Synthesis, Properties, and Biological Activity of Poly[di(sodium carboxylatoethylphenoxy)phosphazene]

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A new water-soluble polyphosphazene polyelectrolyte containing carboxylate functionalities, poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP) was synthesized via reaction of macromolecular substitution. The polymer was characterized using ^{1}H, ^{31}P NMR, and gel permeation chromatography with multiangle laser light scattering detection. PCEP was shown to undergo hydrolytic degradation in aqueous solutions, as indicated by the decrease in the molecular weight and the release of side groups. A series of incompletely substituted copolymers of PCEP containing varying amounts of residual chlorine atoms was also prepared. The rate of degradation for such copolymers increased with the rise in the content of chlorine atoms. In vivo studies demonstrated high potency of PCEP as a vaccine immunoadjuvant. The new polyphosphazene was also shown to be capable of forming microspheres in aqueous solutions via reactions of ionic complexation with physiologically occurring amines, such as spermine.

Introduction

Water-soluble polyphosphazene polyelectrolytes present interest for biomedical applications. They have been studied as hydrogel-forming materials, microencapsulating agents, active components of vaccine formulations, and environmentally responsive polymers.[1−13] The phosphazene backbone used in the construction of such polyelectrolytes offers the advantages of modulated hydrolytic degradation and high flexibility of the polymer skeleton.[14−16] In addition, the macromolecular substitution approach employed in the synthesis of polyphosphazenes gives rise to an unprecedented structural diversity of this class of polymers.[14]

One of the most important potential applications of polyphosphazene polyacids is based on their activity as immunoadjuvants—compounds that enhance the immune response when formulated with vaccine antigens. Poly[di(sodium carboxylatophenoxy)phosphazene], PCPP, has demonstrated potency in vivo with a broad range of bacterial and viral antigens and was advanced into clinical trials.[1−4,6,17−19] It was suggested that the biological activity of PCPP is linked to its ability to form water-soluble noncovalent complexes with antigenic proteins enabling their efficient presentation to immunocompetent cells.[2] The physicochemical properties of such complexes appear to affect their biological behavior.[2] It has been shown that the immunoadjuvant activity of PCPP copolymers is dramatically dependent on the nature of side groups present in the polymer structure.[1−3] However, little is known about the activity of other polyphosphazene polyacids. The establishment of structure−activity relationship in such systems is a critical step in understanding the mechanism of action and in optimization of the existing and development of new potent immunoadjuvants.

In the present Article we report the synthesis, characterization, and studies of the in vivo activity of a new polyphosphazene polyelectrolyte, poly[di(sodium carboxylatoethylphenoxy)phosphazene], PCEP. We also investigate the ability of this polymer to degrade in an aqueous environment and its potential as a microencapsulating agent.

Experimental Section

Materials. Hexachlorocyclotriphosphazene, trimer (Nippon Fine Chemicals, Japan); 2-methoxyethyl ether (diglyme), anhydrous, 99.5%; sodium hydride, 95% (Aldrich Chemical Co., Inc., Milwaukee, WI); potassium hydroxide, hydrochloric acid (38%); sodium chloride (VWR, West Chester, PA); ethanol (EMD Biosciences, Gibbstown, NJ); spermine tetrahydrochloride (*N*,*N*′-bis(3-aminopropyl)-1,4-butanediamine tetrahydrochloride) (TCI America, Portland, OR) were used as received. Methyl 3-(4-hydroxyphenyl)propionate (MHP) (Spectrum Chemical, Gardena, CA) was dried under vacuum at room temperature for 18 h. The macromolecular precursor, poly(diclorophosphazene), PDCP, was synthesized using ring-opening polymerization of hexachlorocyclotriphosphazene in the titanium pressure reactor as described previously.[20]

Analytical Methods. The HPLC system was configured as follows: a Waters 600 HPLC pump (Waters, Milford, MA); two inline filters, a 0.5 μm high-pressure filter (Rainin, Woburn, MA) and a 0.02 μm filter (Anodisc 25, Whatman International Limited, Maidstone, England) in a high-pressure stainless filter holder (Millipore, Bedford, MA); a Waters 717plus autosampler; an Ultrahydrogel Linear column (Waters, Milford, MA); a multiangle laser light scattering (MALLS) detector (DAWN DSP-F, Wyatt Technology, Santa Barbara, CA); a Waters 996 photodiode array detector; and a Waters 410 refractive index detector. Phosphate-buffered saline (PBS, pH 7.4) containing 5% acetonitrile was used as a mobile phase with a flow rate of 0.75 mL/min and an injection volume of 0.05 mL. Method development for determining molecular weights of polyphosphazene polyacids using the GPC-MALLS system was described previously.[21] ^{31}P, and ^{1}H NMR spectra were recorded using a Bruker 400 NMR spectrometer; D_2O was used as a solvent. Elemental analysis was performed by Desert Analytics Laboratory (Tucson, AZ).

Synthesis of PCEP. A solution of methyl 3-(4-hydroxyphenyl)-propionate (108.1 g, 0.6 mol) in diglyme (0.25 L) was heated at 110 °C for 30 min under nitrogen and then cooled to room temperature. A suspension of sodium hydride (14.4 g, 0.57 mol) in diglyme (0.21 L) was slowly added to the solution of methyl 3-(4-hydroxyphenyl)-propionate while stirring under nitrogen to form the sodium salt of

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methyl 3-(4-oxyphenyl)propionate (NaMOP). The reaction mixture, which became clear, was kept at ambient temperature for 1 h. The temperature was then increased to 50 °C, and the solution of PDCP (2.32 g, 0.02 mol) in 0.03 L of diglyme was slowly added (for 30 min). The temperature was increased to 120 °C, and the reaction was continued at this temperature for 10 h while stirring. The reaction mixture was then cooled to 85 °C, and 0.3 L of aqueous potassium hydroxide (12.7 N) was added. The reaction was vigorously stirred for an additional hour at 85 °C. The precipitated polymer was recovered by decanting the liquid layer, dissolved in 0.2 L of deionized water, and precipitated in 1.8 L of ethanol. It was then redissolved in 0.3 L of deionized water (pH was adjusted to 6.3 by addition of 1 N hydrochloric acid) and precipitated again by adding 0.6 L of 30% (w/v) sodium chloride solution. The polymer was then redissolved in 0.3 L of deionized water (pH of the solution was adjusted to 7.4 by the addition of 1 N aqueous sodium hydroxide) and precipitated by addition of 0.9 L of ethanol. The yield after drying in vacuum was 5.3 g (70.7%). ^{1}H NMR, δ (ppm): 2.25, 2.65, 6.6, 6.8; ^{31}P NMR, δ (ppm): −18.3.

Synthesis of incompletely substituted PCEP was conducted using the method described above varying the amounts of NaMOP, reaction temperature, and time. The yields were 26.7% (polymer with 2 mol % chlorine atoms), 58.7% (3 mol %), 47.0% (5 mol %), 88.0% (12 mol %), and 26.7% (15 mol %).

Degradation Study. Polymer solutions were prepared at 1 mg/mL concentration in Tris buffer, pH 7.4 (0.1 M Tris, 4.5% NaCl) and then were filtered using 0.45 μm Millex-HV syringe filters (Millipore, Bedford, MA). Degradation studies were performed at 55 °C; vials containing polymer solutions were incubated in a G24 Environmental incubator shaker (New Brunswick Scientific, Edison, NJ). Two milliliter samples were collected periodically for the determination of molecular weight and degradation products. Analysis was conducted using size exclusion HPLC with multiangle laser light scattering, UV photodiode array, and refractive index detection systems. Absolute molecular weight parameters were determined using light-scattering detection, with the refractive index detector as a mass detector, and ASTRA 2.1 software (Wyatt Technology, Santa Barbara, CA). The concentration of 3-(4-hydroxyphenyl)propionic acid was determined by HPLC with UV detection at 230 nm using Millenium software (Waters, Milford, MA).

In Vivo Evaluations. PCEP was evaluated in vivo for its ability to enhance the immune response (adjuvant activity) to hepatitis B surface antigen, HBsAg (Biodesign International), and X-31 influenza (Charles River Laboratories). PCPP, which was used as a control, was obtained and purified as described previously.[1] PCEP was synthesized using the approach described above. To ensure the high purity of the material, the polymer was purified by preparative size exclusion HPLC (Biocad Perfusion Chromatography Workstation, Applied Biosystems, Foster Hills, CA; Modcol CER 3662 column) with aqueous 0.05 N ammonium carbonate buffer solution (pH 8.5) as a mobile phase. The sample was then isolated by lyophilization.

Immunization was conducted as follows. 0.04, 0.2, and 1 μg doses of HBsAg were used. 100 μL of PBS containing a dose of antigen mixed with 50 μg of the polymer (PCEP or PCPP) was injected in each mouse. BALB/c mice were used (5 mice per group). Formulations containing antigen alone and antigen formulated with 50 μg of PCPP were used as controls. Mice were immunized with a single intramuscular injection. Blood samples were collected 4 weeks postimmunization and serum stored until analysis.

Antigen-specific antibodies (IgG) in mouse serum were determined by ELISA in 96-well Immunolon II plates coated with HbsAg in sodium carbonate buffer, pH 9.6. The plates were washed six times with PBS containing 0.05% Tween 20 (PBST). Two-fold serial dilutions of sera in PBST containing 0.5% gelatin were added to the wells, and the plate was incubated 2 h at ambient temperature. Unbound serum was removed by washing the plates six times with PBST. Biotinylated Goat Anti-Mouse IgG (Caltag Laboratories) was added, and the plates were incubated for 1 h at ambient temperature. The plates were washed six times with PBST, and alkaline phosphatase conjugated streptavidin (BioCan Scientific) was added, and the plates were incubated for 1 h at ambient temperature. Unbound conjugate was removed by washing eight times with deionized water, and serum antibodies were detected by adding 1 mg/mL of *p*-nitrophenyl phosphate di(Tris) salt in 1% diethanolamine−0.5 mM magnesium chloride buffer, pH 9.8. The reaction was allowed to run for 15 min, and the absorbance was measured at 405 nm using a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA). The endpoint titers were the reciprocal of the highest sample dilution producing a signal identical to that of an antibody-negative sample at the same dilution plus 3 times the standard deviation. The average antibody titers for a group of mice were expressed as geometric mean titers (GMT).

Formulations with influenza were evaluated in mice similarly as described, except that X-31 influenza (Charles River Laboratories) was used instead of HBsAg. Doses of 0.2, 1.0, and 5 μg of X-31 were employed.

Microsphere Preparation. A volume of 0.394 mL of 7% (w/v) solution of spermine tetrahydrochloride in PBS (pH 7.4) was added to 105 mL of 0.0167% (w/v) solution of PCEP in PBS (pH 7.4). The mixture was agitated gently by shaking, incubated at ambient temperature for 10 min, and was thereafter examined for the presence of particulates using the Malvern Mastersizer S (Malvern Instruments Inc., Southborough MA) and Olympus CK-2 inverted microscope (Olympus, Japan).

Results and Discussion

Synthesis of PCEP. PCEP was synthesized using macromolecular substitution route as shown in Scheme 1. Reactive polymeric precursor, PDCP, was first prepared using melt polymerization reaction,[20] and then chlorine atoms of the polymer were replaced with methyl phenylpropionate groups. Alkaline hydrolysis of the substituted polymer using potassium hydroxide yielded PCEP. The reactions were carried out without isolation of intermediates using a single-pot/single-solvent procedure described previously.[1]

The substitution reaction was monitored by ^{31}P NMR and assumed to be completed if only one peak was present in the spectrum. The degree of substitution and presence of residual chlorine atoms in the product are important characteristics, which previously have been shown to affect biologically relevant properties of the polymer, such as degradation.[1] To investigate polymers with various contents of residual chlorine atoms, the reaction time, temperature, and the ratio between the nucleophilic reagent and PDCP were varied. As a result of these experiments samples were obtained which displayed more than one signal in the ^{31}P NMR spectrum indicating the presence of unsubstituted chlorines atoms (PCEP-C). The reaction conditions used for their preparation and properties of the resulting polymers are shown in Table 1.

Polymer Characterization. The structure of PCEP was confirmed by ^{1}H NMR and ^{31}P NMR. As described above, the ^{31}P NMR spectrum of PCEP showed one broad singlet at −18.3 ppm (Figure 1, curve 1). In contrast, samples of incompletely substituted polymer (PCEP-C) contained an additional peak at −14.5 ppm, which can be ascribed to Cl−P−$OC_6H_4C_2H_4$-COONa atoms (Figure 1, curves 2 and 3). On the basis of this assumption the amounts of residual chlorine atoms were determined to be in the range of 0−15 mol % (Table 1). The content of chlorine atoms tends to decrease and the second peak in the ^{31}P NMR spectrum eventually disappears (Figure 1) as the substitution conditions are forced, mainly through the increase in the reaction temperature and nucleophile to PDCP ratio. The presence of chlorine atoms in the incompletely substituted samples was confirmed by the results of elemental

Scheme 1. Synthesis of PCEP

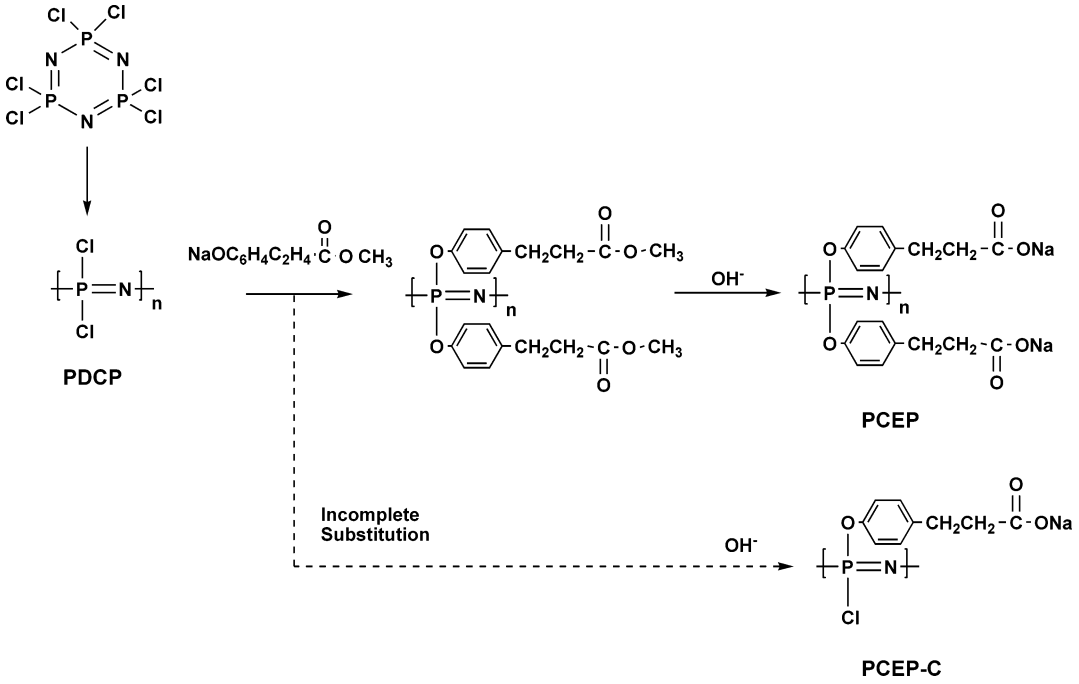


Table 1. Characterization of PCEP and Incompletely Substituted Polymers, PCEP-C

				reaction conditions		
		content of chlorine atoms	$M_w \times 10^{-3}$	NaMOP/ PDCP	T	time
polymer		mol %[a]	g/mol[b]	mol[c]	°C	h
PCEP		0	1800	28:1	120	8
PCEP-C	a	2	1230	20:1	110	10
	b	3	850	10:1	110	10
	c	5	1190	10:1	110	8
	d	12	1270	4:1	70	48
	e	15	1440	4:1	70	5

[a] Calculated based on ^{31}P NMR data. [b] Weight-average molecular weight, calculated based on MALLS-GPC data. [c] Sodium 3-(4-oxyphenyl)propionate (NaMOP).

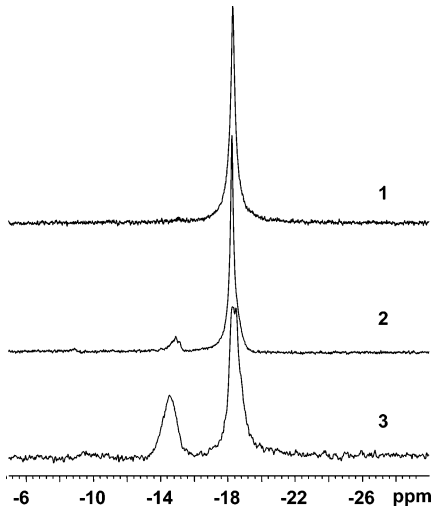


Figure 1. ^{31}P NMR spectra of PCEP (1) and incompletely substituted polymers containing 5 mol % (2) and 15 mol % (3) of residual chlorine atoms (PCEP-C c and e, correspondingly; see Table 1 for details).

analysis. Sample PCEP-C e, for example, was shown to contain 15 mol % of chlorine atoms based on the results of ^{31}P NMR analysis and 14.8 mol % by elemental analysis.

The presence of moisture in the reaction mixture can affect the structure of the incompletely substituted polymers. Use of MHP without drying leads to additional peaks in the ^{31}P NMR spectrum (Figure 2, curve 2) compared to the polymer obtained under the same conditions but with MHP dried under vacuum for 18 h (Figure 2, curve 1). This can be probably explained by the replacement of some of the chlorine atoms with hydroxyl groups in the process of the substitution reaction. Interestingly, the addition of water in the hydrolysis step to polymers that have a degree of substitution of 85 mol % and higher (PCEP-C a−e in Table 1) does not lead to the generation of significant amounts of hydroxyl groups on the polymer. Thus chlorine atoms on the polymer are more susceptible to hydrolytic attack during the first steps of the substitution reaction.

All polymers, including partially substituted samples, had molecular weights in excess of 800 000 g/mol (Table 1) as determined by the MALLS-GPC method.

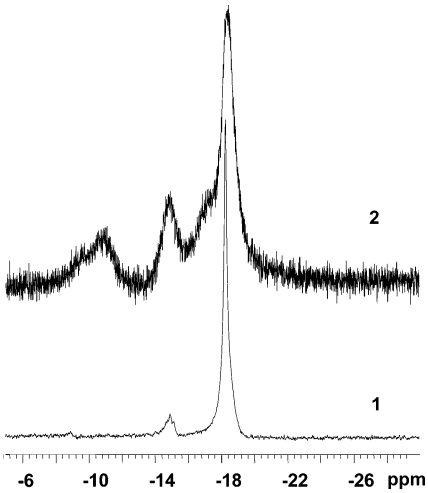


Figure 2. Effect of water in the reaction mixture on the polymer structure. ^{31}P NMR spectra of incompletely substituted polymer PCEP-C c (1) and a polymer obtained under the same conditions but without drying the nucleophilic reagent—MHP. Additional peaks can indicate the presence of the hydroxyl groups attached to the polyphosphazene backbone.

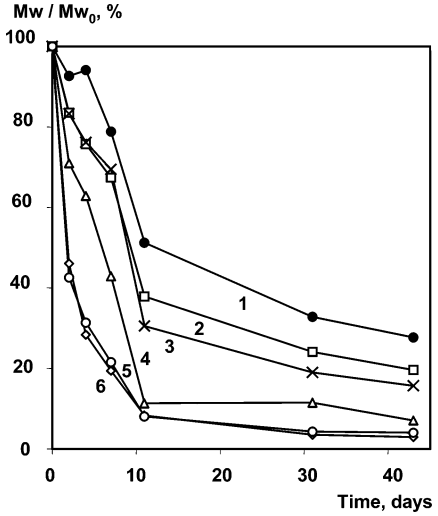


Figure 3. Kinetics of the molecular weight decrease for the degradation of PCEP (1) and incompletely substituted polymers containing various mol % of residual chlorine atoms: 2 mol % (2), 3 mol % (3), 5 mol % (4), 12 mol % (5), and 15 mol % (6) (PCEP and PCEP-C polymers a, b, c, and e, as shown in Table 1; M_{w0}, initial molecular weight; weight-average molecular weights determined by MALLS-GPC; 55 °C; 100 mM Tris buffer, pH 7.4).

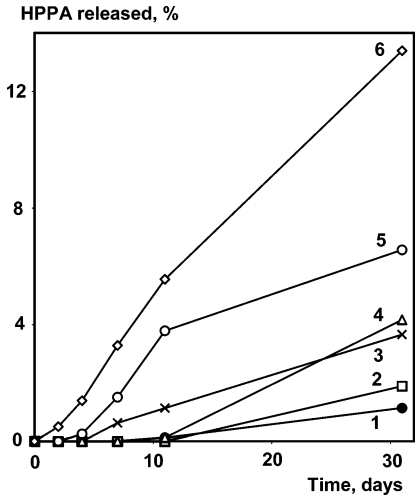


Figure 4. Kinetics of the HPPA release for the degradation of PCEP (1) and incompletely substituted polymers containing various mol % of residual chlorine atoms: 2 mol % (2), 3 mol % (3), 5 mol % (4), 12 mol % (5), and 15 mol % (6) (PCEP and PCEP-C polymers a, b, c, d, and e, as shown in Table 1; M_{w0}, initial molecular weight; weight-average molecular weights determined by MALLS-GPC; 55 °C; 100 mM Tris buffer, pH 7.4).

Hydrolytic Degradation. The ability of some polyphosphazenes to degrade hydrolytically is one of the key properties that make them attractive for biomedical applications.[14] A number of water-soluble polyphosphazenes have been shown to undergo degradation in aqueous solutions.[1,15,16] The rate of hydrolysis typically depends on the structure of the side group; thus, the degradation profile is an essential characteristic of a newly synthesized polyphosphazene. The ability of PCEP to undergo hydrolytic degradation was investigated in aqueous solutions at pH 7.4. Figures 3 and 4 show the kinetic profiles of molecular weight decrease and generation of hydroxyphenylpropionic acid, HPPA, (side group release) for PCEP and polymers containing residual chlorine atoms. For all studied polymers, the decrease in the molecular weight of the polymer with time is accompanied with the increase in the concentration of HPPA in solution. This is consistent with previous reports

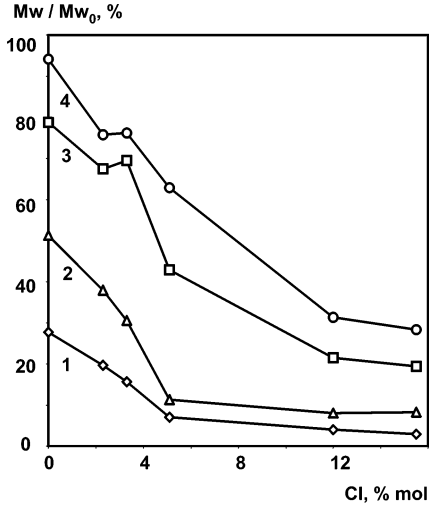


Figure 5. Molecular weight decrease during degradation of PCEP and incompletely substituted polymers PCEP a−e vs the mol % of chlorine atoms in the polymer at days 43 (1), 11 (2), 7 (3), and 4 (4).

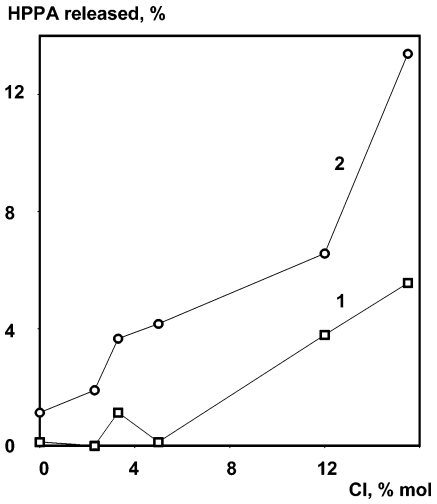


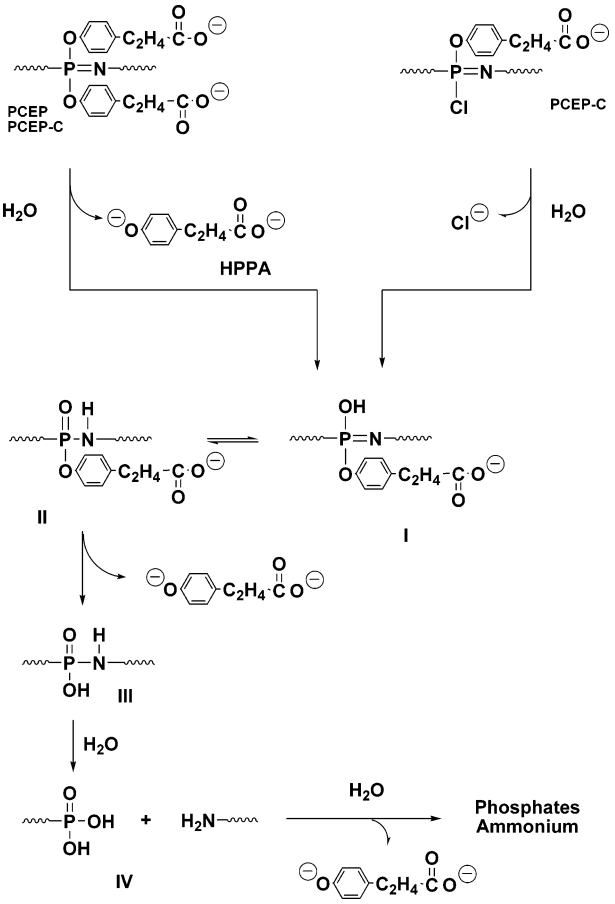
Figure 6. HPPA released during degradation of PCEP and incompletely substituted polymers PCEP a−e vs the mol % of chlorine atoms in the polymer at day 11 (1) and 31 (2).

showing that the mechanism of polyphosphazene degradation involves cleavage of the side group with the formation of the hydroxyl group, which in turn undergoes rearrangement into the unstable phosphazene moiety resulting in the breakdown of the backbone.

There is a clear dependence of the degradation rate on the content of the residual chlorine atoms in the polymer. The extent of degradation at various time points, as indicated both by the molecular weight and HPPA measurements, increases as the content of chlorine in the polymer rises (Figures 5 and 6). This suggests that residual chlorine atoms constitute "weak links" in the polyphosphazene structure and they undergo rapid cleavage thus accelerating the degradation process. It also appears that even if the polymer breakdown is initiated by the hydrolysis of P−Cl bonds, the degradation pathway still involves the cleavage of more stable carboxylatoethylphenoxy− side groups. The dependence of the released HPPA on the mol % of chlorine atoms in the polymer (Figure 6) supports this assumption.

Degradation pathways for various polyphosphazenes, including water-soluble systems, have been suggested previously.[14,16,22,23] A possible mechanism of the degradation of PCEP is outlined in Scheme 2, which summarizes some of the previously reported

Scheme 2. Potential Degradation Pathway



findings and the results of the present study. It appears that the cleavage of either carboxylatoethylphenoxy− side groups or chlorine atoms can be the first step in the degradation process resulting in the formation of hydroxyl derivative (**I**) and unstable phosphazane structure (**II**). As discussed above, hydrolytic cleavage of chlorine atoms of the polymer still leads to the experimentally observed increased release of HPPA, which can suggest the existence of intermediate (**III**). The hydrolysis culminates in the breakdown of the polymer chain (**IV**), and the final polyphosphazene degradation products can also include ammonium and phosphate ions.

Immunoadjuvant Activity. Water-soluble polyphosphazene polyelectrolytes demonstrated potential as compounds capable of enhancing the immune response when administered in vivo with bacterial and viral antigens. PCPP, the most investigated polyphosphazene immunoadjuvant, proved its potency in vivo with various antigens such as trivalent influenza virus vaccine, hepatitis B surface antigen, herpes simplex virus glycoprotein, tetanus toxoid, synthetic peptides, inactivated rotavirus particles, and others.[1−4,6,17−19] It was of interest to evaluate the immunoadjuvant activity of PCEP and compare it to the activity of PCPP.

Water-soluble formulations of PCEP with two antigens, hepatitis B surface antigen (HBsAg) and X-31 influenza (X-31), were prepared for in vivo studies. Each antigen was used at three different doses, while polymer was always kept at the same dose, 50 μg/mouse. Mice were immunized with a single intramuscular injection. The results demonstrate that PCEP is a powerful immunoadjuvant for both antigens (Figures 7 and 8). For all doses, PCEP formulations generated immune responses, which were approximately 10 times higher and more than the titers induced by those containing PCPP. Superior

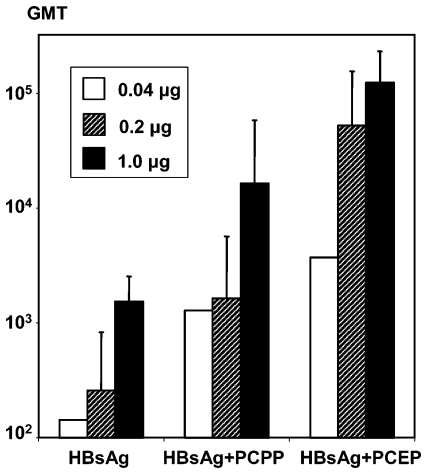


Figure 7. IgG titers after immunization of mice with HBsAg, HBsAg formulated with PCPP, and HBsAg formulated with PCEP at 0.04 μg (white bars), 0.2 μg (shaded bars), and 1 μg (black bars) antigen doses (5 BALB/c mice per group; polymers, 50 μg/mouse; single-dose intramuscular injection; 4 week data).

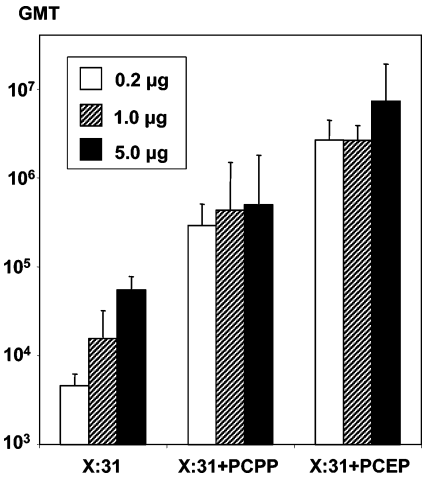


Figure 8. IgG titers after immunization of mice with X-31 influenza, X-31 influenza formulated with PCPP, and X-31 influenza formulated with PCEP at 0.2 μg (white bars), 1.0 μg (shaded bars), and 5.0 μg (black bars) antigen doses (5 BALB/c mice per group; polymers, 50 μg/mouse; single-dose intramuscular injection; 4 week data).

performance of PCEP, as compared to nonadjuvanted antigens and antigens adjuvanted with PCPP, is an important finding, which demonstrates the importance of minor changes in the polymer structure on its biological performance. Further studies are underway to understand the mechanism of such enhancement in the polymer's potency.

Preparation of Microspheres Using PCEP. Formulation of water-soluble immunoadjuvants into microspheric form can extend their utility in the areas of mucosal immunization and controlled vaccine release. Methods have been developed for formulating water-soluble polyphosphazenes into microparticulate delivery systems, while aqueous-based processes attracted special attention because of mild conditions for protein encapsulation.[5,8,10,12] A single-step technique involving ionic complexation of polyphosphazene adjuvants with physiologically benign organic amines, such as spermine and spermidine, was reported recently.[9] It was of interest to evaluate the ability of PCEP to act as a microencapsulation agent in this process. In a simple experiment a solution of spermine was added to a solution of PCEP in PBS (pH 7.4), and the mixture was then shaken and incubated at ambient temperature for 10 min. It was

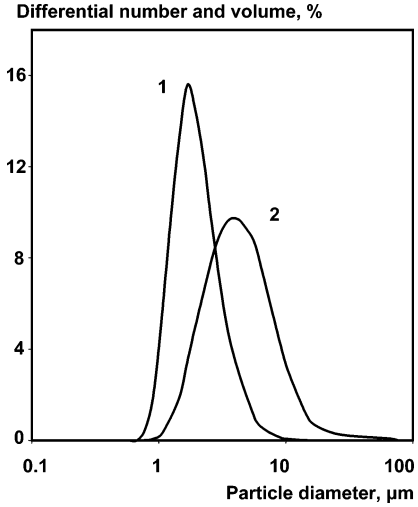


Figure 9. Size distribution profiles by number (1) and by volume (2) in the PCEP−spermine−PBS system (pH 7.4; 10 min incubation time).

then analyzed both by visual examination in an optical microscope and by a laser particle size analyzer. The microspheres were detected with a size in the range of approximately 1−10 μm (Figure 9). Size distribution profiles appeared to be unimodal and relatively narrow when calculated both by number and volume (Figure 9). As seen from the Figure relatively good correlation between these distribution moments was also observed, confirming the absence of large aggregates. These results demonstrate the potential of PCEP as a material for microsphere production.

Conclusion

A new water-soluble polyphosphazene polyelectrolyte containing carboxyl groups was synthesized and proved to be a potent immunoadjuvant in vivo. The new polymer appeared to be capable of undergoing hydrolytic degradation in aqueous solution with the rate of degradation strongly dependent on the presence and the content of the residual chlorine atoms. This finding emphasizes the need for a careful control of the substitution process in the production of the polymer.

Evaluation of PCEP in the reaction of ionic complexation with spermine demonstrated that this polymer, like the previously described PCPP, is capable of producing microspheres in a simple aqueous-based process. This extends the utility of the microencapsulation method and potentially allows preparation of formulations of slow release systems containing both antigen and immunoadjuvant.

One of the most important results was the superior activity of a newly synthesized polymer compared to that of the previously studied PCPP. Efforts on the synthesis and evaluation of biological activity of other polyphosphazene polyelectrolytes are under way with the goal of understanding the effect of polymer structure on its in vivo activity. Establishment of a structure−activity relationship can potentially lead to the discovery of the "superpotent" immunoadjuvants and the elucidation of their mechanism of action.

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