Hydrolytic Degradation Of Ionically Cross-Linked Polyphosphazene Microspheres

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SYNOPSIS

The hydrolytic degradation of gel microspheres based on calcium cross-linked phosphazene polyelectrolytes, poly[di(carboxylatophenoxy)phosphazene] (PCPP) and poly-[(carboxylatophenoxy)(glycinato)phosphazene] (PCGPP), was investigated. These microspheres are of importance as carriers in protein and cell encapsulation. Both PCPP and PCGPP ionotropic polyphosphazene hydrogels are degradable in an aqueous environment (pH 7.4, 37°C). The degradation rates can be increased by incorporation of hydrolysis sensitive glycinato groups as the pendant structures in the polymer (PCGPP). Hydrolysis of these polymer hydrogels led to low molecular weight (< 1,000 Da) products. The erosion and molecular weight profiles varied also according to the molecular weight of the polyphosphazene constituting the gel beads. Another approach to affect the degradation rates consists of coating microspheres with poly-L-lysine. Ionotropic polyphosphazene hydrogels have potential as biodegradable devices for controlled drug delivery systems. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Biodegradable polymers are investigated extensively as materials for controlled release applications.[1] Drug release profiles from biodegradable polymer matrixes can be efficiently tailored by determining the hydrolytic breakdown of the macromolecular system. Many of the synthetic polymers possessing hydrolytically labile backbone bonds (polyiminocarbonates, polyesters, polyanhydrides, polyorthoesters, and polyamides) are hydrophobic. These polymers usually require use of organic solvents or heat for fabrication.[2,3]

Recent developments in genetic engineering have increased the number of available therapeutically important polypeptides. This created a need for hydrophilic and amphiphilic materials that can be used to encapsulate polypeptide drugs. Nonbiodegradable polymer hydrogels have been studied widely as controlled release systems[4-6] because of their biocompatibility, mild conditions of encapsulation, matrix permeability, pH, and thermosensitivity. However relatively few types of synthetic biodegradable hydrogels have been developed and in most cases the degradation is usually limited only to the hydrolysis of the cross-linking agents or specific regions in the macromolecular backbone.[7-10]

Recently the synthesis of a novel polyphosphazene with anionic groups, the sodium salt of poly-[di(carboxylatophenoxy)phosphazene] (PCPP), that undergoes a liquid–gel phase transition upon contact with aqueous solutions of divalent cations, such as calcium ions, at room temperature was reported.[2,11] This has enabled the development of an extremely mild encapsulation method for biologically labile entities, especially proteins, that had not been previously possible with synthetic polymers. Hydrolytic stability or instability of polyphosphazenes is determined not by changes in the backbone structure as for hydrocarbon polymers, but by changes in the side groups attached to the backbone; thus they can be easily modified during polymer synthesis.[12] In the

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present article we investigate the ability of ionotropic polyphosphazene hydrogel microspheres to degrade in aqueous solutions and the possibility of controlling the rate of macromolecular breakdown by introducing a hydrolysis-sensitive glycinato group as a pendant group on the polyphosphazene.

EXPERIMENTAL

Materials

Hexachlorocyclotriphosphazene (mp 110–113°C) was obtained from a tetramer-trimer mixture (Ethyl Corp.), which was purified by two fractional vacuum sublimations.[11] Propyl *p*-hydroxybenzoate (Aldrich Chemical Co.) was purified by recrystallization from methylene chloride and hexane. Triethylamine (Aldrich) was purified by vacuum distillation in the presence of calcium hydride and was stored over molecular sieves before use. Potassium *tert*-butoxide (Aldrich), ethyl glycinate hydrochloride (Aldrich), sodium hydride (Aldrich), poly(L-lysine) (Sigma Chemical Co., St. Louis, MO), and solvents were reagent grade.

PCPP

Poly(dichlorophosphazene) was synthesized by the thermal ring-opening polymerization of hexachlorocyclotriphosphazene at 250°C. PCPP was prepared by chemical modification of poly(dichlorophosphazene) with the sodium salt of propyl *p*-hydroxybenzoate, followed by hydrolysis of ester groups to carboxylic acid.[11]

Preparation of Poly[(carboxylatophenoxy)(glycinato) phosphazene] (PCGPP)

Poly(dichlorophosphazene) (5.0 g, 0.0425 *M*) was dissolved in 300 mL tetrahydrofuran (THF). The sodium salt of propyl *p*-hydroxybenzoate (prepared by reacting propyl hydroxybenzoate, 30.6 g, 0.17 *M*, with 60% sodium hydride, 6.12 g, 0.15 *M*, in mineral oil) was added dropwise to the polymer solution. After addition of the sodium salt, the reaction mixture was stirred under reflux for 2 days and the course of the reaction was monitored by ^{31}P NMR (Brucker EM360 spectrometer).

Ethyl glycinate hydrochloride (23.63 g, 0.17 *M*) was suspended in 50 mL toluene containing triethylamine (23.69 g, 0.17 *M*) and was refluxed for 3.5 h to remove the resulting triethylamine hydrochloride salt. The reaction mixture was cooled in an ice bath and triethylamine hydrochloride, appearing as a white powder, precipitated from the solution. The solution was filtered and added dropwise to the propyl ester polymer mixture at 0°C. The reaction mixture was allowed to warm to room temperature and stirred for 2 days. The polymer was purified by repeated precipitations into 100% ethanol (twice) and hexane (twice).

After purification, the polymer (0.5 g, 1.33 m*M*) was dissolved in dry THF (20 mL). The solution was added slowly to a mixture of potassium *tert*-butoxide and water in dry THF (100 mL). The mixture was cooled to 0°C for the first 5 min, and was then stirred at room temperature. A large excess of ice water (300 mL) was added, and the solution was concentrated by evaporation. The polymer was isolated by acidification of the solution with hydrochloric acid to pH 5.5. The conditions of reactions and weight average molecular weights of obtained polymers were measured by gel permeation chromatography (GPC) in water using an Ultragel 2000 column (Waters, Milford MA) (Table I).

The structures of polymers were confirmed by ^{1}H and ^{31}P NMR (Brucker EM360 spectrometer) and elemental microanalysis (Galbraith Laboratories, Inc., Knoxville, TN).

Gelation of Polyorganophosphazenes, Microsphere Preparation, and Characterization

Aqueous solutions of PCPP and PCGPP were obtained by deprotonation in equimolar amounts of 3% sodium carbonate solution and dissolution in 13

Table I Hydrolyis of Poly(propyl oxybenzoato)(ethyl glycinato)phosphazene]

No.	Concentration of Polymer (% w/v)	Concentration of Potassium Butoxide (mol/L)	Concentration of Water (mol/L)	Reaction Time (h)	M_w (kDa)
1	0.42	0.30	0.1	42	80
2	0.42	0.15	0.05	18	130
3	0.42	0.04	0.05	5	170

m*M* HEPES buffer (pH 7.4). Gel microbeads of controlled size (0.5–1.5 mm) and spherical shape were prepared by spraying the 2.5% (w/w) polymer solution as microdroplets into the gelation solution (7.5% calcium chloride solution in water) using an air jet-syringe pump droplet generator.[2] With this system, the polymer solution is extruded using a modified Razel syringe pump from a 5-mL plastic syringe through a needle located inside a nozzle. The shear forces generated by the flowing air stream inside the nozzle detach the droplets forming at the needle tip and spray them into the calcium chloride solution where they gel and harden for 30 min. The microspheres were collected by draining the calcium chloride solution using a mesh column (Econocolumn, BioRad) and washed three times in deionized water.

Microsphere Coating with Polycation

Calcium cross-linked PCGPP beads were reacted with a positively charged polyelectrolyte by suspension in 30 mL aqueous poly(L-lysine) (PLL) solution at room temperature. The unreacted polycation was removed by washing the beads three times with HEPES buffer, pH 7.4 (total volume of 150 mL) and then with 50 ml phosphate buffered saline (PBS), using the mesh column. The resultant microspheres were suspended in PBS and kept at 4°C until usage.

Degradation Studies

Degradation of calcium-polyphosphazene microspheres was studied at 37°C in an air gravity incubator (Imperial II Incubator, Lab-Line Instruments, Inc.), with gentle agitation on a rotating shaker (ORBIT Shaker, Lab-Line Instruments, Inc.) in vials containing 500 mg of microspheres and 5 mL of 13 m*M* HEPES buffered saline solution (pH 7.4). The degree of swelling was determined gravimetrically. Matrix samples from degradation experiments were washed with deionized water; freeze-dried; weighed to determine mass loss; incubated in 0.2 mL of 2% sodium carbonate for 5 min; dissolved in 1.8 mL of HEPES buffered saline solution (pH 7.4); and filtered through a 0.45-μm polycarbonate filter (Millipore) for GPC analysis.

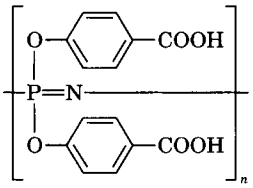
The molecular weight of polyphosphazenes was determined by a Perkin–Elmer Series 10 liquid chromatograph with refractive index and UV detection by using an Ultragel 2000 column (Waters, Milford MA). HEPES buffered saline solution (13 m*M*, pH 7.4) was used as an eluent. Chromatograms were processed by GPC 5 and CHROM 2 software (Perkin–Elmer) to calculate the weight average and number average molecular weights using polyacrylic acid (M_W 1,250–1,100,000 Da, Polymer Laboratories LTD, Ghurch Stretton, UK) as a standard.

RESULTS AND DISCUSSION

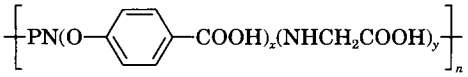
Polymer Synthesis and Microsphere Preparation

Poly(organophosphazenes) have a backbone of alternating nitrogen and phosphorus atoms with two side groups attached to each phosphorus. Molecular structural changes in these polymers are achieved mainly by a macromolecular substitution reaction of poly(dichlorophosphazene), using a wide range of chemical reagents.[13]

PCPP and PCGPP were prepared by the derivatization of poly(dichlorophosphazene) via nucleophilic substitution reactions, with the sodium salt of propyl *p*-hydroxybenzoate (PCPP synthesis) or by subsequent treatment with the sodium salt of propyl *p*-hydroxybenzoate and ethyl glycinate hydrochloride (PCGPP synthesis). Poly[di(aryloxy)-phosphazene] and poly[(aryloxy)(glycinato)-phosphazene] esters were hydrolyzed to the corresponding poly(carboxylic acids). Polymers were characterized by ^{1}H and ^{31}P NMR, elemental analysis, and GPC.



PCPP

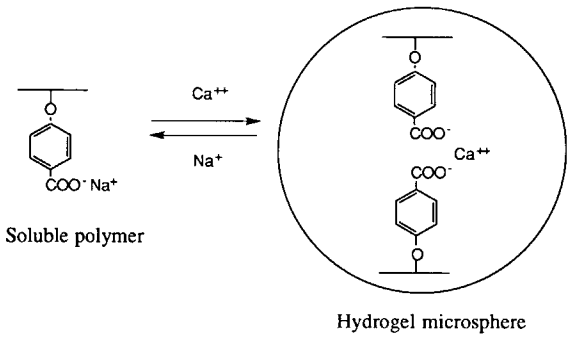


PCGPP

Due to their polyelectrolyte nature, polyphosphazenes can be cross-linked by treatment with dissolved cations such as calcium ions, to form a hydrogel matrix.[11] A process for preparation of gel microspheres of controlled size involves generation of microdroplets of polymer solution by pumping it into a spray nozzle and spraying by pressurized air. A spray cloud impacts a calcium chloride solution in water, that causes cross-linking of the polymer to form gel ionotropic microspheres (Scheme 1).

Degradation of PCPP Microspheres

Degradation of polyphosphazene microspheres was studied in HEPES buffered saline solution (pH 7.4)



Scheme 1 Ionic cross-linking of polyphosphazenes.

by monitoring mass loss, molecular weights of polymer matrices, and formation of soluble products. Erosion profiles for PCPP microspheres prepared from polyphosphazenes of varied molecular weights are shown in Figure 1. No detectable mass loss was observed during 180-days incubation of high molecular weight (3,900 kDa) PCPP microspheres in solution (Fig. 1). However GPC data showed significant decreases in polymer molecular weight during the same time period [Fig. 2(a)]. The mechanism of degradation apparently can involve intramolecular carboxylic group catalysis.[12] For the microspheres prepared from low molecular weight PCPP (400 kDa), decreases in polymer molecular weight [Fig. 2(b)] was accompanied by significant erosion of the hydrogel during the first 10 days. Formation of water-soluble polymeric products of practically the same molecular weight as in the matrix was detected after 4 days of incubation. It appears that chain scission occurs in the cross-linked insoluble polymer and the soluble oligomeric products at the same rates. These data also indicate that there is a molecular weight threshold of approximately 200 kDa in the release of polyphosphazene from the matrix into the solution in this system. This is consistent with data for the degradation of polymers with limited solubility in water. Random hydrolytic chain

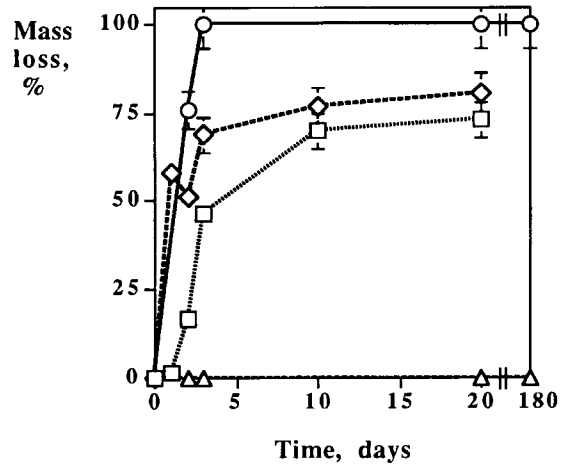
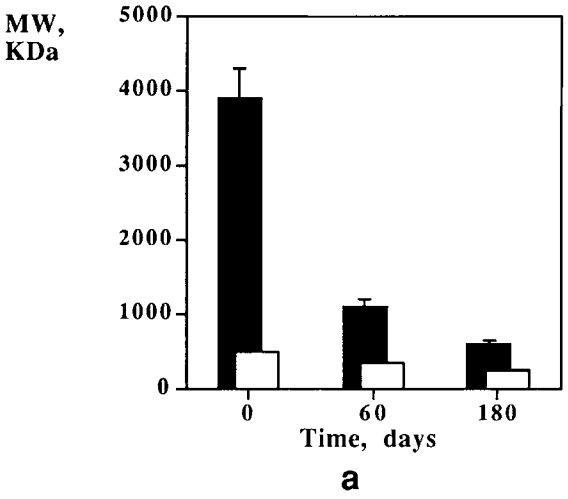


Figure 1 Erosion profiles of calcium polyphosphazene microspheres. Microspheres were obtained by ionic cross-linking of PCPP with molecular weight (△) 3,900 kDa and (□) 400 kDa, and PCGPP with molecular weight (○) 130 kDa and (◇) 170 kDa.



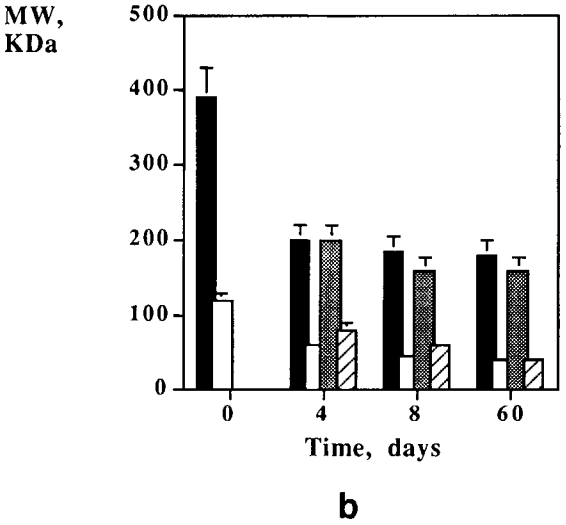


Figure 2 Molecular weight degradation profiles for polyphosphazene hydrogel microspheres formed by ionic cross-linking of PCPP with molecular weight (a) 3,900 kDa and (b) 400 kDa. Microspheres were incubated in HEPES buffered saline (pH 7.4). (□) Weight-average and (▨) number-average molecular weights of water-soluble degradation products in supernatant were determined. Matrix samples were isolated and then dissolved to measure (■) weight average and (□) number average molecular weights.

cleavage of a hydrophobic polymer backbone usually proceeds until a critical molecular weight is reached, from which weight loss begins with the diffusion of cleaved water-soluble fragments out of a matrix.[14] However, it is obvious that polyphosphazene solubility also depends on the amount of calcium ions held by the matrix and the ionization degree of macromolecules. The observed differences in the erosion of PCPP of different molecular weights are of prime importance for the design of drug delivery systems.

Degradation of PCGPP Microspheres

Polyphosphazenes can be efficiently tailored by incorporating appropriate side groups to provide a controllable set of properties, including hydrolytic degradability.[12] It was anticipated that introduction of a hydrolysis-sensitive pendant group would provide sites for rapid hydrolytic chain cleavage and allow the increase of the degradation rate in an aqueous environment.

Polyphosphazenes with amino acid esters, such as PCGPP, are degradable in neutral aqueous media.[12,15,16] Although this polymer is insoluble in water it is a slowly degrading material due to the low accessibility of water into the polymer matrix.[16] The hydrolysis of PCGPP involves initial hydrolysis of the ester to the carboxylic side group, followed by hydrolytic cleavage of an external P—N bond to yield unstable hydroxyphosphazenes.[12] According to the assumed reaction mechanism, hydrolytic susceptibility of the polymer backbone can also be enhanced by using glycine groups instead of ethyl glycinato groups as cosubstituents. It was anticipated also that immobilization of glycinato groups in a hydrophilic matrix, such as an ionotropic polyphosphazene hydrogel, would lead to an increase in their reactivity with water.

Calcium PCGPP microspheres were subjected to *in vitro* degradation under the same conditions as calcium PCPP microspheres. As little as 10% of glycinato side groups has a dramatic effect on microsphere erosion. PCGPP with a weight average molecular weight 130 kDa degraded into completely water-soluble products within 3 days [Figs. 1, 3(b)]. A sample with higher molecular weight, 170 kDa, degraded more slowly [Figs. 1, 3(a)], which is similar to the results obtained earlier for PCPP microspheres. Dependence of the erosion rates on the molecular weight of polymers constituting hydrogel microspheres is also in accordance with reported results for bioerdible alginate matrices.[17] GPC analysis of matrix and soluble products showed [Fig. 3(b)] that a 240-day incubation of 130-kDa PCGPP in an aqueous environment results in breakdown of the polymer backbone leading to fragments with molecular weights lower than 1 kDa and inorganic phosphate.

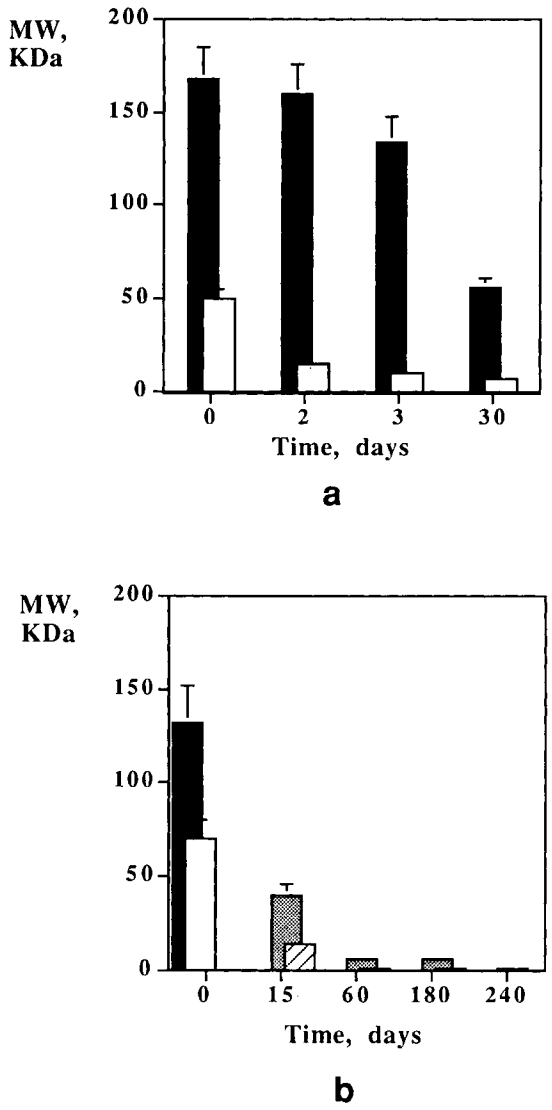


Figure 3 Molecular weight degradation profiles for polyphosphazene hydrogel microspheres formed by ionic cross-linking of PCGPP with molecular weight (a) 170 kDa and (b) 130 kDa. (□) Weight average and (▨) number average molecular weights of water-soluble degradation products in supernatant were determined. Matrix samples were isolated and then dissolved to measure (■) weight average and (□) number average molecular weights.

Degradation of Polyphosphazene Microspheres Coated with PLL

The permeability and release rates from polyphosphazene microspheres can be affected by the creation

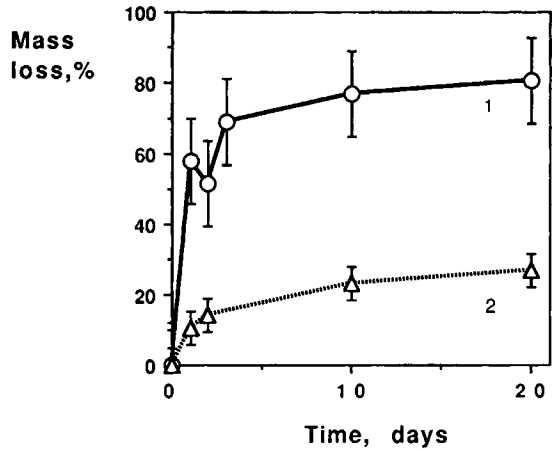


Figure 4 Erosion profiles for (1) calcium PCGPP hydrogel microspheres and (2) the same microspheres coated with PLL (M_W 62 kDa).

of semipermeable membranes around the capsule. These membranes are formed by interacting the microspheres with the positively charged polyelectrolyte, PLL, resulting in the formation of a stable PCPP–PLL polyelectrolyte complex[2] and additional cross-linking of the matrix. This treatment can also contribute to the maintenance of capsule stability in physiological saline solution because the polyelectrolyte complex is much less sensitive to the effects of ionic strength than calcium cross-linked polymer.[18] Coating PCGPP hydrogel microspheres with PLL (M_W 62 kDa) leads to a fourfold decrease of the erosion rate (Fig. 4) apparently because of steric hindrance and an increase of matrix hydrophobicity. This appears to provide an additional approach to control the degradation and stability of polyphosphazene microspheres.

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