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Protein-loaded soluble and nanoparticulate formulations of ionic polyphosphazenes and their interactions on molecular and cellular levels

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Abstract

Nanoparticulate and water-soluble formulations of ionic polyphosphazenes and protein cargo - lysozyme (LYZ) were prepared by their self-assembly in aqueous solutions at near physiological pH (pH 7.4) in the presence and absence of an ionic cross-linker – spermine tetrahydrochloride. Efficiency of LYZ encapsulation, physico-chemical characteristics of formulations, and the effect of reaction parameters were investigated using asymmetric flow field flow fractionation (AF4) and dynamic light scattering (DLS) methods. The effect of both polymer formulations on encapsulated LYZ was evaluated using soluble oligosaccharide substrate, whereas their ability to present the protein to cellular surfaces was assessed by measuring enzymatic activity of encapsulated LYZ against *Micrococcus lysodeikticus* cells. It was found that both soluble and cross-linked polymer matrices reduce lysis of bacterial cells by LYZ, whereas activity of encapsulated protein against oligosaccharide substrate remained practically unchanged indicating no adverse effect of polyphosphazene on protein integrity. Moreover, nanoparticulate formulations display distinctly different behavior in cellular assays when compared to their soluble counterparts. LYZ encapsulated in polyphosphazene nanoparticles shows approximately 2.5-fold higher activity in its ability to lyse cells as compared with water-soluble LYZ-PCPP formulations. A new approach to PEGylation of polyphosphazene nanoparticles was also developed. The method utilizes a new ionic polyphosphazene derivative, which contains graft (polyethylene glycol) chains. PEGylation allows for an improved control over the size of nanoparticles and broader modulation of their cross-linking density, while still permitting for protein presentation to cellular substrates.

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Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

The authors declare no competing financial interest.

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Keywords

self-assembly; polymer - protein interactions; nanoparticles; polyphosphazene; bacterial cells; PEGylation

1. Introduction

Encapsulation of proteins in polymer formulations plays an increasingly important role in delivery of therapeutic drugs and vaccine antigens [1–4]. The material component of such delivery systems is typically designed not only to provide transportation and release modalities for the encapsulated protein cargo, but also to modulate its interactions with important biological targets, such as proteins, nucleic acids, lipids, and cells [5–8]. To that end, delivery of therapeutic agents may benefit from the formation of “steric shields” guarding biomacromolecules against interactions with components of the immune system, which otherwise result in a rapid clearance of therapeutic agents and undesired immunogenicity [9, 10]. Such protection is often achieved through the use of covalently attached water-soluble chains of poly(ethylene glycol), PEG [10–12]. On the contrary, self-assembly of vaccine antigens with ionic macromolecules, such as polyphosphazene immunoadjuvants, leads to enhanced stimulation of dendritic cells, which is highly desirable for the delivery of vaccine antigens [13–16]. These examples emphasize the importance of in depth research in the complex nature of physico-chemical interactions between polymeric nano-carriers and biological targets [8, 17]. Nanoparticulate systems based on biodegradable hydrophobic polymers, such as poly(L-lactic acid), are perhaps the most investigated materials for drug delivery applications [3, 18, 19]. However, it is fair to assume that the barriers preventing interactions of encapsulated protein with biological targets in this case are sufficiently strong, and the release of biologically active molecules is required in order to cause biological responses [19]. The situation is somewhat more obscure when flexible water-soluble macromolecules or ionically cross-linked hydrogels are employed as carriers. In fact, non-covalent nano-assemblies formed between vaccine antigens and polyphosphazene immunoadjuvants appear to be capable of effective presentation of bound antigenic proteins both in vivo and in vitro, apparently without the need for their release [13–16]. However, the impact of materials properties of polyphosphazenes on interactions between bound protein and cellular targets is still not sufficiently understood.

Water-soluble ionic polyphosphazenes, such as poly[di(carboxylatophenoxy)phosphazene], PCPP, have been extensively studied as vaccine and drug delivery systems [20–26], bone replacement composite scaffolds [27–29], carriers for imaging agents [30, 31] and cells [32], and as nanocoatings [33, 34]. Biological activity of ionic polyphosphazenes can be at least partially linked to the ability of these macromolecules to spontaneously form stable non-covalent complexes with proteins and small molecules [13, 16]. Although PCPP is most typically used in a water-soluble state, this polymer can be also easily configured into ionically cross-linked colloid systems using multivalent ions [30, 31, 35, 36]. Therefore, polyphosphazene technology presents an interesting opportunity for comparative evaluation of nanostructures and corresponding water-soluble polymer of the same identity, which is not confined by intermolecular cross-links and lacks defined surface of the hydrogel. The

selection of protein for this study has been mainly directed by the ease of its direct recognition and assessment using both cellular and small molecule substrates. Lysozyme (LYZ) is well suited for such investigation. This enzyme catalyzes hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, which is the major component of gram-positive bacterial cell, and it is usually identified by its lytic activity against *Micrococcus lysodeikticus* cells [37]. Independently, enzymatic activity of LYZ can be “verified” on the molecular level using water-soluble, oligosaccharide analog of the glycan found in cell walls – 4-methylumbelliferyl β -N,N',N'',N'''-tetraacetyl-D-chitotetraoside (MUFD) oligosaccharide [38]. Moreover, it has been already demonstrated that lysozyme can spontaneously self-assemble in aqueous solutions with PCPP [13, 39].

Present paper describes preparation and physico-chemical characterization of LYZ loaded nanoparticulate and water-soluble formulations of PCPP. It evaluates the ability of both formulations to display and present encapsulated enzyme to soluble and cellular substrates demonstrating higher activity of nanoparticulates in cellular assays compared to their soluble formulations. The paper also reports on a new approach for PEGylation of nanoparticles using newly synthesized polyphosphazene derivative, which improves control over the size of nanoparticles while still allowing protein presentation to cells.

2. Materials and methods

2.1. Materials

Lysozyme (LYZ) from chicken egg white, *Micrococcus lysodeikticus* ATCC No. 4698 lyophilized cells (Sigma, St. Louis, MO), spermine tetrahydrochloride (Alfa Aesar, Ward Hill, MA), lysozyme activity Assay kit (BioVision, cat. # K236–100, Milpitas, CA), phosphate buffered saline pH 7.4, PBS (Life Technologies, Carlsbad, CA), heptane, sodium hydride, sodium phosphate monobasic dihydrate, methoxypolyethylene glycol amine (5000 g/mol), PEG-NH₂, bis(2-methoxyethyl) ether, diglyme (Acros Organics, Morris Plains, NJ), propyl ester of p-hydroxybenzoic acid (Spectrum Chemical, Gardena, CA), and acetonitrile (EM Science, Darmstadt, Germany) were used as received.

2.2. Synthesis of polyphosphazenes – PCPP and PCPP-PEG

PCPP (molecular weights: 20,000, 200,000, and 800,000 g/mol) was synthesized using varied ratios of polydichlorophosphazene and nucleophile as described previously [40, 41]. PCPP-PEG (molecular weight 700,000 g/mol) containing 1% (mol) of PEG was synthesized using previously reported reaction pathway [24], but utilizing the following reagents: 0.464 g (4.0 mmol) polydichlorophosphazene, 1.0 g (0.2 mmol) PEG-NH₂, 28 μ L (0.2 mmol) triethylamine, 2.88 g (16.0 mmol) propyl ester of p-hydroxybenzoic acid, and 0.36 g (15.2 mmol) sodium hydride. Polymer structure and composition was confirmed by ¹H NMR (400 MHz, D₂O): δ [ppm] 7.3 (br, 2H, =CH₂-); 6.4 (br, 2H, -CH₂=); 3.6 (br, 4H, -CH₂-CH₂-O). Molecular weights were determined by gel permeation chromatography using a Hitachi high performance liquid chromatography system with L-2450 diode array detector, L-2130 pump, and L-2200 autosampler (Hitachi LaChrom Elite system, Hitachi, San Jose, CA) and Ultrahydrogel Linear size exclusion column (Waters Corporation, Milford, MA). PBS, pH

7.4 with 10% of acetonitrile was employed as a mobile phase with a flow rate of 0.75 mL/min. Samples were prepared at a concentration of 0.5 mg/mL in PBS, pH 7.4 and were filtered using Millex 0.22 µm filters (EMD Millipore, Billerica, MA) prior to the analysis. Molecular weights were calculated using EZ-Chrome Elite software (Agilent Technologies, Santa Clara, CA). A calibration curve was obtained using narrow polyacrylic acid standards (American Polymer Standards Corporation, Mentor, OH).

2.3. Preparation of nanoparticles

Nanoparticles were prepared by combining aqueous solutions of PCPP or PCPP and PCPP-PEG mixtures in 50 mM phosphate buffer (pH 7.4) with spermine tetrahydrochloride in water using microfluidics technique. The microfluidic device comprised herringbone type mixing chamber (Microfluidic ChipShop GmbH, Jena, Germany) to create a steady chaotic flow through repeated sequences of rotational and extensional local flows. The solutions were supplied using syringe pump (Fusion 100, Chemyx Inc., Stafford, TX) with two 5 mL syringes attached to a mixing chamber via silicon tubes with Mini Luer fluid connectors. The flow rate of 0.75 ml/min was used for mixing. For the preparation of protein-loaded nanoparticles, LYZ was added to polymer formulations prior to mixing with spermine.

2.4. Dynamic light-scattering (DLS) measurements

Dynamic light scattering, DLS was carried out using a Malvern Zetasizer Nano series, ZEN3600 and analyzed using Malvern Zetasizer 7.10 software (Malvern Instruments Ltd., Worcestershire, UK). Samples were prepared in a phosphate buffer or PBS, pH 7.4 and filtered using Millex 0.22 µm filters prior to the analysis. Disposable folded capillary cell DTS 1070 were used for z-potential measurements. All samples were analyzed in triplicate.

2.5. Asymmetric Flow Field Flow Fractionation (AF4) measurements

Asymmetric Flow Field Flow Fractionation, AF4 was performed using a Postnova AF2000 MT series (Postnova Analytics GmbH, Landsberg, Germany). The system was equipped with two PN1130 isocratic pumps, PN7520 solvent degasser, PN5120 injection bracket and UV-Vis detector (SPD-20A/20AV, Shimadzu Scientific Instruments, Columbia, MD). A regenerated cellulose membrane with molecular weight cutoff of 10 kDa (Postnova Analytics GmbH, Landsberg, Germany) and a 350-µm spacer were used in a separation micro-channel employing both laminar and cross flows of an eluent - PBS (pH 7.4). The collected data was processed using AF2000 software (Postnova Analytics GmbH).

2.6. Measurement of LYZ activity against 4-methylumbelliferyl β-N,N',N'',N'''-tetraacetyl-D-chitotetraoside (MUFD) oligosaccharide

Enzymatic activity of LYZ against MUFD was analyzed by measuring the release of 4-methylumbelliferone fluorometrically [42] using lysozyme activity assay kit, K236-100 (BioVision, Milpitas, CA). 5–15 µl of analyzed solution was added to each well of 96-well plate containing 50 µL of assay buffer, to which 10 µL of substrate solution was added. The plate was covered by AlumaSeal 96™ foil and kept at 37° C for 2 h, then 50 µl of stop buffer was added to each well and fluorescence (Ex/Em= 360/445 nm) was recorded using BioTek

Synergy Neo2 multi-mode reader (BioTek Instruments, Inc., Winooski, VT). All samples were analyzed in triplicate.

2.7. Measurement of LYZ activity against *Micrococcus lysodeikticus* cells

Enzymatic activity of LYZ against *Micrococcus lysodeikticus* ATCC lyophilized cells was measured as reported previously [43], but with following modifications. A suspension of *Micrococcus lysodeikticus* cells (0.25 mL) in 50 mM potassium phosphate buffer at pH 7.0 was added to each of three wells of 96-well plate. The absorbance (A_{410}) of this suspension was approximately 0.4–0.5 versus a buffer blank. 15 μ L of analyzed solution was added to the cell suspension, immediately mixed by pipetting and a decrease in A_{410} was recorded for 3 minutes. The maximum initial rate ($-A_{410}/\text{minute}$) was calculated for each sample and used for comparison of lysozyme activity in different samples. All samples were analyzed in triplicate. PCPP, PCPP-PEG, and relevant nanoparticulate formulations displayed no cell lysis activity in the absence of LYZ.

3. Results and Discussion

3.1. Preparation of water-soluble formulations and ionically cross-linked nanoparticles

Three types of PCPP formulations were investigated as macromolecular carriers for proteins: water-soluble systems, ionically cross-linked nanoparticles, and PEGylated nanoparticles (Fig. 1). Soluble complexes of PCPP with lysozyme (LYZ) were formulated by simple mixing of the components in aqueous solutions at pH 7.4 (Fig. 1, pathway I). Ionically cross-linked formulations were prepared by adding spermine to LYZ-PCPP formulations as described previously [36] using microfluidics technology (Fig. 1, pathway II). PEGylated nanoparticles were prepared with a new polyphosphazene derivative containing PEG graft chains – PCPP-PEG, which was synthesized using reaction pathway reported previously [24]. PCPP-PEG was admixed to a LYZ-PCPP formulation and the system was cross-linked with spermine as described above (Fig. 1, pathway III).

Complete binding of LYZ with PCPP in all investigated formulations was confirmed by analyzing them for the presence of unbound LYZ using asymmetric flow field flow fractionation (AF4) method. Similarly to size-exclusion HPLC, this elution-based method allows for the separation of macromolecules and nanoparticles by size. However, as opposed to chromatographic methods, the upper size limit for the analyte is suitable for the characterization of nano- and microparticles and can reach as high as 100 μ m [44]. The separation is carried out in a single liquid phase and an external flow of the mobile phase is applied perpendicularly to the direction of sample flow through a channel equipped with semi-permeable membrane [44]. Fig. 2 shows AF4 traces of LYZ-PCPP complex before and after cross-linking with spermine, as well as individual components of formulations – PCPP and LYZ. Both LYZ –PCPP systems display significant shift of their peaks towards larger sizes, however the shape of these peaks suggest some non-specific interactions with the analytical membrane. In fact, DLS profiles of the same formulations confirm this hypothesis displaying similar sizes for spermine cross-linked and spermine free LYZ-PCPP (inset in Fig. 2). Most importantly, all LYZ-PCPP formulations showed no increase in the absorbance at the elution time of 7 minutes - position of LYZ peak in polymer-free formulations. This

demonstrates a complete association of protein with PCPP and PCPP nanoparticles through non-covalent interactions [13], which was confirmed for all systems employed in the present study.

3.2. Effect of reagent concentration and molecular weight of PCPP on size distribution and z-potential of nanoparticles

Investigation of nanoparticles produced by Ionic cross-linking of negatively charged PCPP with multivalent cation – spermine tetrahydrochloride was first carried out in the absence of protein. Mixing of polyphosphazene solutions with spermine was conducted in a microfluidics device using herringbone chip [30, 31]. Fig. 3A demonstrates that the addition of ionic cross-linker in excess of 0.05 mg/mL leads to a rapid increase in the hydrodynamic diameter of the system. This is also accompanied by a dramatic decrease in polydispersity, which may reflect differences in scattering characteristics between soluble state of the polymer and aggregated nanoparticulate form (Fig. 3A). It has to be noted that the formation of particles in the nanoscale and supra nanoscale range (50–200 nm) is limited to a narrow cross-linker concentration. Studies comparing zeta-potential of the system and its hydrodynamic diameter conducted in a broad range of spermine concentrations (0.05 – 10 mg/mL) revealed that the size of particulates is strongly dependent on their surface charge (Fig. 3B, S1 and S2). As seen from Fig. 3B, minimal sizes are achieved at zeta-potential values of –10 mV (low concentration of spermine) and at around +15 mV (high concentration of spermine) with largest size of aggregate observed at electroneutrality. This suggests a dominating role of electrostatic interactions in defining the size of nanoparticulates in this ionotropic system. Large aggregates formed at the concentration of spermine around 5–10 mg/mL (Fig. 3B, S1 and S2) suggest a potential impact of other factors, possibly increase in hydrophobicity of polymer surface due to high cross-linking density. To expand the composition of particles in the nano-scale and supra nano-scale range to a higher content of a cross-linker experiments were conducted on PEGylation of nanoparticles, which are described below.

The ability to control nanoparticle sizes through variations in polymer concentration and its molecular weight parameters was also investigated. Fig. 3C shows the dependence of hydrodynamic diameters of nanoparticles on the amount of cross-linker added for four different concentrations of PCPP. As seen from the Figure, the threshold of nanoparticle formation depends on the concentration of the polymer, with larger amount of spermine required for higher concentration of PCPP. It can be noted, that all profiles display a characteristic plateau corresponding to a size range of 150 – 170 nm for which nanoparticle sizes remained practically unchanged despite the increase in cross-linker concentration. The plateau appears to be more extended for higher concentrations of PCPP. The size of those nanoparticles significantly exceeds the diameter of a single chain PCPP, which suggests multi-chain arrangement.

The effect of polymer molecular weight on nanoparticle sizes is shown in Fig. 3D. As seen from the Figure, the threshold of nanoparticle formation was dependent on the molecular weight with shorter polymer chains requiring larger doses of spermine. Despite the significant differences in hydrodynamic diameters of polymers of different molecular

weights used in these experiments, which ranged from 10 to 60 nm, we have not been able to achieve effective control of particle size in the nanoscale range (below 100 nm).

Macromolecules with lower molecular weights appeared to produce nanoparticles with dimensions similar to their high molecular weight counterparts. This may suggest that surface charge (Fig. 3B), not polymer chain length, was a prevailing factor in determining the dimensions of nanoparticles. Nevertheless, PCPP with molecular weight of 20,000 g/mol was able to generate nanoparticles with hydrodynamic diameters in the vicinity of 100 nm.

3.3. Preparation and characterization of LYZ loaded nanoparticles

Addition of LYZ to aqueous solutions of PCPP led to a fast increase in hydrodynamic diameter of the system as the concentration of protein rose (Fig. 4A). The observed spontaneous binding of LYZ to PCPP can be explained by attractive interactions, which are anticipated for a positively charged protein and anionic polymer [13, 45]. The size of intermolecular complexes was generally below 200 nm for the LYZ-to-PCPP ratios not exceeding approximately twenty protein molecules per polyphosphazene chain, but significant aggregation was observed above that value. The addition of spermine to LYZ-PCPP complex led to a decrease in the size of the system in the molar range of 20 – 45 protein molecules per polymer chain essentially preventing the aggregation (Fig. 4A). Spermine cross-linked LYZ-PCPP nanoparticles (NP) were also characterized by significantly lower polydispersity compared to their “cross-linker free” formulations (Fig. S3).

3.4. Water-soluble LYZ-PCPP complexes and LYZ-PCPP nanoparticles display distinctly different behavior in their interactions with soluble and cellular substrates

Enzymatic activity of water-soluble LYZ-PCPP complexes and LYZ loaded PCPP nanoparticles was evaluated using assays, which employed either bacterial cells or soluble oligosaccharide MUFD as substrates. It has been previously reported that polyelectrolytes can modulate activity of LYZ and other enzymes by shielding their active sites or changing microenvironmental concentration of the substrate [46–51]. Fig. 4B demonstrates that although both formulations resulted in a significant, over 60%, loss of activity when compared with activity of unmodified LYZ, this reduction was less pronounced for nanoparticles. Interestingly, this effect cannot be explained simply in terms of effect of polymer scaffold on the enzyme. Comparison of nanoparticulate and water-soluble PCPP formulations conducted with soluble MUFD substrate showed less than 20% decrease in activity, with nanoparticulate formulations displaying practically no loss of activity (Fig. 4C). Fold increase of LYZ activity in nanoparticles over LYZ activity in water-soluble complexes was only slightly higher than unity for MUFD substrates, but was approximately 2.5 when activity was measured against bacterial cells (Fig. 4D). This may indicate that the observed differences did not result from changes in the activity of the enzyme itself, but stemmed from the effect of the macromolecular carrier on interactions of the enzyme with these substrates. It is possible to speculate that bacterial cells have a greater “appetite” for particulate formulations containing bactericidal enzyme and therefore display self-destructive activity. A potential reason for this is differences in the physical and conformational behavior of soluble polyphosphazene and the same macromolecule conformed into a solid nanoparticle by ionic cross-linking. Previously, it has been reported

that nanoparticles with high energy of the defined surface generally display greater affinity to cell membranes than water-soluble macromolecules, such as proteins [52–54]. It is conceivable that the enhancement of the activity observed for nanoparticulate formulations as compared to their soluble polyphosphazene counterparts is also a result of a better adsorption of nanoparticles to cell membranes. Importantly, since no release of LYZ was observed in Franz cell under these conditions, it can be concluded that both systems were capable of displaying LYZ in the way that allowed the enzyme to interact with cellular substrates.

3.5. PEGylation of nanoparticles allows effective control of nanoparticle sizes and modulates their activity against cell substrate

To explore the possibility of modulating the size of particles in the nanoscale range and to further elucidate the role of physical interactions between nanoparticles and bacterial cells in regulating activity of encapsulated enzyme, a covalent modification of polyphosphazene carrier with graft poly(ethylene glycol) (PEG) chains was carried out. The pathway for the synthesis of PEGylated nanoparticles was similar to the formation of spermine cross-linked PCPP nanoparticles, but involved the use of binary polymer mixture – PCPP and PCPP-PEG (Fig. 1, pathway III). PCPP-PEG was designed to contain 1% (mol/mol) of 5,000 g/mol PEG side groups. As seen from Fig. 5A binary polymer system displays an extended plateau over a broad range of spermine concentration indicating the stabilizing effect of PEG chains against aggregation. These results are in dramatic contrast with previously observed data for PCPP alone. In that system preparation of particles in a nano-scale range was only possible in a very narrow concentration range of a cross-linker (Fig. 2A). Moreover, the size of PEGylated particulates in the nanoscale range can be effectively controlled through varying the content of PEGylated polyphosphazene (Fig. 5B). As seen from the Figure, increasing concentration of PEGylated polymer leads to a reduction in nanoparticle sizes. This can indicate that PEG chains reduce intermolecular interactions of spermine modified PCPP, which otherwise result in the formation of larger particles.

PEGylation – a technique of covalent derivatization with PEG has been successfully applied to multiple proteins, liposomes, and nanoparticles to improve their stability and reduce their interactions with components of the immune system [11, 12, 55, 56]. “Stealth” properties enabled by the formation of PEG shield around the nanoparticle or biomacromolecule can be also efficient in reducing their interactions with cells [57, 58]. The use of PEGylated polyphosphazene polyacids for non-covalent PEGylation of proteins was also recently reported [24, 59].

To investigate the effect of PEGylation on biological activity of nanoparticles, LYZ loaded PCPP nanoparticles with variable content (10–50 % (w/w)) of PCPP-PEG were prepared under conditions that allowed maintaining the same particle sizes (Fig. 5C). The effect of PEGylation on the enzymatic activity of LYZ containing nanoparticles was strongly dependent on the substrate utilized in the analysis. While activity against cell substrate was clearly suppressed by addition of PCPP-PEG in a dose dependent manner and eventually resulted in a 2-fold reduction compared to unmodified nanoparticles, the rate of oligosaccharide (MUFD) hydrolysis was maintained at the level of at least 80% of that value

(Fig. 5D). As was suggested above, the formation of the surface upon ionic cross-linking of LYZ-PCPP complex may lead to a pronounced increase in the activity of the system against cellular substrates. Modification of nanoparticle surface with water-soluble “stealth” polymer – PEG was undertaken as an established approach to reduce interactions between nanoparticles and cells [60–62]. As expected, such treatment of the surface resulted in a decrease of enzymatic activity (up to two fold reduction), which appears to confirm the suggested importance of nanoparticle surface – cell membrane interactions in understanding of overall availability of the encapsulated protein. The results also appear to indicate that the proposed PEGylation method expands the composition range of nanoparticles in terms of their cross-linking density, but still allows for effective presentation of the protein to cells, which suggests a potential utility of the approach for vaccine delivery applications.

4. Conclusions

The ability to load, display or release proteins is an important feature of polymer based delivery systems and optimization of these properties is critical for developing a new generation of drugs and vaccines. Present study included polyphosphazene protein carrier in its water-soluble form, as well as the same macromolecule configured into ionically cross-linked nanoparticles. The ionic cross-linking process was carried out at near physiological conditions and manifested itself in an increase of hydrodynamic diameter (from 60 to 100–150 nm) and a steep drop in dispersity as measured by DLS. LYZ, a model protein cargo, was incorporated both into soluble PCPP and its nanoparticles. The activity of protein-loaded materials was assessed using bacterial cells as substrates, thereby providing important information on the ability of polymer carrier to display and present protein cargo to cell surfaces, and on the molecular level using soluble oligosaccharide. Both polyphosphazene carriers showed only marginal effect on the activity of encapsulated LYZ against molecular substrate, which suggests preservation of protein integrity in polymer matrices. However, the reduction in enzymatic activity was more pronounced for cellular substrates resulting in a loss of bactericidal activity over 60%. Furthermore, despite similar activity of encapsulated LYZ on the molecular level, nanoparticles displayed higher efficiency in lysing cellular substrates - an approximately 2.5-fold increase in activity over water-soluble formulations. PEGylation of nanoparticles, the approach, which utilizes a new polyphosphazene derivative – PCPP-PEG, improves modulation of nanoparticle size and cross-linking density, while still allowing for protein presentation to cellular substrates.

Findings on the improved presentation of proteins to cell surfaces enabled by ionically cross-linked hydrogel nanoparticles, rather than by structurally identical soluble macromolecules, can potentially provide some valuable guidance in the development of formulations with enhanced or reduced cellular activity. Although the above results were obtained for bactericidal enzyme using bacterial cells, the findings may also present a broader interest since PCPP can be used in vaccine delivery, regenerative engineering, or in its PEGylated form, as a carrier for therapeutic and imaging agents. These potential uses mandate further studies of polyphosphazene delivery vehicles using application specific protein and cellular systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Nanoparticulate and soluble polyphosphazenes allow for efficient lysozyme loading
- Polyphosphazenes do not significantly alter enzyme activity with molecular substrate
- Enzyme loaded nanoparticles are more active against cells than soluble formulations
- Graft polyphosphazene enables single-step synthesis of PEGylated nanoparticles

Fig. 1.
Schematic presentation of water-soluble and nanoparticulate formulations of PCPP with lysozyme (LYZ) and pathways to their preparation.

Fig. 2.

AF4 fractograms of LYZ, PCPP, LYZ-PCPP complex, and nanoparticles loaded with LYZ (NP-LYZ); inset shows DLS profiles for the same formulations (0.5 mg/mL PCPP, 0.15 mg/mL LYZ, 0.07 mg/mL spermine, pH 7.4)

Fig. 3.

Effect of spermine concentration on (A) z-average hydrodynamic diameter and (B) zeta potential of PCPP-spermine system - plotted as z-average hydrodynamic diameter vs. z-potential (0.15 mg/mL PCPP, concentration of spermine: 0.05 – 10 mg/mL; pH 7.4). Z-average hydrodynamic diameter as a function of spermine concentration for (C) 0.1 (1), 0.2 (2), 0.3 (3) and 0.5 mg/mL PCPP (4) (pH 7.4) and (D) for PCPP with molecular weights of (1) 800,000, (2) 200,000, and (3) 20,000 g/mol (0.5 mg/mL PCPP, pH 7.4)

Fig. 5.

(A) Z-average hydrodynamic diameter as a function of spermine concentration for nanoparticle formation in the presence of (A) 0.045 mg/mL PCPP-PEG (0.15 mg/mL PCPP, pH 7.4). (B) Effect of PCPP-PEG concentration on Z-average hydrodynamic diameter - 0.015 mg/mL (1), 0.03 mg/mL (2), and 0.075 mg/mL (3) PCPP-PEG (0.15 mg/mL PCPP, pH 7.4). Effect of PCPP-PEG content in LYZ loaded nanoparticles on (C) hydrodynamic diameter (1) and polydispersity index (2) and (D) enzymatic LYZ activity of nanoparticles against cell (1) and MUFD oligosaccharide (2) substrates (0.05 mg/mL LYZ, 0.5 mg/mL PCPP, 0.07 mg/mL spermine, 50 mM phosphate buffer, pH 7.4).