Polyphosphazene Microspheres: Preparation by Ionic Complexation of Phosphazene Polyacids with Spermine

Alexander K. Andrianov, Jianping Chen

Parallel Solutions Inc., 763D Concord Avenue, Cambridge, Massachusetts 02138

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ABSTRACT: Polyphosphazene polyacids are potent immunostimulating compounds. Formulation of these water-soluble macromolecules into a microparticulate form can extend their utility in vaccine delivery applications. A simple method for the preparation of microspheres and nanospheres using polyphosphazene polyacids is reported. The key element of the process is ionic complexation of polyphosphazenes in aqueous solutions with physiologically occurring amines, such as spermine. A phase diagram of poly[di(carboxylatophenoxy)phosphazene] (PCPP)–spermine–water system is established, which shows an extensive area of microsphere formation. Microsphere size distribution is studied as a function of reaction conditions and concentrations of reactants. This method can be applied to polyphosphazene polyacids containing carboxylic acid and sulfonic acid functionalities and can be used for protein encapsulation. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 101: 414–419, 2006

Key words: polyphosphazenes; microencapsulation; polyelectrolytes; biological applications of polymers; drug delivery systems

INTRODUCTION

Polyphosphazenes possess unique potential for biomedical applications because of the tunable biodegradability of their backbone and unprecedented structural diversity.[1] Polyphosphazenes with ionic side groups are of particular interest, since they also demonstrate biological activity as immunostimulants.[2–4] Activity of these synthetic macromolecules has been extensively studied for their water-soluble formulations with antigens. Microparticulate systems, however, can provide additional advantages for vaccine delivery, especially for sustained release applications and alternative routes of administration, such as delivery to mucosal surfaces.[5,6] Formulating these polymers in a microparticulate form can lead to a new generation of vaccine delivery vehicles, in which matrix material is also an immunostimulant and can be released in a sustained mode along with the antigen. This is especially important in view of our recent studies, which suggest correlation between the *in vivo* activity of poly[di(carboxylatophenoxy)phosphazene] (PCPP) and its ability to form noncovalent water-soluble complexes with the antigen.[7]

Previously, we reported a method for protein microencapsulation based on aqueous coacervation of PCPP, using sodium salts as coacervation agents and calcium chloride as a crosslinker.[8] The scope of this methodology is narrowed by the specificity of PCPP–sodium ion interactions[9] and the inability to produce nanospheres. In addition, ion-exchange reactions between calcium-crosslinked hydrogel matrix and the media can lead to undesirable fast destruction of microspheres.

This article investigates the behavior of PCPP in the presence of other potential "coacervating agents," such as multivalent organic amines. Condensation of DNA and some synthetic polyelectrolytes induced by multivalent amines has been extensively studied.[10] Polymer collapse in such systems is described by the "ion-bridging" model and is frequently manifested in the aggregation and formation of precipitate.[11] We discovered that the addition of physiologically occurring spermine to aqueous solutions of polyphosphazene polyacids can result in the generation of microparticulate systems. The manuscript describes this new approach to polyphosphazene microsphere and nanosphere preparation and evaluates its potential for protein microencapsulation.

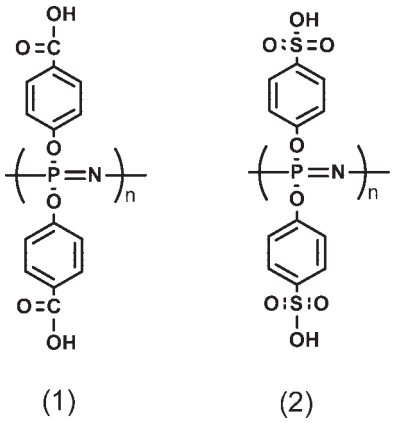
EXPERIMENTAL

Materials

PCPP (1) and sulfonated polyphosphazene (2) were synthesized as described previously (Scheme 1).[9,12]

Spermine, tetrahydrochloride (*N*,*N*′-bis(3-aminopropyl)-1,4-butanediamine tetrahydrochloride); Dulbecco's phosphate-buffered saline, PBS (pH 7.4); and

Correspondence to: A. K. Andrianov (aandrianov@parallelsolutionsinc.com).



(1) (2)

Scheme 1. Structures of PCPP (1) and sulfonated polyphosphazene (2).

Fluorescein isothiocyanate labeled bovine albumin, FITC-BSA were purchased from Sigma (Sigma Chemical Co., St. Louis, MO) and were used as received. FITC-BSA contained 11.2 mol of FITC per 1 mol of FITC-BSA.

Analytical methods

The HPLC system was configured as follows: Shodex DEGAS KT-37 on-line vacuum degasser (Showa Denko K.K., Tokyo, Japan), Waters 600 HPLC pump (Waters, Milford, MA), two inline filters (0.5 μm high pressure filter; Rainin, Woburn, MA) and 0.02 μm filter (Anodsc 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 MALLS detector (DAWN DSP-F, Wyatt Technology, Santa Barbara, CA), a Waters 996 Photo Diode Array detector, and a Waters 410 refractive index detector. PBS (pH 7.4) was used as a mobile phase, and the flow rate was 0.75 mL/min. Mobile phase was filtered through a 0.02 μm filter (Anodisc 47, Whatman International Limited, Maidstone, England) into a 4-valve ULTRA-WARE filtration reservoir (Kontes, Vineland, New Jersey). Sodium azide (0.01%) was added to mobile phase to prevent biological degradation of the columns. The molecular weight of the polymer was determined as described previously.[13]

Particle size distribution of microspheres was analyzed by a Malvern Mastersizer S (Malvern Instruments Inc., Southborough MA). Microspheres were also studied using an Olympus CK-2 inverted microscope (Olympus, Japan).

Phase diagram

A phase diagram of a polyphosphazene–spermine–water system was prepared as follows. PCPP, sodium salt (weight average molecular weight of 8.4×10^5 g/mol) was dissolved in deionized water to prepare a series of solutions ranging in concentration from 0.002 to 3.6% (w/v). Solutions of spermine in deionized water were prepared, ranging in concentration from 0.02 to 12% (w/v). The polymer solutions were then mixed with the spermine solutions in the ratio of 1.0–0.2 mL, so that the concentration of PCPP and spermine in the resulting solutions varied in the 0–2% (w/v) range. The solutions or dispersions were agitated by gentle shaking and then examined by microscope to determine the presence of microparticulate dispersions or precipitate. The phase diagram was then established by plotting the physical state of the system versus composition of the tertiary system: spermine, PCPP, and water.

Preparation of microspheres

A typical example of microsphere preparation is described below. 0.07 mL of 7% (w/v) solution of spermine in PBS (pH 7.4) was added to 5 mL of 0.19% (w/v) aqueous PCPP solution (PBS, pH 7.4). The mixture was agitated gently by shaking and incubated at ambient temperature for 30 min. The suspension of microspheres was then diluted with threefold excess of PBS buffer, let to stand for an additional 30 min, and examined thereafter for the presence of particulates, using the particle size analyzer and optical microscope. Supernatant was collected, filtered through a 0.2 μm Millex HV syringe filter (Millipore, Bedford, MA), and then analyzed for the presence of polymer by HPLC.

Microencapsulation of FITC-BSA

0.017 mL of 0.3% (w/v) FITC-BSA solution in PBS (pH 7.4) was added to 1 mL of 0.05% (w/v) PCPP solution in PBS (pH 7.4). The mixture was incubated at ambient temperature for 30 min. 0.034 mL of 0.7% (w/v) spermine solution in deionized water was added to the mixture. The final concentrations of components in the system were the following: PCPP, 0.048% (w/v); FITC-BSA, 0.005% (w/v); and spermine, 0.023% (w/v). The sample was incubated at ambient temperature for 30 min. and then the microspheres were isolated by centrifugation. Supernatant was collected, filtered through a 0.2 μm Millex HV syringe filter (Millipore, Bedford, MA), and then analyzed for the presence of polymer and protein by HPLC.

Release studies

Experiments were performed at 37°C in New Brunswick G24 Environmental Incubator Shaker (New Brunswick Scientific, Edison, New Jersey), in vials containing 5 mL of PBS. The medium was sampled

periodically and the amount of protein released was evaluated by monitoring the absorbance at 495 nm for FITC-BSA.

RESULTS AND DISCUSSION

Ionic complexation reactions between PCPP and spermine in aqueous solutions

Methods have been developed for formulating water-soluble polyphosphazenes into microparticulate delivery systems. Aqueous based processes attracted special attention because of mild conditions for protein encapsulation. Earlier techniques involved spraying protein–polyphosphazene mixtures into aqueous calcium chloride.[14–16] They required sophisticated spraying equipment and allowed only limited control over the microsphere size distribution. Method utilizing aqueous coacervation as an alternative way for making microdroplets[8] addressed some of these problems. Microspheres were formed through the addition of sodium chloride to a solution of polyphosphazene polyelectrolyte (coacervation step) and through crosslinking of generated coacervate microdroplets with calcium chloride (stabilization step). However, sodium ions used in the process had an adverse effect on the stability of microspheres, dictating the need for careful control of the reaction conditions. There have also been reports on the cleavage of ionic crosslinks and the microsphere disintegration in aqueous solutions.[17] Stability of microspheres can be improved through the treatment of microspheres with polycations such as poly(L-lysine); however, this method often leads to the formation of large aggregates or precipitation.[14,16] It was of interest to combine advantage of these systems: stability of multivalent interactions between the organic compounds and simplicity of the coacervation approach. We focused our studies on the ionic complexation reactions between polyphosphazene polyelectrolytes and low molecular weight polyamines, such as spermine.

A phase diagram was established for PCPP–spermine–water system (Fig. 1) in the area of low concentrations of spermine and PCPP (below 2% (w/v)). It showed the existence of three regions corresponding to amorphous precipitate (region 1), microparticulate dispersion (region 2), and solution (region 3). Inspection of microparticulates using optical microscopy revealed their spherical morphology and the absence of large aggregates. Dispersion was observed in a relatively broad range of concentrations (PCPP, 0.05–0.5% w/v and spermine, 0.01–1.7% w/v).

Polyphosphazene polyacid containing a different type of acidic functions (sulfonic acid groups) was also tested for its ability to interact with spermine. This polymer with a structure similar to the structure of PCPP (Scheme 1) was described previously.[12] We were not able to form microspheres with this polymer, using sodium chloride coacervation method described previously.[8] However, mixing this polymer with spermine under conditions similar to that described for PCPP resulted in polyphosphazene microspheres.

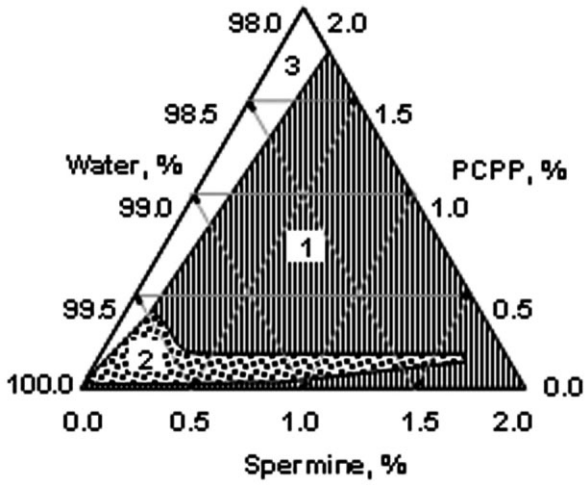


Figure 1 Phase diagram in PCPP–spermine–water system: precipitate (region 1), microparticulate dispersion (region 2), solution (region 3) (20°C and, 30 min incubation time).

Ionic complexation of polyphosphazene macromolecules with spermine in aqueous solutions can also be described as a "complex coacervation" process. Coacervation is a phenomenon in which a macromolecular aqueous solution separates into two immiscible liquid phases.[18] Phase concentrated in macromolecules is called the coacervate and is in equilibrium with a dilute solution of polymer. Complex coacervation, frequently called "associative phase separation," typically involves a system with two oppositely charged molecules.[18] It appears that in PCPP–spermine mixture, amine acts both as a coacervating agent for polyphosphazene and as a crosslinker of the coacervate phase, allowing for the formation of stable microspheres.

Preparation of microspheres

Typical microsphere preparation procedure included addition of spermine to solutions of PCPP in water or PBS (pH 7.4) and subsequent mild agitation (by inversion or shaking) for 5–60 min. The kinetics of microsphere formation appeared to be fast, and in most systems, no changes were observed after the initial 2 min (aqueous dispersions of microspheres were monitored for 48 h). In some systems (typically in the area of high spermine concentration), dilution with water or buffer was necessary to prevent aggregation or precipitation. Microspheres were sufficiently stable to be collected by filtering or centrifuging. They could be then redispersed at the desired concentration. Micro-

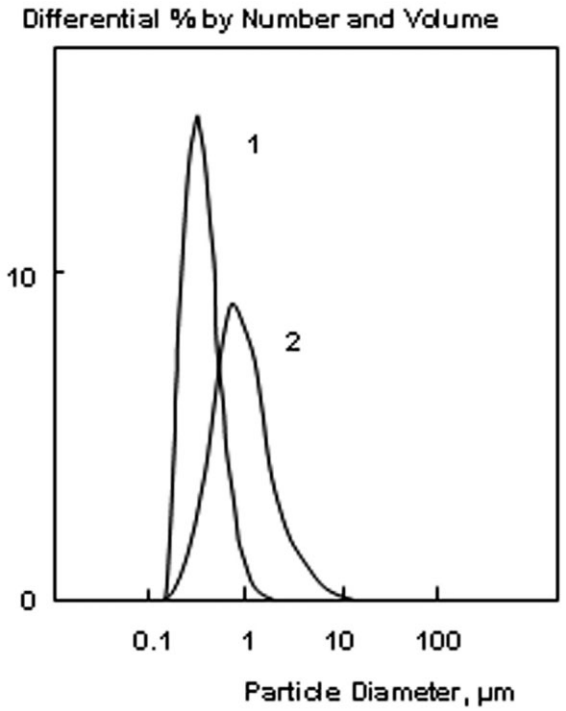


Figure 2 Size distribution profiles by number (1) and by volume (2) in PCPP–spermine–PBS system (concentration of PCPP, 0.187%; spermine, 0.097%; pH 7.4; 30 min incubation time).

spheres were stable in PBS and addition of salts, such as sodium chloride or potassium chloride (up to 5%), did not result in their degradation because of ion-exchange reactions.

Studies of microsphere size distribution

Ionic complexation of aqueous PCPP with spermine led to the formation of dispersions, with particle sizes in the range of 200 nm–100 μm (Figs. 2-4). Size distribution profiles appeared to be unimodal and relatively narrow when calculated by number (Fig. 2). However, presence of larger particles and aggregates can be detected with higher sensitivity by monitoring size distribution by volume. In general, good correlation between these distribution moments was observed, confirming a narrow size range and the absence of large aggregates (Fig. 2). Occasionally, bimodal profiles were obtained for distribution based on volume, indicating some aggregation, although this appeared to be limited to the area of higher polymer concentration (Fig. 3).

The ability to modulate microsphere size through the reaction conditions was also investigated. The results demonstrate the existence of "size–reactant concentration" relationship (Fig. 4). It appears that polymer concentration is the most effective parameter in the microsphere size control. Mean diameter–polymer concentration dependence is characterized by a maximum at ~0.35% (w/v) of PCPP. Particle size is less sensitive to the concentration of spermine; however, higher content of amine in the system tends to produce larger microspheres.

Efficiency of polymer incorporation

The efficiency of polyphosphazene incorporation in microspheres is an important process characteristic. Microencapsulation systems were analyzed for the presence of water-soluble PCPP by size-exclusion HPLC. Figure 5 shows the percentage of polymer incorporation as a function of its initial concentration in solution. Systems with low PCPP concentrations (below 0.5% w/v) are characterized by nearly complete integration of polymer in microspheres. Dramatic decrease in the efficiency of incorporation is observed at the higher polymer concentration. Interestingly, it appears that this decrease in the efficiency of polymer incorporation (0.5–0.7% w/v) corresponds to the overlap concentration of PCPP (0.6% w/v). As mentioned earlier, a region of concentrated polymer solutions is also characterized by some aggregation in the system. Thus, it appears that the region of low PCPP concentrations (below 0.35%) allows size control in the range of 200 nm–100 μm and characterized with minimal aggregation and polymer loss (Figs. 3–5).

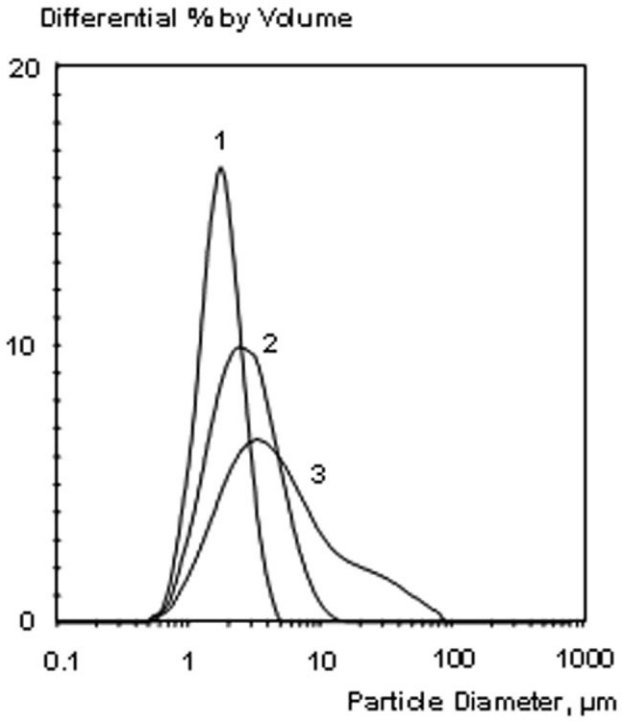


Figure 3 Size distribution by volume in PCPP–spermine–PBS system as a function of PCPP concentration: 0.08% (1), 0.2% (2), 0.4% (3) (concentration of spermine, 0.15%; pH 7.4; 20°C; 30 min incubation time).

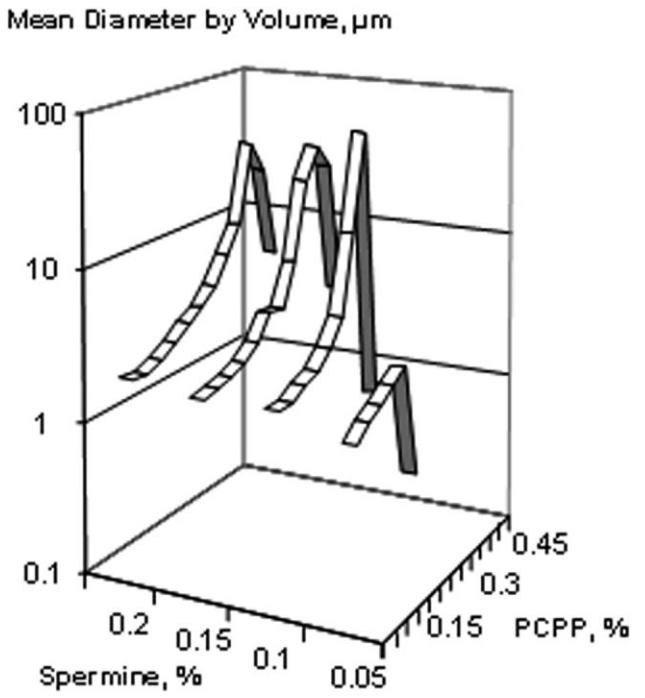


Figure 4 Effect of PCPP and spermine concentration in aqueous solutions on the mean diameter of resulting microspheres (PBS; pH 7.4; reaction time 30 min).

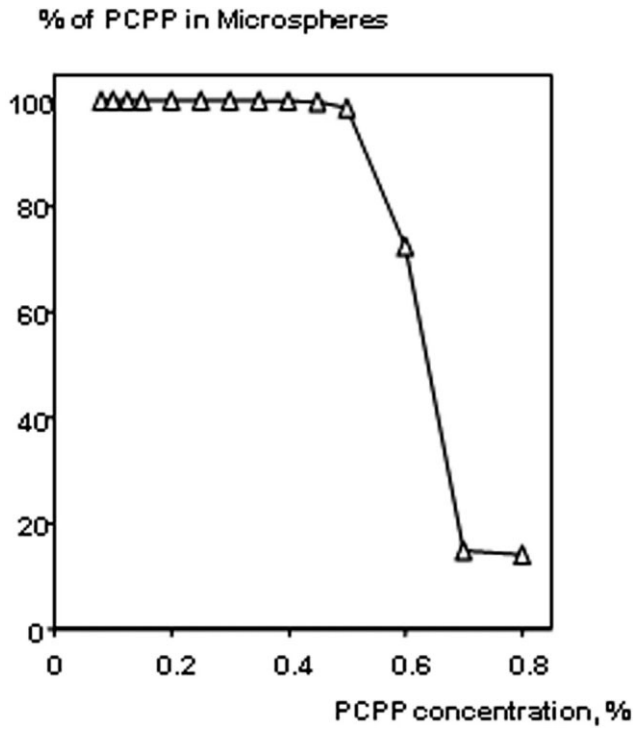


Figure 5 Efficiency of polymer incorporation in microspheres as a function of its concentration in solution (concentration of spermine, 0.15% w/v; PBS; pH 7.4; 20°C).

Microencapsulation of FITC-BSA

To evaluate the suitability of PCPP–spermine system for protein microencapsulation, studies were performed using model protein, FITC-BSA. Microencapsulation was carried out by mixing FITC-BSA and PCPP solutions, incubating the mixture at room temperature for 30 min, and then adding spermine to induce microsphere formation. The efficiency of protein encapsulation was then determined by filtering microspheres and detecting protein in the supernatant by size exclusion HPLC. This "soluble" protein was then compared to the initial amount of the FITC-BSA used for microencapsulation, and the efficiency of encapsulation was determined as follows:

$$\text{Encapsulation efficiency (\%)} = \{[\text{FITC-BSA}]_{\text{initial}} - [\text{FITC-BSA}]_{\text{solution}}\}/[\text{FITC-BSA}]_{\text{initial}}$$

In our experiments, the highest efficiency, 76%, was obtained under the following conditions: PCPP, 0.48 mg/mL; FITC-BSA, 0.05 mg/mL; spermine, 0.23 mg/mL; and coacervation time, 30 min. Size distribution was similar to the profile obtained for "empty" microspheres.

FITC-BSA release profiles

FITC-BSA release profiles from spermine-crosslinked PCPP microspheres are presented in Figure 6. Micro-

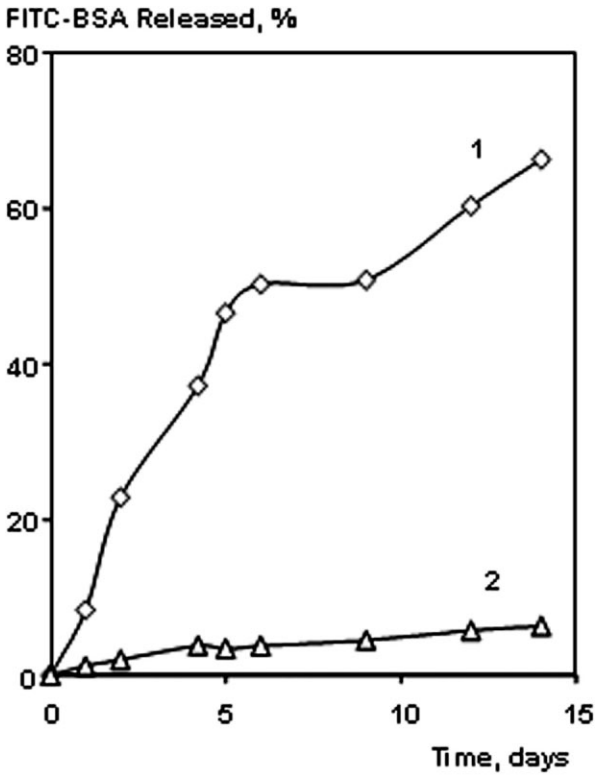


Figure 6 Release profiles for FITC-BSA loaded microspheres based on calcium-crosslinked (1) and spermine-crosslinked (2) PCPP (PBS, pH 7.4, 37°C).

sphere samples were incubated in PBS (pH 7.4) and supernatant was analyzed periodically to determine concentration of FITC-BSA in solution. The percentage of FITC-BSA released was calculated relative to the total amount of FITC-BSA in microspheres immediately after encapsulation. The results were compared to release profiles from calcium-crosslinked microspheres prepared as described previously.[8] Spermine-crosslinked microspheres demonstrated significantly slower release profiles than their calcium-crosslinked counterparts (Fig. 6).

This dramatic decrease in the protein release rate for spermine-crosslinked microspheres as compared to their calcium-crosslinked counterparts (Fig. 6) can be probably explained by the differences in stability. Higher stability of spermine-crosslinked microspheres in solutions containing monovalent cations can expand the utility of polyphosphazene microspheres, especially in the applications where prolonged release is desired. Combining calcium and spermine as crosslinkers, control of their distribution in microspheres, can lead to new systems with more sophisticated release profiles.

CONCLUSIONS

A simple method for formulating polyphosphazene immunostimulants in microspheric form is developed. The method relies on physiologically occurring crosslinker spermine, which is capable of condensing polyphosphazenes into microparticulate systems with a size range of 200 nm–100 μm. The process is based on aqueous solutions and does not require solvents or elevated temperatures. In such systems, polyphosphazene immunostimulants serve as "wall-forming materials," and microspheres can be used for modulated release of antigenic proteins and as a potential supply of the immunostimulant.

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