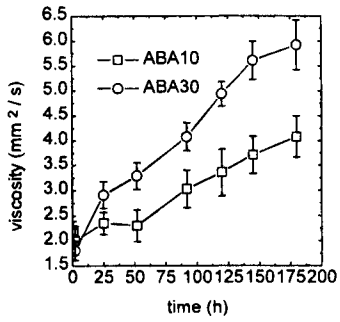

Fig. 7. Change of the viscosity of the albumin microenvironment inside degrading ABA block polymers.

days. The release pattern and the detection of water solubilized albumin suggests that a part of the albumin forms an own phase within the polymer matrix. Water penetrates rapidly into this phase, solvating the albumin, which is released very rapidly. Thereafter, a decrease in the release rate to close to zero is observed and the spectral shape indicates that the protein molecule is localized in a highly viscous matrix.

A very rapid hydration of the encapsulated albumin was also observed for the ABA microparticles (Fig. 6b and 6c).

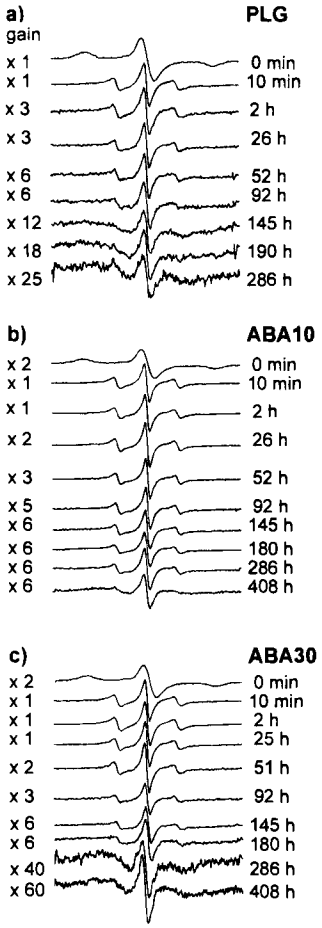

Fig. 6. 2.1 GHz EPR spectra of PLG (a), ABA10 (b) and ABA30 (c) microparticles loaded with spin labeled albumin before and after exposure to phosphate buffer.

However, the burst release was 1/5 compared to PLG (Fig. 5). The experimental data prove that the block polymers immediately generate an aqueous environment for the encapsulated protein still providing a controlled release. The ratio of the signal amplitude between the central and the third peak indicates a microenvironment with a viscosity of 4 mm^2/s, which is comparable to a 1:1 glycerin-water mixture (23°C) (Fig. 7). The release rate was higher from ABA30 microparticles compared to ABA10, indicating that higher amounts of hydrophilic PEO segments will provide a faster release. In contrast to PLG, the polymer matrix did provide this hydrophilic environment for albumin for a very long time. A continuous decrease of the albumin mobility was observed with time (Fig. 7). This finding could indicate the presence of several hydrophilic microdomains with distinct microviscosities where the albumin localized in a low viscous environment is released faster compared to the albumin localized in an environment of higher viscosity. However, another process may also cause or at least contribute to the decreased mobility: Previous experiments demonstrated that the content of the hydrophilic PEO decreases faster in degrading ABA polymers compared to polylactide (6,15). Therefore, the overall amount of hydrophilic microdomains will decrease with time. It has been shown that the release from

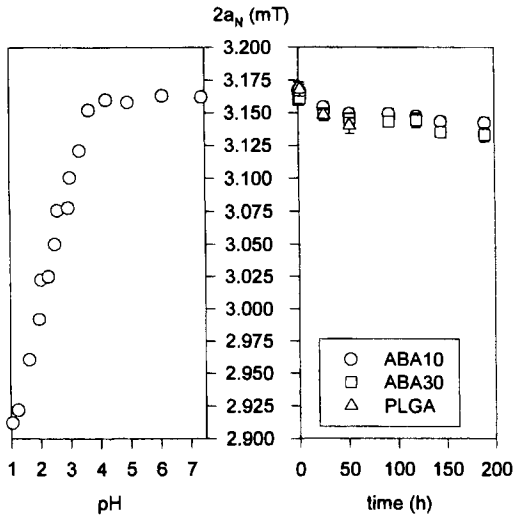

Fig. 8. Influence of the pH on the constant of hyperfine coupling $2a_N$ (left) and change of $2a_N$ of albumin inside the microspheres after exposure to phosphate buffer, pH 7.4.

macromolecules from ABA polymers does strongly depend from the molecular weight (7) and therefore, we expect a faster release of the cleaved PEO segments compared to the albumin due to the smaller molecular weight of the PEO (10 kD). Our data show that the preferential release of PEO segments may result in an increased microviscosity of the microenvironment of incorporated proteins, comparable to an environment of 80:20 glycerin/water mixtures. The observed drop in pH suggests that buffer ions are not able to compensate the polymer degradation induced increase in microacidity inside the particles.

In conclusion, our study demonstrates that important parameters of the protein environment can be studied continuously and nondestructively by EPR inside protein loaded microparticles. The characterization of microviscosity and microacidity gives unique information about the mechanisms of drug release. The approach is very useful to obtain experimental data on the influence of polymer composition on the state of the encapsulated protein. The experimental data prove or disprove the success of the strategies of protein stabilization. For example, our results clearly demonstrate that ABA-block polymers provide a hydrophilic and low viscous protein environment for a long time. Therefore, it can be concluded that one major aim of the development of the ABA block polymers has been realized. The observed decrease in pH suggests that the polymers used in this study were not able to preserve a neutral environment inside the particles. Further studies will investigate which factors determine the microacidity inside the particles in more detail.

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