Degradation of Polyaminophosphazenes: Effects of Hydrolytic Environment and Polymer Processing

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Polyphosphazenes with amino acid ester side groups show potential as hydrolytically degradable materials for biomedical applications. This study focuses on practical aspects of their use as biodegradable materials, such as effects of the hydrolytic environment and sample processing. Poly[di(ethyl glycinato)phosphazene], PEGP, and poly[di(ethyl alaninato)phosphazene], PEAP, were prepared by macromolecular substitution reaction, ensuring the absence of the residual chlorine atoms to avoid their influence on the hydrolysis. The kinetics of polymer degradation was studied by simultaneously measuring polymer mass loss, molecular weight decrease, and the release of phosphates and ammonia. The effect of pH, buffer composition, temperature, casting solvents, and film thickness were investigated.

Introduction

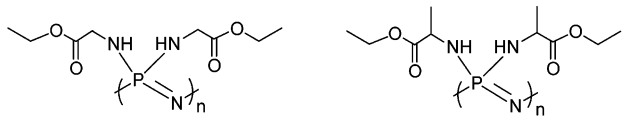
Polyaminophosphazenes show potential as hydrolytically degradable materials for use in biomedical applications. Their ability to degrade stems out of the properties of their inorganic backbone and the degradation rate is determined by the characteristics of the organic side group. Ultimate breakdown products predictably include phosphates and ammonia, originating from the phosphorus and nitrogen backbone, as well as the product of the side group cleavage. In addition, the unsurpassed structural diversity of this class of polymers offers advantages in achieving the properties desired for a specific application.

Since the first report on the synthesis of polyaminophosphazenes with side groups containing amino acid derivatives,[1] they have been attracting much attention.[2−9] The studies have been focusing on the synthesis of new derivatives, elucidation of structure−degradation rate relationship, and investigation of these polymers for drug release applications. However, the mechanism of hydrolytic breakdown in these systems is not completely understood and thus requires further investigation.[10] From the standpoint of their practical use for biomedical applications, it is imperative to conduct comprehensive studies to include simultaneous monitoring of various degradation parameters, such as changes in polymer mass and molecular weight and the release of low molecular degradation products.

This is especially important since contradicting results have been reported on the effect of pH and other factors on the degradation kinetics.[4] Notably, not all studies provide critical characteristics of starting materials, including the content of synthetic irregularities, such as residual chlorine atoms, which can have a profound effect on the mechanism and kinetics of degradation.[10−12] The effects of other important degradation parameters, such as buffer composition or casting solvent, are also often overlooked in these studies.

The present paper focuses on two polyaminophosphazenes: poly[di(ethyl glycinato)phosphazene], PEGP, and poly[di(ethyl alaninato)phosphazene], PEAP (Chart 1). Both of them have been reported previously and were selected in the present study as reliable systems for further investigation of the effect of various environmental factors, such as pH, temperature, and buffer composition, as well as sample processing parameters. To obtain maximum information on the peculiarities of the degradation mechanism, the hydrolysis process was investigated by simultaneous measurement of polymer mass, molecular weight, and the release of low molecular degradation products, such as phosphates and ammonia.

Chart 1. Structures of PEGP and PEAP



Poly[di(ethyl glycinato)phosphazene] PEGP

Poly[di(ethyl alaninato)phosphazene] PEAP

Experimental Section

Materials. L-Alanine ethyl ester hydrochloride (Aldrich Chemical Co., Inc., Milwaukee, WI) and glycine ethyl ester hydrochloride (TCI, America) were dried under vacuum at ambient temperature for 2 days. Triethylamine (Mallinckrodt Baker, Inc., Phillipsburg, NJ) was distilled before use. Hexachlorocyclotriphosphazene, trimer (Nippon Fine Chemicals, Japan); 2-methoxyethyl ether (diglyme), anhydrous; tetrahydrofuran (THF), anhydrous (EMD Chemicals Inc., Gibbstown, NJ); toluene, anhydrous, 99.8% (Aldrich Chemical Co., Inc., Milwaukee, WI); chloroform-*d*, 100% (isotopic); tetra-*n*-butylammonium bromide, 98% (Alfa Aesar, Ward Hill, MA), 99.5%; L-ascorbic acid, 99%+; potassium phosphate monobasic, 99%; sodium phosphate dibasic, heptahydrate; sodium phosphate, monobasic, monohydrate, Nessler's reagent (Aldrich Chemical Co., Inc., Milwaukee, WI); and boric acid, sodium tetraborate decahydrate, tris(hydroxymethyl)aminomethane (EMD Biosciences, Gibbstown, New Jersey) were used as received. The macromolecular precursor poly(diclorophosphazene), PDCP, was synthesized by ring-opening polymerization of hexachlorocyclotriphosphazene in the titanium pressure reactor as described previously.[13]

Synthesis of PEGP. Triethylamine (0.0422 L, 0.28 mol) was added to a suspension of glycine ethyl ester hydrochloride (39.08 g; 0.28 mol)

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in THF (0.35 L) at ambient temperature under nitrogen, the temperature was increased to 60 °C, and the reaction mixture was stirred for 5 h to form glycine ethyl ester. The reaction mixture was then cooled to ambient temperature and precipitate was removed by filtration under nitrogen. Polydichlorophosphazene (1.62 g, 0.014 mol) in diglyme (0.03 L) was slowly added to the filtrate and the reaction was kept at ambient temperature for 1 h. Then the temperature was increased to 60 °C and the reaction was continued for an additional 4 h. The reaction mixture was then cooled to ambient temperature and the precipitate was removed by filtration. The polymer was then purified by precipitating in 1.2 L of deionized water. Twenty milliliters of 30% NaCl was added to the mixture to facilitate precipitation. Polymer then was dried under vacuum for 4 days. The yield was 2 g (57%).

Synthesis of PEAP. Triethylamine (0.0973 L, 0.7 mol) was added to a suspension of L-alanine ethyl ester hydrochloride (107.53 g; 0.7 mol) in THF (0.7 L) at ambient temperature under nitrogen, the temperature was increased to 60 °C, and the reaction mixture was stirred for 5.5 h to form L-alanine ethyl ester. The reaction mixture was then cooled to ambient temperature, and precipitate was removed by filtration under nitrogen. Polydichlorophosphazene (4.06 g, 0.035 mol) in diglyme (0.052 L) was slowly added to the filtrate and the reaction was kept at ambient temperature for 1 h. Then the temperature was increased to 50 °C and the reaction was continued for an additional 48 h. The reaction was then cooled to ambient temperature and the precipitate was recovered by filtration. The polymer was purified by precipitating in 2.1 L of hexane and then twice by redissolving in 0.2 L of THF and precipitating in 0.6 L of deionized water. The polymer then was dried under vacuum for 4 days. The yield was 3.2 g (32%).

High-Throughput Synthetic Methods. Automated parallel synthesis was performed on a Quest 205 synthesizer (Argonaut Technologies, San Carlos, CA). The synthesizer contains a series of 10 100-mL Teflon reaction vessels equipped with vertical oscillating agitation, temperature control, and filtration system. Typically, 0.1 g of PDCP was taken for the reaction with L-alanine ethyl ester or glycine ethyl ester; total reaction volume of each tube was 30 mL. Reaction was conducted under nitrogen atmosphere; temperature, time, and reactant ratios were varied. Upon completion of the reaction, polymer was precipitated in hexane, redissolved in THF, precipitated in water, dried, and analyzed by gel-permeation chromatography (GPC) and NMR.

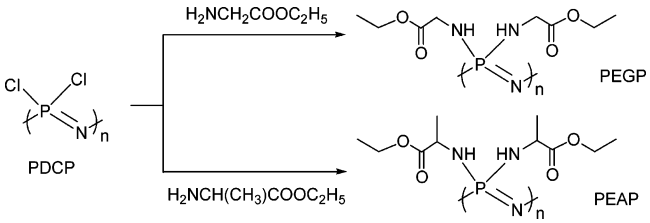
Analytical Methods. The GPC system included Waters 510 HPLC pump, Waters 717plus autosampler, and Waters 410 refractive index detector (Waters, Milford, MA). Waters Styragel HT6E column was used in a combination with Waters Styragel guard column (Waters, Milford, MA). THF (BHT-stabilized) containing 0.1% tetra-*n*-butylammonium bromide was used as a mobile phase, the flow rate was 0.8 mL/min, and an injection volume of 0.1 mL was used. Molecular weights were calculated on the basis of the polystyrene standards (Alfa Aesar, Ward Hill, MA). The results were processed with Millenium[32] version 3.20 software (Waters, Milford, MA).

^{31}P and ^{1}H NMR spectra were recorded on a Bruker 400 NMR spectrometer; $CDCl_3$ was used as a solvent.

Preparation of Polymer Films. Polymer films were prepared by solvent casting method. Polymer solutions (7−8% w/w) were prepared by dissolving the polymer in THF or toluene at room temperature. Aliquots (0.10 or 0.30 mL) were placed in stainless steel plates with a flat bottom (internal diameter 15 mm). Samples were kept at ambient temperature for 1 h to slowly evaporate the solvent and then dried under vacuum at room temperature for 24 h (THF) or for 72 h (toluene). Thickness of the films was estimated to be 30 μm (for 0.10 mL) or 90 μm (for 0.30 mL).

Degradation Studies. Degradation studies were conducted in heterogeneous systems containing polymer films and hydrolytic media: (1) deionized water; (2) 100 mM phosphate buffer, pH 5.5; (3) 100 mM phosphate buffer, pH 7.4; (4) 100 mM phosphate buffer, pH 8.0; (5) 100 mM Tris buffer, pH 8.0; (6) 100 mM borate buffer, pH 8.0.

Scheme 1. Synthesis of PEGP and PEAP



Polymer samples were placed in sealed plastic vials containing 10 mL of hydrolytic medium and incubated in a G24 environmental incubator shaker (New Brunswick Scientific, Edison, NJ). Studies were performed at 37 and 55 °C. Samples were collected periodically and dried under vacuum for 24 h. Polymer films incubated in buffer solutions were immersed in 10 mL of deionized water for 30 min to remove salts. They were then rinsed twice with deionized water before drying. One sample was used for each data point ($n = 1$).

Mass losses were determined gravimetrically. Molecular weights were measured by dissolving samples in THF (5 mg/mL) and analyzing them by GPC as described above.

Inorganic phosphate and ammonia were quantitatively determined in the degradation medium for samples incubated in water. For a phosphate determination, an aliquot was mixed with ammonium molybdate in an acidic medium to form phosphomolybdic acid, which was reduced by ascorbic acid to form a blue complex suitable for photometric measurement.[14] A reagent solution was prepared by mixing 5 mL of 0.4% ammonium molybdate, 12.5 mL of 0.2 M sulfuric acid, 5 mL of 0.7% ascorbic acid, and 2.5 mL of 0.018% potassium antimonyl tartrate. An aliquot (0.4 mL) of the solution to be analyzed was mixed with 1 mL of the reagent solution; the mixture was incubated at ambient temperature for 15 min and its optical density was measured at 885 nm. Solutions of potassium phosphate, monobasic (concentration range 7.5−60 μM), were used as standards.

Analytical determination of ammonia in the degradation medium was carried out by spectrophotometric measurement of the optical density after a color reaction with Nessler's reagent.[15] An aliquot (0.04 mL) of the sample to be analyzed was diluted with 0.86 mL of deionized water, then 0.1 mL of Nessler's reagent was added, and the solution was mixed by inversion and analyzed at 436 nm. Ammonium chloride solutions (concentration range 3−19 mM) were used as standards.

Results and Discussion

Polymer Synthesis and Characterization. The macromolecular substitution approach, commonly used in polyphosphazene synthesis, allows access to a vast variety of polymer structures.[10] Polyorganophosphazenes, such as PEGP and PEAP, are constructed through the replacement of reactive chlorine atoms of polyphosphazene precursor, PDCP, with the appropriate nucleophiles (Scheme 1). Although such an approach presents a convenient way for the introduction of a wide variety of different side groups into a polymer, it can also lead to incompletely substituted polymers and potential variability of their properties. Most importantly, it is well documented that residual chlorine atoms in such polymers can have a dramatic effect on the degradation kinetics of polyphosphazenes.[10,11,16] This can provide a potential explanation for the conflicting results that have been reported on the effect of hydrolytic environment on the degradation of PEGP[4] and emphasizes the need for careful control of polymer characteristics.

Thus, a prerequisite for the study of biodegradable polyphosphazene for biomedical applications is the achievement of a maximum polymer substitution, which is indicated by the presence of a single peak in ^{31}P NMR (−0.8 ppm for PEGP; −4.2 ppm for PEAP). This can be realized by forcing the

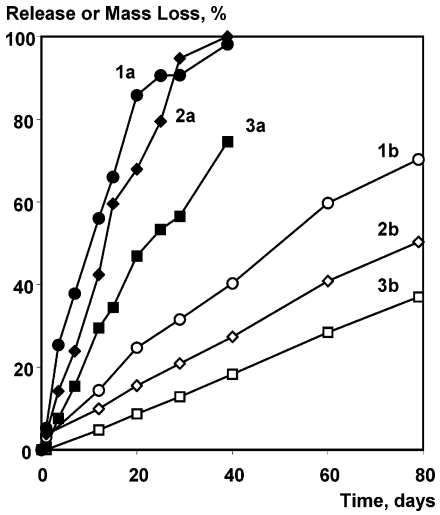


Figure 1. Mass loss (1) and release of ammonia (2) and inorganic phosphate (3) for PEGP at 55 °C (a) and 37 °C (b) vs time (deionized water).

reaction conditions through the increase of the reaction temperature and time, which can in turn lead to the degradation of the polymer during the synthesis. Thus, yet another important requirement was to achieve maximum molecular weight as indicated by the GPC analysis.

Synthesis of PEGP and PEAP (Scheme 1) was first optimized on a small scale by a high-throughput approach for the complete substitution and highest molecular weight. In our experiments the best results for PEGP were achieved at 60 °C and 4 h reaction time. The corresponding parameters for PEAP were 50 °C and 48 h. These conditions led to polymers characterized by a single peak in ^{31}P NMR and polymer molecular weight of 400 000 (polydispersity index 1.4).

Degradation Kinetics. Hydrolytic degradation of PEGP and PEAP was studied by monitoring basic characteristics of the polymeric material: changes in mass and weight-average molecular weight, and release of low molecular compounds, such as ammonia and inorganic phosphate, in the hydrolytic medium. The latest two are the ultimate products in the destruction of the phosphorus and nitrogen backbone and are unique indicators of the degree of polyphosphazene degradation. To allow for an accurate determination of these compounds, hydrolysis was first studied in deionized water. Degradation at physiological temperature, 37 °C, and accelerated hydrolysis at 55 °C were investigated. The yields of ammonia and phosphate were calculated as percentages of the theoretical amounts that can be released from the samples.

Figure 1 shows the kinetics of mass loss, as well as the release of ammonia and inorganic phosphate, for PEGP. As seen from the figure, at 55 °C practically complete erosion of the polymer occurred within 40 days. The half-life of the sample at 37 °C was approximately 50 days. The decrease in the mass of the film was always accompanied by the generation of ammonia and phosphate. Both of these compounds were found at levels somewhat lower than expected on the basis of the weight of the degraded polymer at the same time point, which can suggest presence of other degradation byproducts. Since no water-soluble macromolecular species were detected in the medium, the existence of low molecular compounds was assumed. At either temperature, the amounts of phosphates were lower than those of the ammonia, which probably indicated presence of organic phosphates not detectable by the assay (less than 10% of the theoretical amount of phosphate expected from the ammonia analysis).

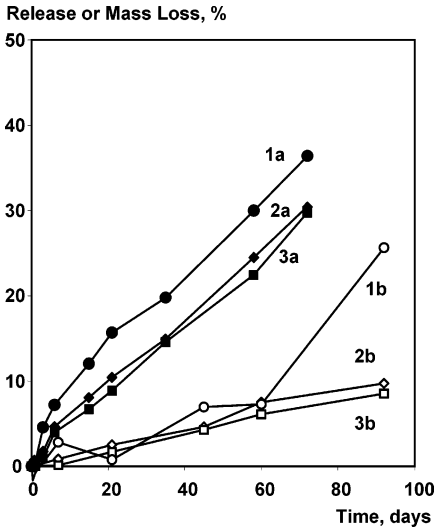


Figure 2. Mass loss (1) and release of ammonia (2) and inorganic phosphate (3) for PEAP at 55 °C (a) and 37 °C (b) vs time (deionized water).

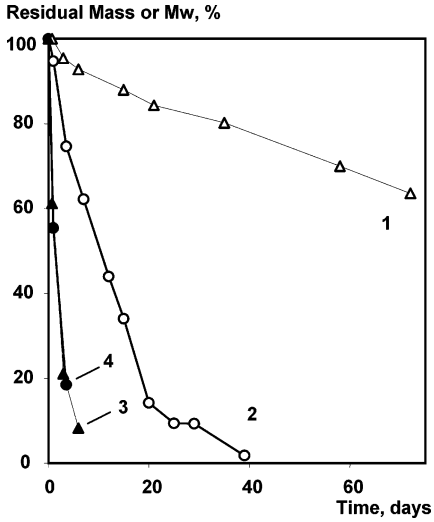
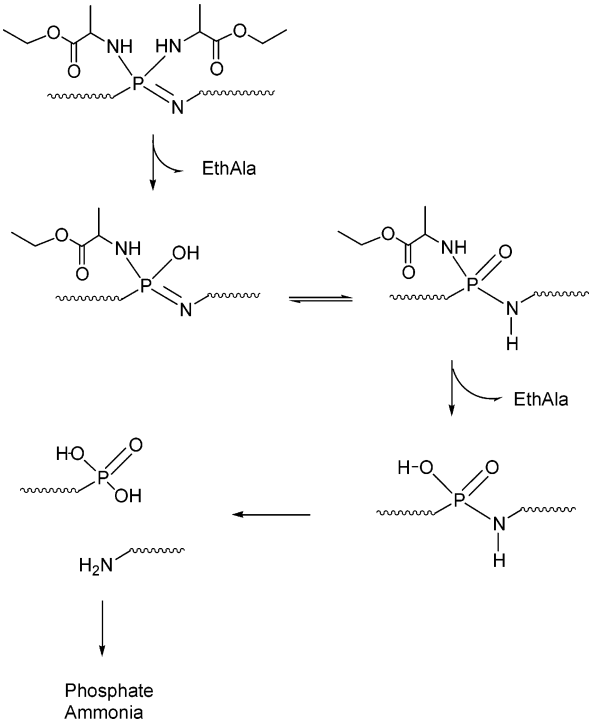


Figure 3. Residual mass vs time for PEAP (1) and PEGP (2), and molecular weight loss vs time for PEAP (3) and PEGP (4) (deionized water, 55 °C).

The kinetics of PEAP degradation (Figure 2) was significantly slower than for PEGP, which was expected on the basis of the previous findings and can be explained by the more hydrophobic nature of this polymer. The same trends as for the system described above were observed at 55 °C, although the differences between the kinetic curves were less significant. Interestingly, slower degradation at 37 °C showed overly good correlation between the mass loss and the release of ammonia and phosphate, at least in the first 60 days. This probably suggests that other low molecular byproducts have a relatively short life.

Comparison of mass and molecular weight kinetic profiles revealed striking differences between the rates of their decline (Figure 3). Much faster decrease in the molecular weight was observed for both systems, which typically indicates a bulk degradation mechanism.[17] The distinction in two parameters was especially pronounced for PEAP, where less than 10% mass loss in the first 5 days was accompanied with 90% drop in the molecular weight. Somewhat unexpectedly, there was no significant difference in the molecular weight reduction profiles for the two polymers.

Scheme 2. General Degradation Pathway for Polyaminophosphazenes



In general, the results support the previously suggested pathway for the degradation of polyaminophosphazenes (Scheme 2).[1,2]

Effect of pH and Buffer Composition. The effect of pH on the rate of polyphosphazene degradation is one of the most discussed in the literature.[2,4,12,18] From the biological standpoint, pH sensitivity of biodegradable polyphosphazenes opens opportunities for the design of systems, which can potentially discriminate between extracellular fluids and endosomes.[18] This can also provide important information on the mechanism of degradation. A commonly accepted hypothesis suggests either intramolecular or intermolecular acid catalysis as a first stage in the degradation pathway.[2] This is consistent with the previously observed increase in the degradation rate in the presence of acids;[5] however, findings on the higher hydrolysis rate at basic pH were also reported.[4]

Figures 4 and 5 show polymer mass and molecular weight decrease profiles at pH 5.5, 7.4, and 8.0. In this pH range, the effect was only observed for PEGP, where the rate of mass loss increased with the decrease in pH (Figure 4). The pH dependence of the molecular weight decrease was difficult to establish due to a rapid drop in the initial stage of the degradation.

There was no pronounced pH effect on the molecular weight or mass loss profiles of PEAP (Figure 5). This might be due also to the more hydrophobic nature of this polymer. Lower water uptake by this hydrophobic film (data not shown) leads to a higher overall density of protonizable groups in the polymer compared to PEGP. This can significantly affect the concentration gradient of hydrolysis catalyzing protons and diminish the effect of a buffer. The phenomenon is somewhat similar to the diffusion of cation through the matrix of the oppositely charged polyelectrolyte in ionically cross-linkable hydrogels.[19]

Degradation kinetics was also studied in different buffers. There were no detectable differences in the degradation rates for both PEGP and PEAP in phosphate, borate, and Tris buffers of the same ionic capacity at pH 8.0 (Figure 6). The kinetics was not affected by the presence of organic component (Tris buffer), which has a potential for stronger interactions with hydrophobic polymer films.

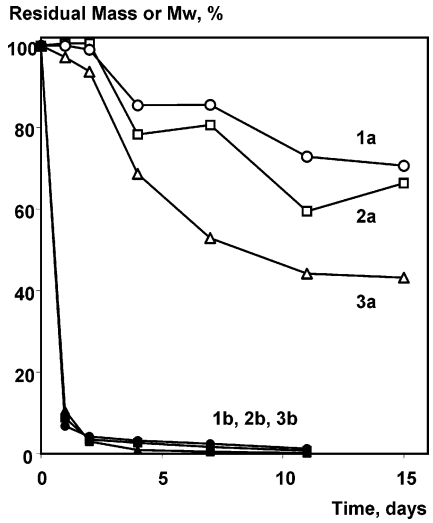


Figure 4. Residual mass (a) and residual molecular weight (b) of PEGP vs time at pH 8.0 (1), 7.4 (2), and 5.5 (3) (100 mM phosphate buffer, 55 °C).

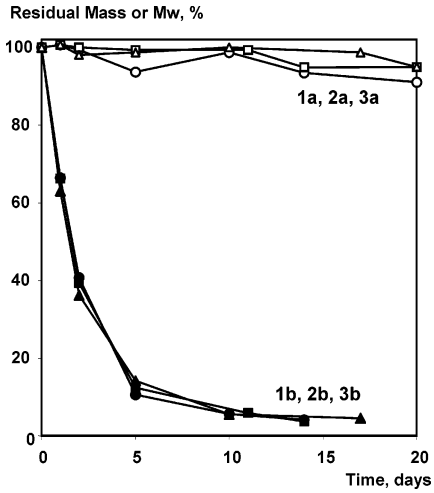


Figure 5. Residual mass (a) and residual molecular weight (b) of PEAP vs time at pH 8.0 (1), 7.4 (2), and 5.5 (3) (100 mM phosphate buffer, 55 °C).

Effect of Polymer Film Processing. The effects of polymer film thickness and casting solvents on the degradation kinetics were also investigated. PEAP films 30 and 90 μm thick were prepared and their degradation was studied in phosphate buffer at pH 7.4 (Figure 7). No significant effect of film thickness was observed on the kinetics of PEAP hydrolysis as measured by the percent of mass loss. This confirms a "bulk" mechanism of erosion, when the entire polymer film, not just the surface, is affected by the hydrolysis from the early stages. Once again, significantly faster decrease in the molecular weight was observed as compared to the mass loss for both films. The molecular weight drop appeared to be slightly slower for 90 μm film, which might indicate some diffusion limitations for the hydrolysis of a thicker film (Figure 7).

Degradation studies reported in the present study were conducted on polymer films prepared with THF as a casting solvent. Films were also formed with toluene and their hydrolysis was compared with the ones prepared with THF (Figure 8). There was no noticeable effect on the degradation in phosphate buffer, pH 7.4, as measured by either mass loss or molecular weight decrease (Figure 8).

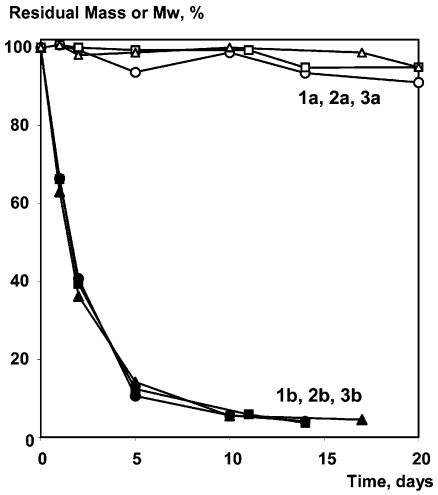


Figure 6. Residual mass (a) and residual molecular weight (b) of PEAP vs time in phosphate (1), borate (2), and Tris (3) buffers (pH 8.0, 100 mM buffers, 55 °C).

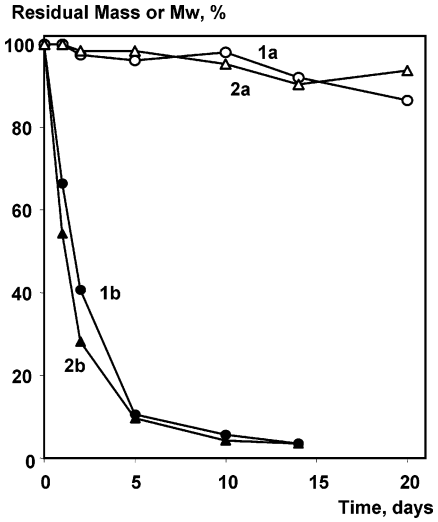


Figure 7. Residual mass (a) and residual molecular weight (b) of PEAP vs time for films 90 μm (1) and 30 μm (2) thick (pH 7.4, 100 mM phosphate buffer, 55 °C).

Conclusion

Hydrolytic degradation of two polyaminophosphazenes, PEGP and PEAP, formulated in films was studied in aqueous solutions. The hydrolysis process was characterized by a simultaneous decrease in polymer mass, molecular weight, and the release of low molecular degradation products: phosphates and ammonia. In accordance with previous findings, the degradation rate was higher for PEGP and, as expected, increased with temperature. Dramatically faster decrease of the molecular weight with time was observed as compared to the mass loss of the samples. Decrease in the pH of the buffer from 8.0 to 5.5 has led to a higher degradation rate of PEGP, as measured by mass loss, but had no noticeable effect on PEAP. The hydrolysis rate remained unaffected by various types of buffers, both inorganic and organic. The degradation rate of PEAP was not sensitive to the type of casting solvents; however, there was a slightly faster kinetics of the molecular weight decrease for thinner films.

Present findings provide conclusive evidence of a bulk mechanism of degradation for both of the studied polyphosphazenes. Since many drug delivery applications dictate the need for a surface-controlled erosion,[20] it can be desirable to study

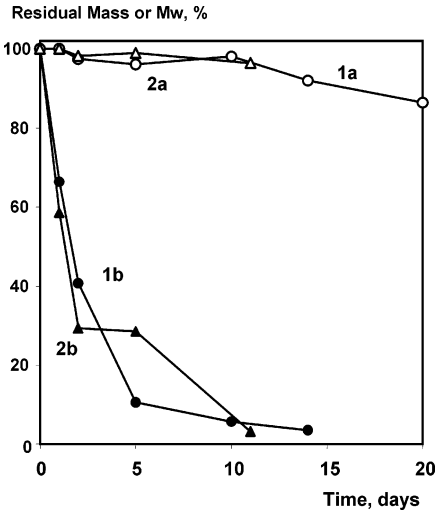


Figure 8. Residual mass (a) and residual molecular weight (b) of PEAP vs time for film cast from THF (1) and film cast from toluene (2) (pH 7.4, 100 mM phosphate buffer, 55 °C).

the existing compounds and synthesize new derivatives containing more hydrophobic substituents. The versatility of polyphosphazene synthetic methods open remarkable opportunities for such endeavors, with dozens of more hydrophobic degradable polyphosphazene derivatives already synthesized.[10] While it is difficult to expect any polymer to be hydrophobic enough for water not to penetrate the bulk and for the erosion process to be confined to surface layers only,[21] some polymers, such as poly(ortho esters), polyanhydrides, and certain polyester copolymers, are capable of displaying predominant surface degradation.[21−23] Thus hydrophobic polyphosphazenes, in all likelihood, will be the focus of new research efforts. Considerable interest demonstrated recently in degradable water-soluble and hydrophobic polyphosphazenes[1−12,16,20,23] will most likely be sustained also due to the predictability of their degradation products, remarkable flexibility of their backbone, and ease of their biological functionalization.

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