Next Generation Polyphosphazene Immunoadjuvant: Synthesis, Self-Assembly and *In Vivo* Potency with Human Papillomavirus VLPs-Based Vaccine

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Abstract

Poly[di(carboxylatomethylphenoxy)phosphazene], PCMP - a new member of polyphosphazene immunoadjuvant family is synthesized. *In vitro* assessment of a new macromolecule revealed hydrolytic degradation profile and immunostimulatory activity comparable to its clinical stage homologue - PCPP, however PCMP was characterized by a beneficial reduced sensitivity to the ionic environment. *In vivo* evaluation of PCMP potency was conducted with Human Papillomavirus (HPV) virus-like particles (VLPs) based RG1-VLPs vaccine. In contrast with previously reported self-assembly of polyphosphazene adjuvants with proteins, which typically results in the formation of complexes with multimeric display of antigens,

PCMP surface modified VLPs in the composition dependent pattern, which at a high polymer-to VLPs ratio led to stabilization of antigenic particles. Immunization experiments in mice demonstrated that PCMP adjuvanted RG1-VLPs vaccine induced potent humoral immune responses, in particular, on the level of highly desirable protective cross-neutralizing antibodies, and outperformed PCPP and Alhydrogel adjuvanted formulations.

Key words: polyphosphazenes; vaccine delivery; self-assembly; virus-like particles; immunostimulating compounds

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Introduction

New challenges in the development of contemporary vaccines, which increasingly rely on highly purified, but weakly immunogenic antigens, intensify quest for novel immunoadjuvant and vaccine delivery mechanisms.[1, 2] The ability of immunoadjuvant systems to shape humoral and cellular responses to vaccine antigens plays a critical role in the induction of strong and robust protective immunity.[3] Although the majority of immunoadjuvants are based on emulsion, hydrogel, and nanoparticulate formulations, and frequently include multiple components, there is a growing interest to well-defined synthetic systems, in which immunostimulatory activity can be controlled and modulated through changes in their molecular structures.[4, 5] To that end, a family of polyphosphazene immunoadjuvants, which are based on water-soluble macromolecules consisting of biodegradable inorganic backbone and negatively charged organic side groups, presents particular interest due to apparent simplicity of their macromolecular structure and clinically demonstrated potency.[6-14] The most advanced representative of this group - poly[di(carboxylatophenoxy)phosphazene], PCPP (Figure 1) is a high molecular weight linear polyelectrolyte, which attains its biological performance by spontaneously self-assembling itself with vaccine antigens into water-soluble supramolecular constructs and exerting immunostimulatory activity.[15-19] Another polyphosphazene adjuvant - poly[di(carboxylatoethylphenoxy)phosphazene], PCEP, which only differs from PCPP by two methylene groups (Figure 1) was also synthesized, and induced, somewhat unexpectedly, not only higher antibody levels to vaccine antigens, but also shifted the response towards desirable balanced Th1/Th2 immunity.[10, 20-22] The mechanistic cause of such different biological behavior of these two structurally similar macromolecules is still not evident. It is established that the physical and biological properties of polyphosphazenes are critically dependent on the types of side groups present and on the ratios of different side groups along the same chain. This inspires synthesis of new macromolecules of this homologous series, in particular a polymer with a single methylene

group bridge between phenoxy and carboxylic acid moiety - poly[di(carboxylatomethylphenoxy)phosphazene], PCMP (Figure 1), which can potentially facilitate the establishment of structure activity relationship (SAR) in this family of adjuvants. PCMP is also promising candidate in terms of potential clinical safety. It is well-established that the degradation pathway for polyphosphazene immunoadjuvants, such as PCPP, leads to the release of side group and small amounts of ammonium phosphate.[23] Thereby, rationally designed macromolecules should rely on side groups, which are physiologically benign and display known metabolism profile. To that end, PCMP side group - 4-hydroxyphenylacetic acid, HPA is a constituent of numerous foods, such as olives, cocoa beans, oats, beer, and is well-known for its antioxidant properties.[24-29] It can be also found throughout all human tissues and biofluids.[26, 27, 30, 31]

Antigens based on virus-like particles (VLPs), which are formed by structural viral proteins and mimic the morphology of the pathogen, present an increasingly popular strategy for the development of contemporary vaccines.[32-35] VLPs are efficiently taken up by dendritic cells (DCs), followed by DC activation, maturation, and stimulation of CD4+ T helper cells, leading to the induction of strong humoral and cellular immune responses.[32, 33] Recently, a novel chimeric VLP-based human papillomavirus (HPV) vaccine was developed, which uses RG1-VLPs as an antigenic component, and is comprised of 72 HPV16-L1 pentamers with each L1 subunit engineered to express a 20 amino acid sequence from the HPV16-L2 capsid protein termed RG1 representing a type-common cross-neutralization epitope.[36] When adjuvanted with aluminum salt formulations such as Alhydrogel,[36] the RG1-VLP vaccine provides protection against a broad repertoire of HPV types.

Present paper reports synthesis of a novel homologous member of polyphosphazene immunoadjuvant family - PCMP and compares its *in vitro* activity with parent macromolecule of this class - PCPP. *In vivo* potency of both adjuvants is evaluated with novel HPV VLPs-based

vaccine. We observe spontaneous self-assembly of polyphosphazene adjuvants with VLPs and describe the mechanism as surface modification, which is in contrast with previously reported multimerization of conventional protein antigens by polyphosphazenes. We demonstrate that *in vivo* immunoadjuvant potency of this new rationally designed PCMP macromolecule with HPV VLPs is superior to its PCPP counterpart and other adjuvants used in the study.

Methods

Synthesis of PCMP

All anhydrous procedures were carried out under an atmosphere of dry nitrogen using Labstar Pro glovebox workstation (M. Braun Inertgas-Systeme GMBH, Garching, Germany) and standard laboratory glassware. Ethyl 4-hydroxyphenylacetate, EHPA (24.9 g; 0.138 mol) in diglyme (100 mL) was heated at 110°C for 30 min under nitrogen and then cooled to room temperature. A suspension of sodium hydride (3.15 g, 0.131 mol) in diglyme (0.01 L) was slowly added to the solution of EHPA while stirring under nitrogen. 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 polydichlorophosphazene, PDCP (0.53 g, 0.0046 mol) in 0.03 L of diglyme was slowly added. The mixture was brought to 120°C, and the reaction was let to continue at this temperature for 10 h while stirring. The reaction mixture was then cooled to 90°C, and 0.3 L of aqueous potassium hydroxide (12.7 N) was added. The reaction was vigorously stirred for an additional 30 min at 90°C. The precipitated polymer was recovered by decanting the liquid layer, dissolved in 0.1 L of deionized water, and precipitated by adding 6N hydrochloric acid. It was then redissolved in deionized water by adding 1 N aqueous sodium hydroxide. The purification was repeated. The pH of resulting solution was adjusted to pH 8.5 by adding 1 N hydrochloric acid. The solution was filtered using 0.45 mm polyether sulfone membrane unit, dialyzed using biotech-grade cellulose ester membrane with 50 kDa cut-off, and lyophilized. The yield was 0.98 g (55 % of theoretical).

Physico-chemical characterization methods

NMR spectra were recorded using 400 MHz AscendTM instrument (Bruker Biospin Corp, Billerica, MA). Gel permeation chromatography (GPC) characterization was carried out using La Chrome Elite system (Hitachi, San Jose CA) with PBS as a mobile phase and poly(ethylene oxide) as molecular weight standards (American Polymer Standards Corporation, Mentor, OH).[37] Analysis of formulations was conducted by DLS using Zetasizer Nano series (Malvern Instruments Ltd., Worcestershire, UK) instrument and Asymmetric Flow Field Flow Fractionation, AF4 with AF2000 MT instrument (Postnova Analytics GmbH, Landsberg, Germany).[37]

In vitro immunostimulatory activity and hemocompatibility

Mouse macrophage reporter (RAW BLUE) cells (Invivogen, San Diego, CA, USA) were maintained in culture in 1X DMEM (containing glucose and L-glutamine) supplemented with 10% Fetal Bovine Serum (FBS), 1% Penn Strep and 100 µg/ml normocin. Samples and controls were added to assay wells with 100,000 cells/well in a 96 well plate and incubated at 37°C, 5% carbon dioxide for 20 h. Culture supernatant at one-tenth total volume was added to pNPP reagent and color development read at 405 nm using a Thermo scientific SpectraMax plate reader (Molecular Devices, San Jose, CA, USA). The hemocompatibility of PCMP was evaluated using a modified hemolysis test with porcine red blood cells (RBCs).[38, 39]

Formulations for in vivo studies

RG1-VLPs were manufactured by Paragon Bioservices and were combined with Alhydrogel (InvivoGen, San Diego, CA) at 40 µg/ml RG1-VLPs and 1 mg/ml Alhydrogel and incubated on a rocking platform for 1 h at 4 °C with final dose of 2 µg RG1-VLPs and 50 µg Alhydrogel per injection before equilibration to ambient temperature and administration to mice. Stock solutions of PCMP and PCPP were prepared by dilution into ambient temperature 1X PBS, combined with RG1-VLPs, and vortexed 30 sec for final dosing of 2 µg RG1-VLPs and 25 or 50 µg PP adjuvant. Gardasil-9 (Merck, recombinant 9-valent HPV vaccine) was used as a positive control comparator at 2 µg HPV16-L1 VLPs per dose.

In vivo mouse vaccination studies

8-weeks-old female BALB/c mice (Charles River, Wilmington, MA) were randomized into groups of 8 animals/group and immunized on days 0, 21, 42 (3-weeks intervals). Mice were anesthetized with isoflurane before intramuscular (i.m.) injection into the quadriceps muscle with 50 µl dose volumes. No adverse effects were detected. Terminal bleeds on isoflurane-anesthetized mice were performed via cardiac puncture on day 56 and were collected in serum separator tubes (Fisher Scientific, Waltham, MA) at ambient temperature and centrifuged at 6000 g for 1.5 min. Cell-free sera was collected and stored at -80 °C.

Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 2011; National Academy Press; Washington, D.C.). NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals.

HPV16-L1 VLP and HPV16-L2 RG1 peptide ELISAs

Mouse sera were subjected to quantitation by ELISA of antibodies specific to HPV16-L1 VLPs and to the HPV16-L2 RG1 epitope.[40] For HPV16-L1 antibody quantitation, Maxisorp 96-well plates (Thomas Scientific, Swedesboro, NJ) were coated with HPV16-L1 VLPs at 2.7 µg/ml in coating buffer (1X PBS + 0.2% Proclin 300 (Sigma-Aldrich, St. Louis, MO) and used within 3-5 days after incubation at 4 °C. ELISA plates were incubated with blocking buffer (4% skim milk, 0.2% Tween 20 in 1X PBS) 1.5 h then washed 5 times with wash buffer (0.25% Tween 20 in saline buffer) using a BioTek EL405 plate washer. Sera samples were diluted in blocking buffer at 1:2500 dilution and then serially diluted 1:2 on the plate for 7 more wells for a final volume of 100 µl/well. Sera used for standards and positive controls were generated from mice vaccinated with RG1-VLPs + Alhydrogel, and BALB/c naïve mouse sera (Innovative Research) was used for negative controls. Incubation of sera samples was for 1 h at ambient temperature, gently shaking (300 rpm), followed by plate washing. The secondary antibody conjugate goat anti-mouse IgG-

horseradish peroxidase (HRP) (Sigma-Aldrich, St. Louis, MO) was added to the plates at a dilution of 1:10,000 at a volume of 100 µl/well and plates were incubated again 1 h, ambient temperature, gently shaking. After washing, freshly prepared TMB solution was added at 100 µl/well and plates were incubated 25 min at ambient temperature protected from light. Reactions were stopped by the addition of 100 µl/well of 0.36N H2SO4. Plate optical density (OD) values were measured at 450/620 nm with a SpectraMax M5 (Molecular Devices, San Jose, CA) instrument and data processed by SoftMax Pro 6.3 (Molecular Devices, San Jose, CA). Antibody levels, expressed as ELISA units (EU/ml), were then calculated by interpolation of OD values from the standard curve by averaging the calculated concentrations from all dilutions which fell within the range of the standard curve.

For HPV16-L2 RG1 epitope-specific Ab quantitation, the ELISA procedure is virtually the same as for the L1 ELISA except for the use of NUNC streptavidin-coated 96-well plates (Thermo-Fisher, Waltham, MA) that were coated with 250 ng/ml N-terminal-biotinylated L2 peptide (L2 a.a. 17-36) (JPT) in coating buffer (0.1 M Tris buffer, 0.15 M NaCl, 0.1% Tween 20) at 100 µl/well. Plates were used after 1-day incubation at 4 °C. Sera samples were diluted in blocking buffer 1:5,000 and then serially diluted 1:2. The secondary antibody conjugate was diluted 1:20,000 before being added to plates. All other procedural steps were as in the L1 ELISA protocol. The use of polyclonal antibodies

Furin-cleaved pseudovirion-based neutralization assay (fc-PBNA). LoVo-T cells (ATCC CCL-229, human colorectal adenocarcinoma line) grown to 70-90% confluency were removed by Trypsin/EDTA treatment and seeded at 7500 cells/well in a 96-well flat-bottom plate and incubated for 24 h at 37 °C, 5% carbon dioxide.[41] Pre-diluted (1:25) mouse sera samples were serially diluted 4-fold in DMEM + 10% FBS media in another 96-well plate, including positive and negative control samples derived from RG1-VLP/Alhydrogel-vaccinated mice and naïve mice, respectively. Furin-cleaved pseudovirion (fc-PsV) particles (from HPV types 16, 18, 39, 6) were

synthesized and diluted to pre-determined concentrations (1:1500 for HPV16/39, 1:500 for HPV6, 1:125 for HPV18 based on titration assays) and added to 96-well round-bottom plates followed by equal volume of serially diluted serum samples, and the plates were then incubated for 2 h at 37°C. After incubation, the serum/fcPsV particle mixtures were added to the 96-well flat-bottom plates previously seeded with LoVo T cells, and the plates were then incubated at 37 °C 5% carbon dioxide for 72 h, after which cell supernatants were transferred to 96-well Optiplates (Perkin-Elmer, Waltham, MA) and incubated at 70 °C for 45 min. Optiplates were then incubated on ice for 5 min and centrifuged briefly, before SEAP (secreted alkaline phosphatase) substrate (Cayman Chemical, Ann Arbor, MI) was added, followed by 30 min incubation at ambient temperature, protected from light. Plates were read on a SpectraMax M5 microplate reader. The PBNA titers are reported as the reciprocal of the dilution that caused a 50% reduction in SEAP activity in comparison to the fcPsV-infected cells without added sera.

Results

Synthesis and physico-chemical characterization of PCMP

The synthetic pathway to PCMP involved polymerization of hexachlorocyclotriphosphazene (I) to produce macromolecular precursor PDCP (II), substitution of diglyme-stabilized PDCP with EHPA to produce water-insoluble polyorganophosphazene (III), and basic hydrolysis of ethyl ester groups of this polymer to yield water-soluble PCMP (Figure 2). This approach, and hydrolytic deprotection of (III) using potassium hydroxide in heterogeneous system in particular, allowed simple and efficient recovery of precipitated PCMP from the reaction mixture. GPC analysis of the polymer revealed high molecular weight peak with unimodal distribution (Supplementary materials, Table S1 and Figure S1). DLS measured z-average hydrodynamic diameter of PCMP in PBS (75 nm), which is typically important for applications of polyphosphazenes as vaccine delivery system, was in the same range of that for PCPP - 60-80 nm.[19, 42] PCMP structure was confirmed by ^{1}H and ^{31}P NMR (Supplementary materials, Table S1, Figures S2 and S3).

Biologically relevant in vitro properties of PCMP

PCMP was first evaluated for *in vitro* properties, which are relevant to its potential application as an immunoadjuvant. The ability to undergo hydrolytic degradation is one of the key characteristics of polyphosphazene adjuvants.[23] Kinetics and mechanism of degradation has been extensively studied for clinical stage macromolecule, PCPP, and involves pH and temperature dependent hydrolysis with the release of side group and ammonium phosphate as degradation products.[23] The potential of PCMP to degrade under physiological conditions was assessed by monitoring the decrease in weight average molecular weight of the polymer in PBS, pH 7.4 (Figure 3A). Similarly, to PCPP studies, we investigated hydrolysis under accelerated (80°C), physiological (37°C), and storage (4°C) conditions.[23] The half-life of the polymer in solution at 80°C was approximately two days (Figure 3A), which proves its ability to completely degrade with the release of low molecular weight products. As expected, the degradation rate was significantly reduced at physiological temperature and the molecular weight loss was less than 25% at 4°C within the timeframe of the experiment (Figure 3A). The half-life under near physiological conditions (PBS, pH 7.4, 37ºC) was 16 days and the weight average molecular weight of degradation products after six months was 18 KDa - well below the general threshold of renal filtration of polymers - 30-50 kDa.[43] Overall, the results demonstrate the ability of PCMP to degrade in a PCPP-like mode, which is generally characterized by long solution shelf-life under refrigerated conditions, adequate *in vivo* half-life to exert vaccine delivery capacity, and complete eventual destruction of the backbone with the release of low-molecular weight products.[23]

We investigated the effect of various concentrations of sodium chloride on hydrodynamic diameters of PCPP and PCMP in solutions (Figure 3B). As seen from the Figure, while PCPP severely aggregates in the presence of two-fold of physiological sodium chloride concentration, PCMP does not display similar vulnerability and remains completely free of aggregates even in 50 mg/ml sodium chloride solutions. The hemocompatibility of a new polyphosphazene derivative

was verified using hemolysis test with porcine RBCs.[38, 39] The results indicate the absence of inherent cytotoxicity of both anionic polyphosphazenes at neutral pH: PCPP and PCMP. This is in a contrast with pronounced hemolytic activity of a typical cationic polymer - polyethyleneimine, which was used for comparison purposes (Supplementary materials, Figures S4 and S5).

Immunostimulatory activity of polyphosphazene adjuvants is yet another characteristic that is important for their physiological performance.[16, 19] To that end, PCMP was compared to PCPP using RAW Blue cells - an engineered mouse macrophage cell line, which has been increasingly used for evaluation of immunoadjuvants.[44-50] These immune cells contain Toll-Like Receptors (TLRs) and integrate Secreted Alkaline Phosphatase (SEAP) reporter inducible by Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) to allow screening of innate immune activation. The ability of polyphosphazene adjuvants to non-specifically interact with TLRs was demonstrated previously.[16] Figure 3C confirms dose-dependent immunostimulatory activity of PCMP in these assays, which is generally of the same level as that of PCPP. It has to be noted though, that the immunostimulatory effect of polyphosphazenes is only one of two arms, in which they exercise their immunoadjuvant potency. Perhaps, even more important is the way these macromolecules assemble with and deliver vaccine antigens,[15, 16, 18, 19] which is investigated next.

Self-assembly of polyphosphazene adjuvants with HPV VLPs

Adjuvant and vaccine delivery capacity of PCMP and PCPP were evaluated and compared in formulations with RG1-VLPs, which is the antigenic component of a novel chimeric HPV vaccine.[36] It was important to understand if similar self-assembly processes will occur in such systems and if so, what will be the potential spatial arrangement and size of such assemblies. DLS analysis of both, PCPP and PCMP formulations with VLPs (Figures 4A and 4B), showed no aggregation with only minor increase in size compared to the size of either component, when the polymer was used in excess (Figures 4A, 4B, and 4C). The lack of aggregation was independently confirmed by electron microscopy (Figure S6). However, the results were dramatically different

when the deficiency of either PCPP, or PCPP was used (Figure 4C). The inset in Figure 4C shows severe aggregation in both systems when VLPs were present in excess.

RG1-VLP formulations with polyphosphazene adjuvants were further investigated using asymmetric flow field flow fractionation (AF4) method.[16, 19, 51] This technique performs effective separation of analytes by size, but unlike most chromatography methods, allows analysis of supramolecular assemblies of practically unlimited sizes.[51] AF4 fractograms of PCMP and PCPP (Figures 4D and S7) were consistent with previously reported results on formulations of polyphosphazene adjuvants.[16, 19] Somewhat unexpectedly, we were not able to detect any signals for RG1-VLP using this technique (Figure 4D). This may be attributed to non-specific interactions of VLPs with analytical membrane and resulting adsorption of particles under conditions of the experiment. However, modification of RG1-VLPs with PCMP leads to the emergence of higher elution time - larger size shoulder on the polymer peak (Figures 4D). Likewise, AF4 fractogram of VLP-PCPP formulation displays a separate peak (26 min) with a larger elution time than PCPP (17 min) indicating presence of larger size PCPP modified VLPs. Both of these results appear to be consistent with size increase observed based on DLS analysis (Figures 4A and 4B).

Short-term stability of PCMP-VLPs formulations

Short-term stability of polyphosphazene formulations with RG1-VLPs was studied by DLS. Figures 5A and 5B show representative DLS profiles of PCPP-VLPs and PCMP-VLPs formulations before and after 44-hour incubation in PBS (pH 7.4) at 4°C. As can be seen from the Figure, no changes were observed in the size distribution profile by intensity for PCMP adjuvanted formulation. Although no aggregation was observed for PCPP formulation either, some increase in the diameter of the formulation was detected (Figure 5A). The dependences of hydrodynamic diameters of polymer-modified VLPs on the incubation time in the 70-h range are shown in Figure 5C. The results indicate that PCMP based formulations show superior stability that its PCPP based counterparts.

In vivo immunoadjuvant activity of PCMP

To investigate the *in vivo* potency of PCMP, we compared immunoadjuvant activities of PCMP and PCPP using HPV vaccination in mice. We immunized BALB/c mice intramuscularly (prime and two boosts, three-week intervals between injections) with RG1-VLPs formulated with PCPP or PCMP (25 or 50 μg doses). All formulations were prepared at excess of polyphosphazenes and were free of aggregates. Polyphosphazene formulations were benchmarked against RG1-VLPs formulated with 50 μg Alhydrogel (alum), unadjuvanted VLPs and Gardasil-9 vaccine. Both VLP/polyphosphazene formulations promoted statistically higher geomeans of HPV16-L1 antibody levels compared to VLPs alone, which was not achieved in formulations adjuvanted with alum (Figure 6A). Furthermore, the 50-μg dose of both PCPP and PCMP achieved statistical relevance compared to alum. Induction of high L2-specific antibody levels is less challenging presumably due to the high affinity of the RG1 epitope, and all three adjuvants, alum, PCPP, and PCMP, were able to boost L2 antibody levels significantly above VLPs alone (Figure 6B). Moreover, the 50 ug dose of PCMP also increased L1 Ab levels higher than those achieved by Gardasil-9, which was also unable to induce any Abs specific to L2, due to the absence of the RG1 epitope.

Pseudovirion-based neutralization assays (PBNA) were conducted to determine the extent of neutralizing antibody titers generated versus HPV16 and cross-neutralizing antibody titers versus HPV18, HPV39, and HPV6. PCPP and PCMP outperformed alum in the induction of HPV16 type-specific neutralizing antibody titers, and the 50 μg PCMP group achieved statistical superiority compared to alum and Gardasil-9 (Figure 7A). PCMP-adjuvanted formulations also demonstrated high magnitude neutralizing titers to both HPV18 and HPV39 which were not achieved by either PCPP or alum (Figure 7B and 7C). Polyphosphazene-adjuvanted groups showed higher HPV6-neutralizing titers, and although the levels did not achieve statistical superiority over alum, unlike with alum, no non-responding mice were observed in the 50 μg polyphosphazene groups (Figure

7D). These data demonstrate that PCMP not only may readily substitute for Alhydrogel in the context of a mouse VLP-based vaccine model, but can attain higher magnitude humoral responses, especially in the levels of highly desirable protective cross-neutralizing antibodies.

Discussion

Synthesis of PCMP was carried out using a reaction pathway, which was originally designed as a multi-step bench scale procedure and later modified for the three-step production process for a clinical-grade PCPP.[20, 52, 53] The adaptation of synthetic processes, which were used in the manufacturing of clinical grade PCPP, to the synthesis of new members of this homologous series, is highly beneficial in terms of successful development of this class of immunoadjuvants.

The new adjuvant demonstrated similar properties to PCPP in terms of its clinically relevant hydrolytic degradation and immunostimulatory properties, but showed superior stability in solutions with higher ionic content. Previous studies have shown that PCPP displays unusual sensitivity to sodium ions,[53] which can result in undesirable agglomeration of its supramolecular complexes with some vaccine antigens, eventually leading to inferior *in vivo* potency.[15] This "hyper" sensitivity manifests in phase separation and appears to be restricted to sodium ions only. Interestingly, it was not observed for formulations with other monovalent ions, such as potassium and lithium.[53] It was hypothesized that the sensitivity results from the unique spatial arrangements of neighboring side groups in PCPP structure,[53] which are stabilized by aromatic-aromatic (π - π) interactions of benzene rings.[54, 55] Demonstrated lower sensitivity of PCMP to ionic environment appears to support this hypothesis. Moreover, it can be an important factor for improving stability of vaccine formulations as protein loaded polymer chains can become prone to aggregation even at a lower salt concentration.[15] In fact, PCMP formulations display superior short-term stability compared to those of PCPP (Figure 5). It has to be noted that the general capacity of polyphosphazene polyelectrolytes to stabilize protein-based vaccine antigens has been previously reported.[17, 56, 57]

The ability of PCPP to spontaneously self-assemble with vaccine antigens via physiologically stable non-covalent interactions has been well-established in its formulations with antigenic proteins. PCPP coils, which can be compared to viruses in terms of their dimensions - 60-80 nm in diameter, can usually assemble and display multiple copies of proteins, which are significantly smaller in size compared to the polymer itself.[15, 16, 18, 19] However, in formulations of VLPs with PCPP and PCMP, both the antigen and the polymer are characterized by comparable dimensions (Figures 4A and 4B) and are not likely to result in the established pattern of multimeric complexes (Figure 8A).[15, 16, 18, 19] Both DLS and AF4 results suggest the occurrence of interactions between soluble polyphosphazene adjuvants and VLPs with resulting assemblies displaying increase in dimensions compared to initial components (Figure 4). At low polyphosphazene-to-VLPs ratios, these non-covalent interactions manifest themselves in a drastic increase in size of assemblies (over 10-fold) indicating undesirable aggregation in the formulation. It can be hypothesized, that attachment of multiple particles to the same polyphosphazene chain causes significant destabilization in the system due to charge neutralization therefore resulting in aggregation effects shown in Figure 4C (inset). The suggested pathway can be schematically presented in Figure 8A (low PPZ:VLP ratio). The situation improves drastically when higher concentrations of polyphosphazenes are used (Figure 4C) and only minor (approximately 20%) increase in size compared to initial components is detected, which is unlikely to be caused by particle aggregation. Recent studies on covalent surface modification of VLPs with synthetic polymers also report an increase in size of modified particles describing the phenomenon as the formation of polymer shell around VLPs.[58, 59] Similarly, it can be suggested that excess of polyphosphazenes compared to VLPs lead to the formation of polymer coatings around VLPs, although via non-covalent bonds. Since both PCPP and PCEP are negatively charged, electrostatic repulsions between such surface modified particles can effectively prevent aggregation. The ‘visualization’ of polymer treated VLPs in AF4 analysis (Figures 4D and Supplementary materials, S7) also supports this hypothesis, as negatively charged polymer coating should be effective in the prevention of non-

specific interactions with analytical membrane - the phenomenon observed for untreated VLPs. The discussed mechanism can be schematically presented in Figure 8B (high PPZ:VLP ratio). From the practical perspective, especially when it concerns further preclinical development, the results clearly demonstrate that formulation conditions are critical for the performance of polyphosphazene adjuvants, and they have to be controlled to avoid undesirable aggregation and potential loss of formulation activity.

In vivo immunoadjuvant activity of PCMP was evaluated using HPV VLPs vaccine. RG1-VLPs are the antigenic component of a novel chimeric VLP-based HPV vaccine comprised of 72 HPV16-L1 pentamers, each L1 subunit engineered to express a 20 amino acid sequence from the HPV16-L2 capsid protein termed RG1.[36] Unlike L1 capsid sequences, the RG1 peptide of the L2 sequence is well-conserved among HPV types and is known to present a high-affinity B cell epitope for the generation of cross-neutralization antibodies in vivo. When adjuvanted with aluminum salt formulations such as Alhydrogel (alum),[36] the RG1-VLP vaccine provides protection against a broad repertoire of HPV strains. In mice, PCMP adjuvanted formulations induced superior HPV16 type-specific neutralizing antibody responses compared to alum adjuvanted VLPs and Gardasil-9. Since HPV16-L1 serves as the backbone of the RG1-VLP vaccine, protection versus non-HPV16 types must rely on the cross-neutralizing potential of the RG1 epitope. To that end, PCMP, but not PCPP, formulations outperformed alum adjuvanted VLPs and Gardasil-9 in attaining high levels of specific neutralizing types of HPV (HPV18; HPV39 and HPV39, correspondingly) demonstrating potential in the induction of highly desirable protective cross-neutralizing antibodies.

The above results demonstrate an inspiring *in vivo* potency of a new PCMP adjuvant, its ability to form defined supramolecular assemblies with the antigen, and lay out the foundation for future development of structure-activity relationship in this important family of immunoadjuvants.

Appendix A. Supplementary data

Supplementary materials for this article can be found online at

Figure Legends

Figure 1. Schematic presentations of PCMP and other members of homologous series of polyphosphazene immunoadjuvants - PCPP and PCEP.

Figure 2. Three-step synthetic pathway to PCMP.

Figure 3. (A) Hydrolytic degradation of PCMP expressed as a percent of weight-average molecular weight (Mw) change over time at 80°C, 37°C, and 4°C (0.5 mg/mL PCMP, PBS, pH 7.4); (B) hydrodynamic diameter of PCMP and PCPP as a function of added sodium chloride (DLS, polymer concentration 0.5 mg/mL, PBS, pH 7.4), and (C) immunostimulatory activity of PCMP and PCPP (n =3, error bars - standard deviation).

Figure 4. DLS profiles of (A) PCPP, VLP, and their formulation at PCPP:VLP=2.5:1 (w/w) ratio; (B) PCMP, VLP, and their formulation at PCMP:VLP=2.5:1 (w/w) ratio; (C) hydrodynamic diameter of PCPP and PCMP complexes with VLP versus polyphosphazene (PPZ) -to-VLP ratio; and (D) AF4 fractograms of PCMP, VLP, and their formulation (0.25 mg/mL PCMP, 0.1 mg/mL VLPs, poly(styrene sulfonic acid) standards, PBS, pH 7.4,).

Figure 5. DLS profiles of (A) PCPP-VLP and (B) PCMP-VLP at 0 h and 44 h and (C) and hydrodynamic diameter of PCPP-VLP and PCMP-VLP assemblies versus time of incubation (PBS, pH 7.4, 4°C, 0.5 mg/mL PCPP or PCMP, 0.02 mg/mL VLPs).

Figure 6. PCMP adjuvant for higher magnitude HPV16-L1- (A) and L2-specific (B) antibody (Ab) levels compared to alum. Mice were immunized i.m. with 2 μg RG1-VLP alone or adjuvanted with 50 μg alum +/- 25, 50 μg PCPP, 25, 50 μg PCMP, or Gardasil-9 on days 0, 21, 42 and peripheral blood sera samples derived on day 56. Sera samples were tested for HPV16-L1- and HPV16-L2

RG1-specific IgG via ELISA. Data are reported as geometric means + 95% CI. Statistical comparisons are between VLPs alone and all groups or between alum and all groups (upper tier for (A)) and were generated using one-way ANOVA nonparametric analysis with the Kruskal-Wallis multiple comparisons test. ns, not significant ($p > 0.05$); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Figure 7. HPV-specific neutralization titers induced by PCMP are superior to Alhydrogel. Mice were immunized i.m. with 2 μg RG1-VLP alone or adjuvanted with 50 μg alum +/- 25, 50 μg PCPP, 25, 50 μg PCMP, or Gardasil-9 on days 0, 21, 42 and peripheral blood sera samples derived on day 56. Day 56 sera samples were analyzed for neutralizing titers via fc-PBNA specific for PsV16-(A), PsV18-(B), PsV39-(C), and PsV6-SEAP (D). Data are reported as geometric means + 95% CI. Statistical comparisons are between VLPs alone and all groups or between alum and all groups (upper tier for (A)) and were generated using one-way ANOVA nonparametric analysis with the Kruskal-Wallis multiple comparisons test. ns, not significant ($p > 0.05$); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Figure 8. Suggested mechanism of spontaneous self-assembly of polyphosphazenes (PPZs) with protein (A) and VLP (B) antigens.

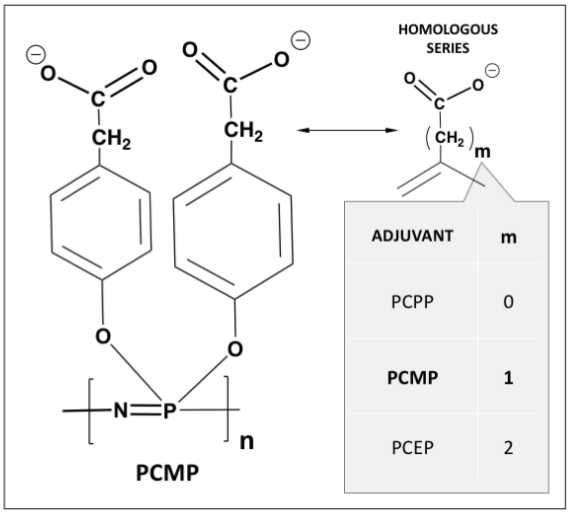

Figure 1.

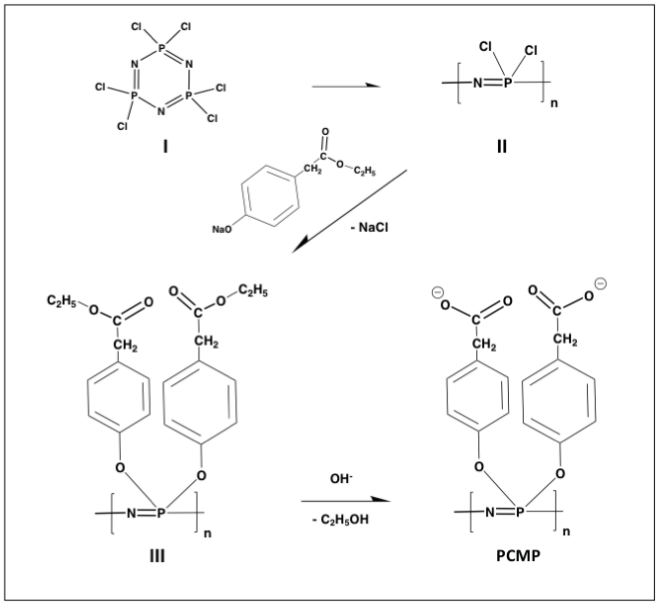

Figure 2.

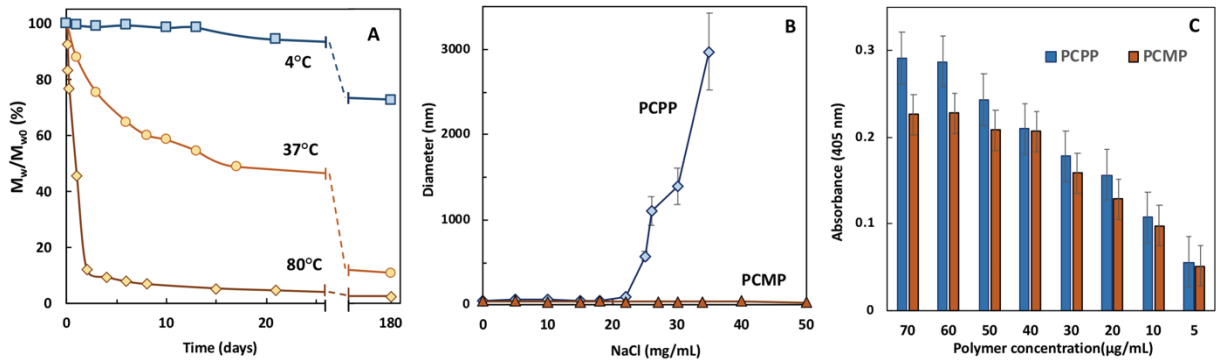
A
4°C
37°C
80°C
M_w/M_{w0} (%)
Time (days)
B
PCPP
PCMP
Diameter (nm)
NaCl (mg/mL)
C
PCPP
PCMP
Absorbance (405 nm)
Polymer concentration(μg/mL)

Figure 3.

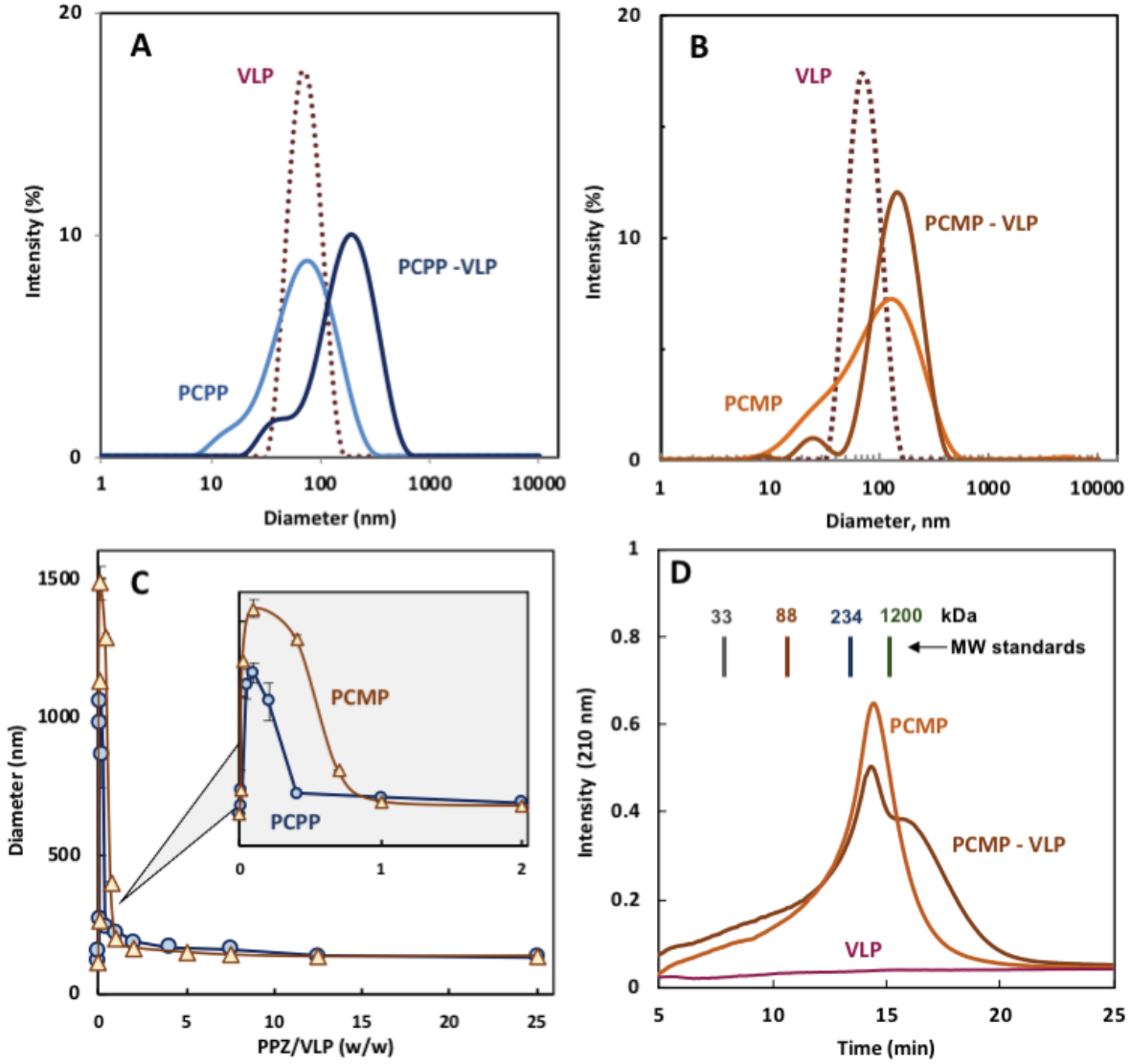
A
VLP
PCPP -VLP
PCPP
Intensity (%)
Diameter (nm)
B
VLP
PCMP - VLP
PCMP
Intensity (%)
Diameter, nm
C
PCMP
PCPP
Diameter (nm)
PPZ/VLP (w/w)
D
33
88
234
1200
kDa
MW standards
PCMP
PCMP - VLP
VLP
Intensity (210 nm)
Time (min)

Figure 4.

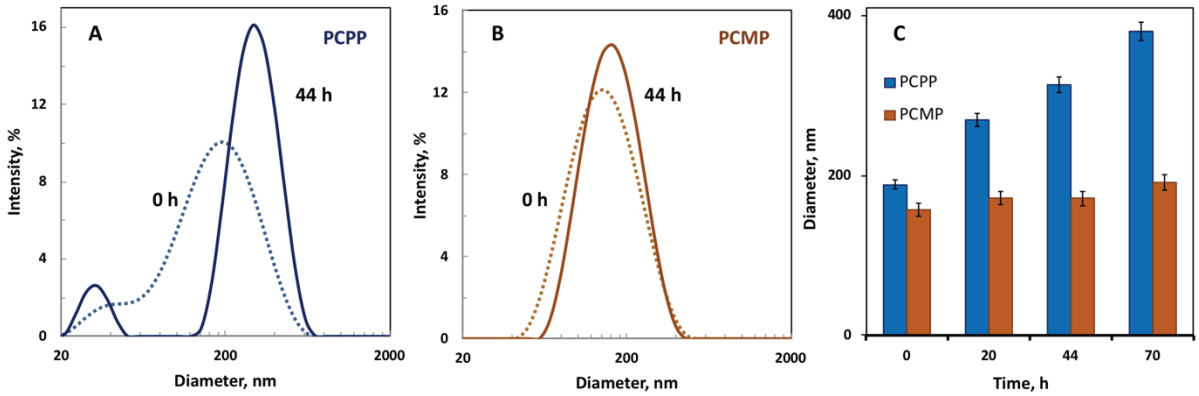

Figure 5.

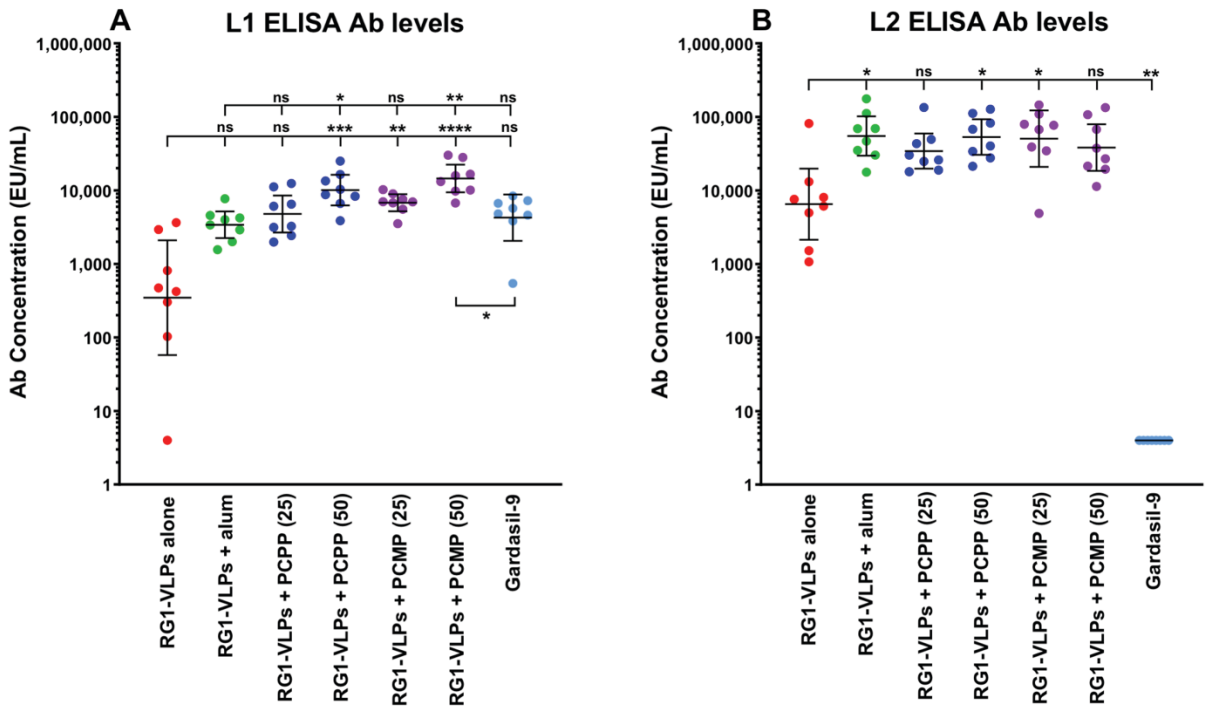

Figure 6.

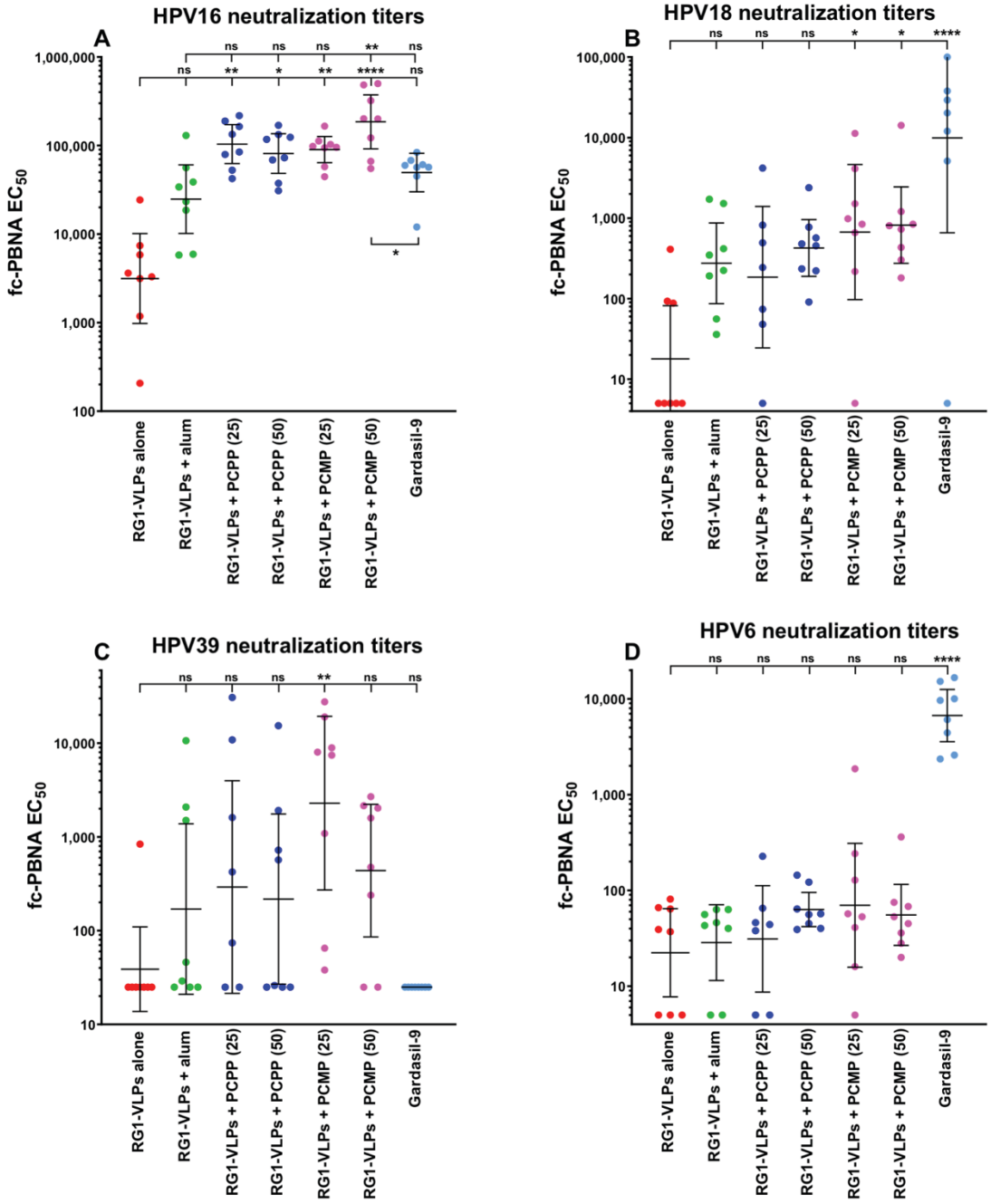
A
HPV16 neutralization titers
fc-PBNA EC50
1,000,000
100,000
10,000
1,000
100
ns ns ns ** ns
ns ** * ** **** ns
*
RG1-VLPs alone
RG1-VLPs + alum
RG1-VLPs + PCPP (25)
RG1-VLPs + PCPP (50)
RG1-VLPs + PCMP (25)
RG1-VLPs + PCMP (50)
Gardasil-9
B
HPV18 neutralization titers
fc-PBNA EC50
100,000
10,000
1,000
100
10
ns ns ns * * ****
RG1-VLPs alone
RG1-VLPs + alum
RG1-VLPs + PCPP (25)
RG1-VLPs + PCPP (50)
RG1-VLPs + PCMP (25)
RG1-VLPs + PCMP (50)
Gardasil-9
C
HPV39 neutralization titers
fc-PBNA EC50
10,000
1,000
100
10
ns ns ns ** ns ns
RG1-VLPs alone
RG1-VLPs + alum
RG1-VLPs + PCPP (25)
RG1-VLPs + PCPP (50)
RG1-VLPs + PCMP (25)
RG1-VLPs + PCMP (50)
Gardasil-9
D
HPV6 neutralization titers
fc-PBNA EC50
10,000
1,000
100
10
ns ns ns ns ns ****
RG1-VLPs alone
RG1-VLPs + alum
RG1-VLPs + PCPP (25)
RG1-VLPs + PCPP (50)
RG1-VLPs + PCMP (25)
RG1-VLPs + PCMP (50)
Gardasil-9

Figure 7

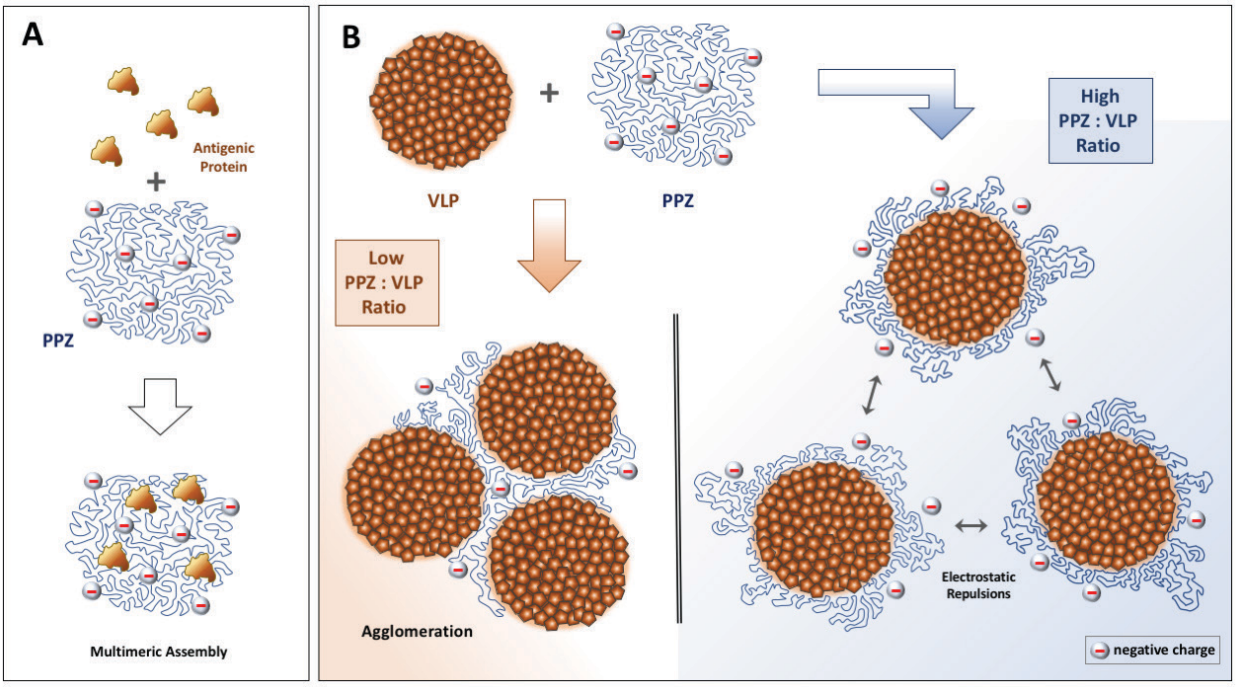

Figure 8.

References

1. Pashine, A.; Valiante, N. M.; Ulmer, J. B. Targeting the innate immune response with improved vaccine adjuvants, *Nat Med* 2005;***11***:S63-S68.

2. Liang, T. J. Current progress in development of hepatitis C virus vaccines, *Nat Med* 2013;***19***:869-78.

3. Duthie, M. S.; Windish, H. P.; Fox, C. B.; Reed, S. G. Use of defined TLR ligands as adjuvants within human vaccines, *Immunol Rev* 2011;***239***:178-96.

4. Del Giudice, G.; Rappuoli, R.; Didierlaurent, A. M. Correlates of adjuvanticity: A review on adjuvants in licensed vaccines, *Semin Immunol* 2018;***39***:14-21.

5. Brito, L. A.; Malyala, P.; O'Hagan, D. T. Vaccine adjuvant formulations: A pharmaceutical perspective, *Semin Immunol* 2013;***25***:130-45.

6. Andrianov, A. K. Polyphosphazenes as vaccine adjuvants: In: Singh, M., editor. *Vaccine Adjuvants and Delivery Systems*. Hoboken, New Jersey: John Wiley & Sons, 2007, 355-78.

7. Andrianov, A. K. Polyphosphazene vaccine delivery vehicles: state of development and perspectives: In: Andrianov, A. K., editor. *Polyphosphazenes for biomedical applications*. Hoboken, N.J.: Wiley, 2009, 47-63.

8. Powell, B. S.; Andrianov, A. K.; Fusco, P. C. Polyionic vaccine adjuvants: another look at aluminum salts and polyelectrolytes, *Clin Exp Vaccine Res* 2015;***4***:23-45.

9. Eng, N. F.; Garlapati, S.; Gerdts, V.; Potter, A.; Babiuk, L. A.; Mutwiri, G. K. The Potential of Polyphosphazenes for Delivery of Vaccine Antigens and Immunotherapeutic Agents, *Curr Drug Delivery* 2010;***7***:13-20.

10. Magiri, R.; Mutwiri, G.; Wilson, H. L. Recent advances in experimental polyphosphazene adjuvants and their mechanisms of action, *Cell Tissue Res* 2018;***374***:465-71.

11. O'Connell, R. J.; Excler, J.-L.; Polonis, V. R., et al. Safety and Immunogenicity of a randomized Phase I prime-boost trial with ALVAC-HIV (vCP205) and Oligomeric gp160 MN/LAI-2 Adjuvanted in Alum or Polyphosphazene, *J Infect Dis* 2016;***213***:1946-54.

12. O'Connell, R.; Polonis, V.; Ratto-Kim, S., et al. Safety and immunogenicity of a randomized phase I prime-boost trial with ALVAC-HIV (vCP205) and gp160 MN/LAI-2 adjuvanted in alum or polyphosphazene, *Retrovirology* 2012;***9***:O50.

13. Thongcharoen, P.; Suriyanon, V.; Paris, R. M., et al. A Phase 1/2 Comparative Vaccine Trial of the Safety and Immunogenicity of a CRF01_AE (Subtype E) Candidate Vaccine: ALVAC-HIV (vCP1521) Prime With Oligomeric gp160 (92TH023/LAI-DID) or Bivalent gp120 (CM235/SF2) Boost, *J Acquired Immune Defic Syndr* 2007;***46***:48.

14. Ison, M. G.; Mills, J.; Openshaw, P.; Zambon, M.; Osterhaus, A.; Hayden, F. Current research on respiratory viral infections: Fourth International Symposium, *Antiviral Res* 2002;***55***:227-78.

15. Andrianov, A. K.; Marin, A.; Roberts, B. E. Polyphosphazene polyelectrolytes: A link between the formation of noncovalent complexes with antigenic proteins and immunostimulating activity, *Biomacromolecules* 2005;***6***:1375-79.

16. Andrianov, A. K.; Marin, A.; Fuerst, T. R. Molecular-Level Interactions of Polyphosphazene Immunoadjuvants and Their Potential Role in Antigen Presentation and Cell Stimulation, *Biomacromolecules* 2016;***17***:3732-42.

17. Palmer, C. D.; Ninković, J.; Prokopowicz, Z. M., et al. The effect of stable macromolecular complexes of ionic polyphosphazene on HIV Gag antigen and on activation of human dendritic cells and presentation to T-cells, *Biomaterials* 2014;***35***:8876-86.

18. Cayatte, C.; Marin, A.; Rajani, G. M., et al. PCPP-Adjuvanted Respiratory Syncytial Virus (RSV) sF Subunit Vaccine: Self-Assembled Supramolecular Complexes Enable Enhanced Immunogenicity and Protection, *Mol Pharmaceutics* 2017;***14***:2285-93.

19. Andrianov, A. K.; Marin, A.; Wang, R., et al. Supramolecular assembly of Toll-like receptor 7/8 agonist into multimeric water-soluble constructs enables superior immune stimulation in vitro and in vivo, *ACS Appl Bio Mater* 2020;***3***:3187-95.

20. Andrianov, A. K.; Marin, A.; Chen, J. Synthesis, properties, and biological activity of Poly[di(sodium carboxylatoethylphenoxy)phosphazene], *Biomacromolecules* 2006;***7***:394-99.

21. Mutwiri, G.; Benjamin, P.; Soita, H., et al. Poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP) is a potent enhancer of mixed Th1/Th2 immune responses in mice immunized with influenza virus antigens, *Vaccine* 2007;***25***:1204-13.

22. Dar, A.; Lai, K.; Dent, D., et al. Administration of poly[di(sodium carboxylatoethylphenoxy)]phosphazene (PCEP) as adjuvant activated mixed Th1/Th2 immune responses in pigs, *Vet Immunol Immunopathol* 2012;***146***:289-95.

23. DeCollibus, D. P.; Marin, A.; Andrianov, A. K. Effect of Environmental Factors on Hydrolytic Degradation of Water-Soluble Polyphosphazene Polyelectrolyte in Aqueous Solutions, *Biomacromolecules* 2010;***11***:2033-38.

24. Papadopoulos, G.; Boskou, D. Antioxidant effect of natural phenols on olive oil, *J Am Oil Chem Soc* 1991;***68***:669-71.

25. Nardini, M.; Ghiselli, A. Determination of free and bound phenolic acids in beer, *Food Chem* 2004;***84***:137-43.

26. Nardini, M.; Natella, F.; Scaccini, C.; Ghiselli, A. Phenolic acids from beer are absorbed and extensively metabolized in humans, *J Nutr Biochem* 2006;***17***:14-22.

27. Villaño, D.; Fernández-Pachón, M. S.; Troncoso, A. M.; García-Parrilla, M. C. Comparison of antioxidant activity of wine phenolic compounds and metabolites in vitro, *Anal Chim Acta* 2005;***538***:391-98.

28. Sroka, Z.; Cisowski, W. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids, *Food Chem Toxicol* 2003;***41***:753-58.

29. Vissiennon, C.; Nieber, K.; Kelber, O.; Butterweck, V. Route of administration determines the anxiolytic activity of the flavonols kaempferol, quercetin and myricetin — are they prodrugs?, *J Nutr Biochem* 2012;***23***:733-40.

30. Bouatra, S.; Aziat, F.; Mandal, R., et al. The Human Urine Metabolome, *PLOS ONE* 2013;***8***:e73076.

31. Zhang, K.; Zuo, Y. GC-MS Determination of Flavonoids and Phenolic and Benzoic Acids in Human Plasma after Consumption of Cranberry Juice, *J Agric Food Chem* 2004;***52***:222-27.

32. Kushnir, N.; Streatfield, S. J.; Yusibov, V. Virus-like particles as a highly efficient vaccine platform: Diversity of targets and production systems and advances in clinical development, *Vaccine* 2012;***31***:58-83.

33. Chackerian, B. Virus-like particles: flexible platforms for vaccine development, *Expert Rev Vaccines* 2007;***6***:381-90.

34. Lua, L. H. L.; Connors, N. K.; Sainsbury, F.; Chuan, Y. P.; Wibowo, N.; Middelberg, A. P. J. Bioengineering virus-like particles as vaccines, *Biotechnol Bioeng* 2014;***111***:425-40.

35. Zeltins, A. Construction and Characterization of Virus-Like Particles: A Review, *Mol Biotechnol* 2013;***53***:92-107.

36. Schellenbacher, C.; Kwak, K.; Fink, D., et al. Efficacy of RG1-VLP Vaccination against Infections with Genital and Cutaneous Human Papillomaviruses, *J Invest Dermatol* 2013;***133***:2706-13.

37. Andrianov, A. K.; Marin, A.; Martinez, A. P.; Weidman, J. L.; Fuerst, T. R. Hydrolytically Degradable PEGylated Polyelectrolyte Nanocomplexes for Protein Delivery, *Biomacromolecules* 2018;***19***:3467-78.

38. Andrianov, A. K.; Marin, A.; Fuerst, T. R. Self-assembly of polyphosphazene immunoadjuvant with poly(ethylene oxide) enables advanced nanoscale delivery modalities and regulated pH-dependent cellular membrane activity, *Heliyon* 2016;***2***:Article e00102.

39. Yessine, M.-A.; Lafleur, M.; Meier, C.; Petereit, H.-U.; Leroux, J.-C. Characterization of the membrane-destabilizing properties of different pH-sensitive methacrylic acid copolymers, *Biochim Biophys Acta, Biomembr* 2003;***1613***:28-38.

40. Tsang, S. H.; Basu, P.; Bender, N., et al. Evaluation of serological assays to monitor antibody responses to single-dose HPV vaccines, *Vaccine* 2020;***38***:5997-6006.

41. Wang, J. W.; Jagu, S.; Kwak, K., et al. Preparation and properties of a papillomavirus infectious intermediate and its utility for neutralization studies, *Virology* 2014;***449***:304-16.

42. Andrianov, A. K.; Marin, A.; Deng, J.; Fuerst, T. R. Protein-loaded soluble and nanoparticulate formulations of ionic polyphosphazenes and their interactions on molecular and cellular levels, *Mater Sci Eng, C* 2020;***106***:110179.

43. Fox, M. E.; Szoka, F. C.; Fréchet, J. M. J. Soluble polymer carriers for the treatment of cancer: the importance of molecular architecture, *Acc Chem Res* 2009;***42***:1141-51.

44. Shafique, M.; Meijerhof, T.; Wilschut, J.; de Haan, A. Evaluation of an intranasal virosomal vaccine against respiratory syncytial virus in mice: effect of TLR2 and NOD2 ligands on induction of systemic and mucosal immune responses, *PloS one* 2013;***8***:e61287-e87.

45. Shafique, M.; Wilschut, J.; de Haan, A. Induction of mucosal and systemic immunity against respiratory syncytial virus by inactivated virus supplemented with TLR9 and NOD2 ligands, *Vaccine* 2012;***30***:597-606.

46. Rees, P. A.; Lowy, R. J. Measuring type I interferon using reporter gene assays based on readily available cell lines, *J Immunol Methods* 2018;***461***:63-72.

47. Li, X.; Szewczuk, M. R.; Malardier-Jugroot, C. Folic acid-conjugated amphiphilic alternating copolymer as a new active tumor targeting drug delivery platform, *Drug Des Devel Ther* 2016;***10***:4101-10.

48. Pizzuto, M.; Bigey, P.; Lachagès, A.-M., et al. Cationic lipids as one-component vaccine adjuvants: A promising alternative to alum, *J Controlled Release* 2018;***287***:67-77.

49. Yu, C.; An, M.; Li, M.; Liu, H. Immunostimulatory Properties of Lipid Modified CpG Oligonucleotides, *Mol Pharm* 2017;***14***:2815-23.

50. Cai, H.; Shukla, S.; Steinmetz, N. F. The Antitumor Efficacy of CpG Oligonucleotides is Improved by Encapsulation in Plant Virus-Like Particles, *Adv Funct Mater* 2020;***30***:1908743.

51. Messaud, F. A.; Sanderson, R. D.; Runyon, J. R.; Otte, T.; Pasch, H.; Williams, S. K. R. An overview on field-flow fractionation techniques and their applications in the separation and characterization of polymers, *Prog Polym Sci* 2009;***34***:351-68.

52. Allcock, H. R.; Kwon, S. An ionically cross-linkable polyphosphazene: Poly[bis(carboxylatophenoxy)phosphazene] and its hydrogels and membranes, *Macromolecules* 1989;***22***:75-79.

53. Andrianov, A. K.; Svirkin, Y. Y.; LeGolvan, M. P. Synthesis and biologically relevant properties of polyphosphazene polyacids, *Biomacromolecules* 2004;***5***:1999-2006.

54. Martinez, C. R.; Iverson, B. L. Rethinking the term “pi-stacking”, *Chemical Science* 2012;***3***:2191-201.

55. Grimme, S. Do Special Noncovalent π–π Stacking Interactions Really Exist?, *Angew Chem, Int Ed Engl* 2008;***47***:3430-34.

56. Marin, A.; DeCollibus, D. P.; Andrianov, A. K. Protein Stabilization in Aqueous Solutions of Polyphosphazene Polyelectrolyte and Non-Ionic Surfactants, *Biomacromolecules* 2010;***11***:2268-73.

57. Andrianov, A. K.; Decollibus, D. P.; Marin, A.; Webb, A.; Griffin, Y.; Webby, R. J. PCPP-formulated H5N1 influenza vaccine displays improved stability and dose-sparing effect in lethal challenge studies, *J Pharm Sci* 2011;***100***:1436-43.

58. Manzenrieder, F.; Luxenhofer, R.; Retzlaff, M.; Jordan, R.; Finn, M. G. Stabilization of virus-like particles with poly(2-oxazoline)s, *Angew Chem Int Ed Engl* 2011;***50***:2601-05.

59. Lee, P. W.; Isarov, S. A.; Wallat, J. D., et al. Polymer Structure and Conformation Alter the Antigenicity of Virus-like Particle–Polymer Conjugates, *J Am Chem Soc* 2017;***139***:3312-15.

Supplementary Materials

Next Generation Polyphosphazene Immunoadjuvant: Synthesis, Self-Assembly and In Vivo Potency with Human Papillomavirus VLPs-Based Vaccine

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Materials

Ethyl 4-hydroxyphenylacetate, EHPA, diglyme, anhydrous, sodium phosphate monobasic dihydrate, potassium hydroxide, sodium hydride (Sigma-Aldrich, St. Louis, MO); hexachlorocyclotriphosphazene (Fushimi Pharmaceutical, Kagawa, Japan); normocin (InvivoGen, San Diego, CA, USA); p-nitrophenylphosphate, pNPP (Millipore Sigma, St. Louis, MO, USA); phosphate buffered saline (PBS, pH 7.4); Dulbecco's modified eagle medium, DMEM; penicillin streptomycin, Pen Strep (Thermo-Fisher Scientific, Grand Island, NY, USA) were used as received. PDCP and PCPP were synthesized as described previously.[1, 2]

Table S1. Physico-Chemical Characterization of PCMP.

Mw* (kg/mol)	456
Đ†	3.0
^{1}H NMR:	6.7 (br, 2H, C6H4); 6.6 (br, 2H, C6H4); 3.26 (br, 2H, −CH2−) ppm
^{31}P NMR:	−19.0 ppm
Dz‡ (nm)	75

Polydispersity index, DLS	0.45
Z-potential	-11.2

*Weight average molecular weight; †Đ = M_w/M_n – molecular weight dispersity (GPC, PBS, pH 7.4, polyethylene oxide standards), ‡Z-average hydrodynamic diameter (DLS, PBS, pH 7.4).

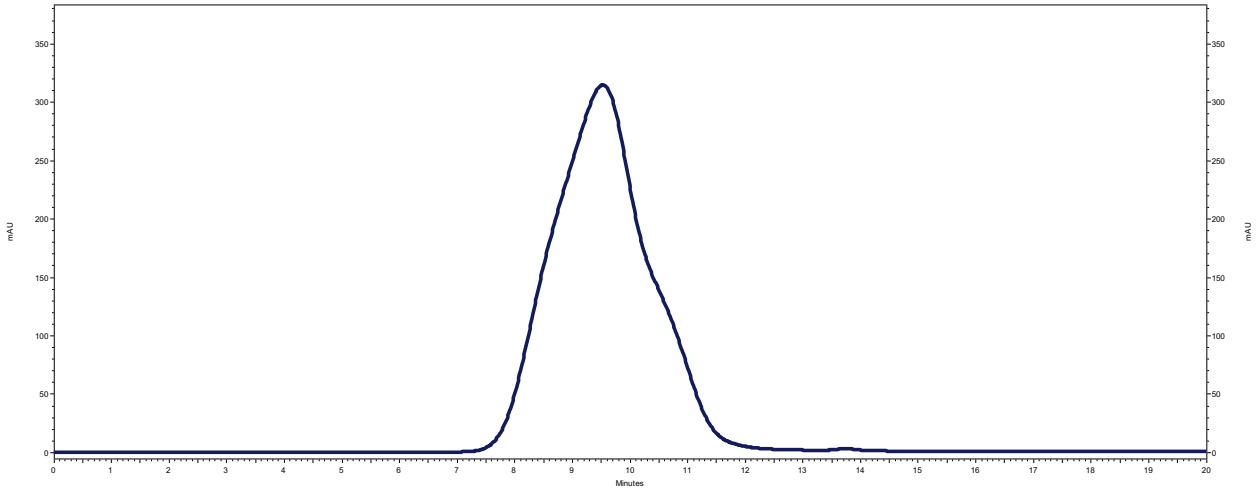

Figure S1. GPC profile of PCMP in PBS (pH 7.4).

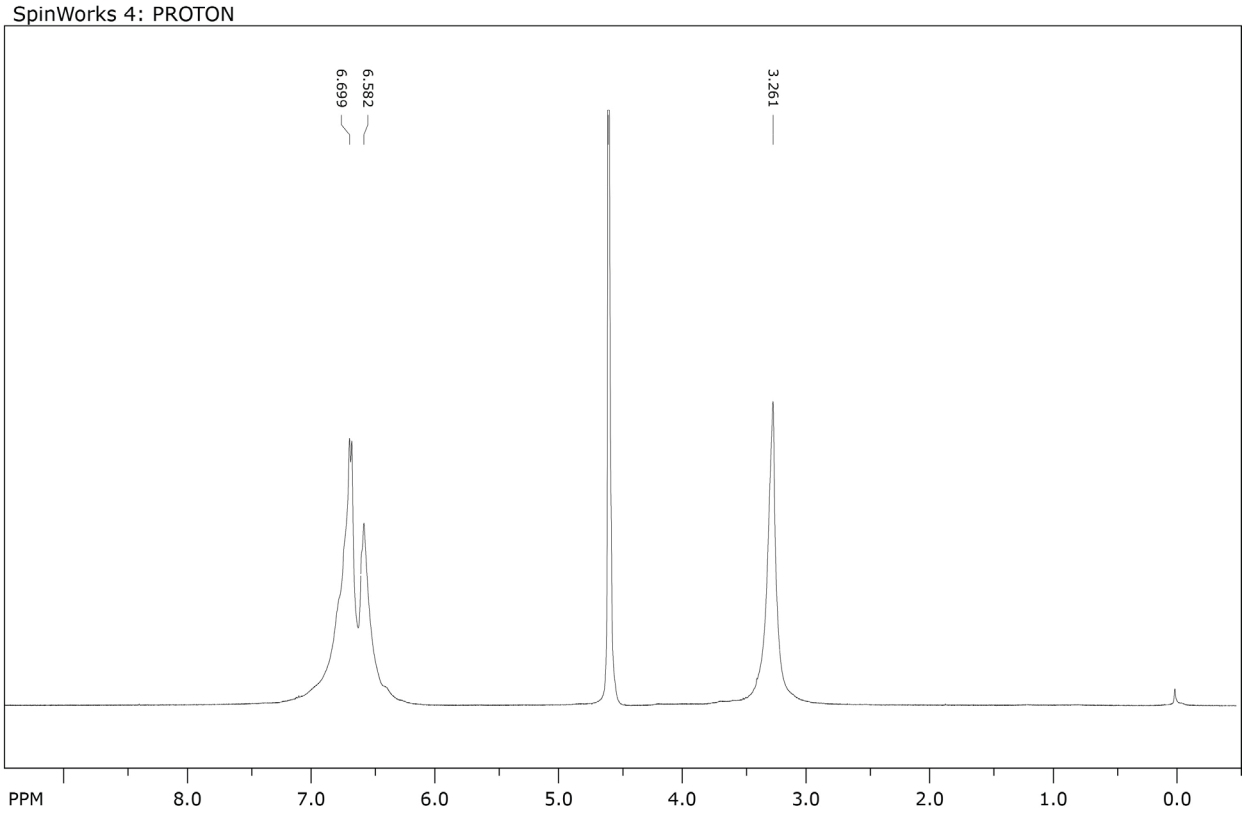

Figure S2. ^{1}H NMR of PCMP (D_2O)

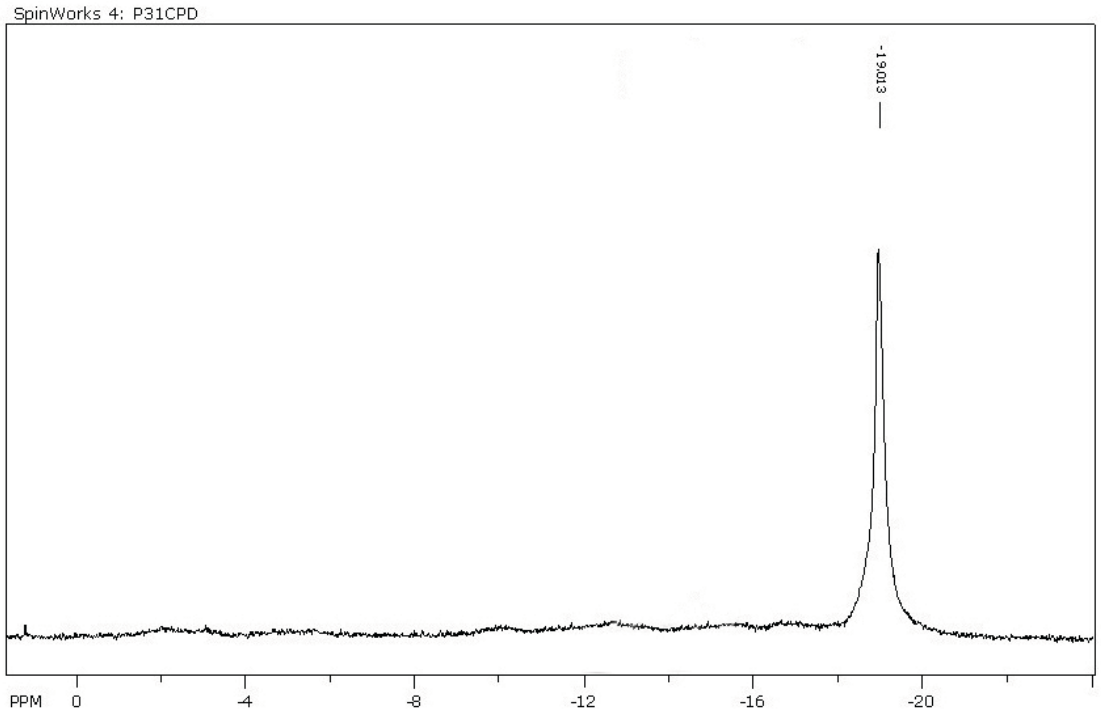

Figure S3. ^{31}P NMR of PCMP (D_2O).

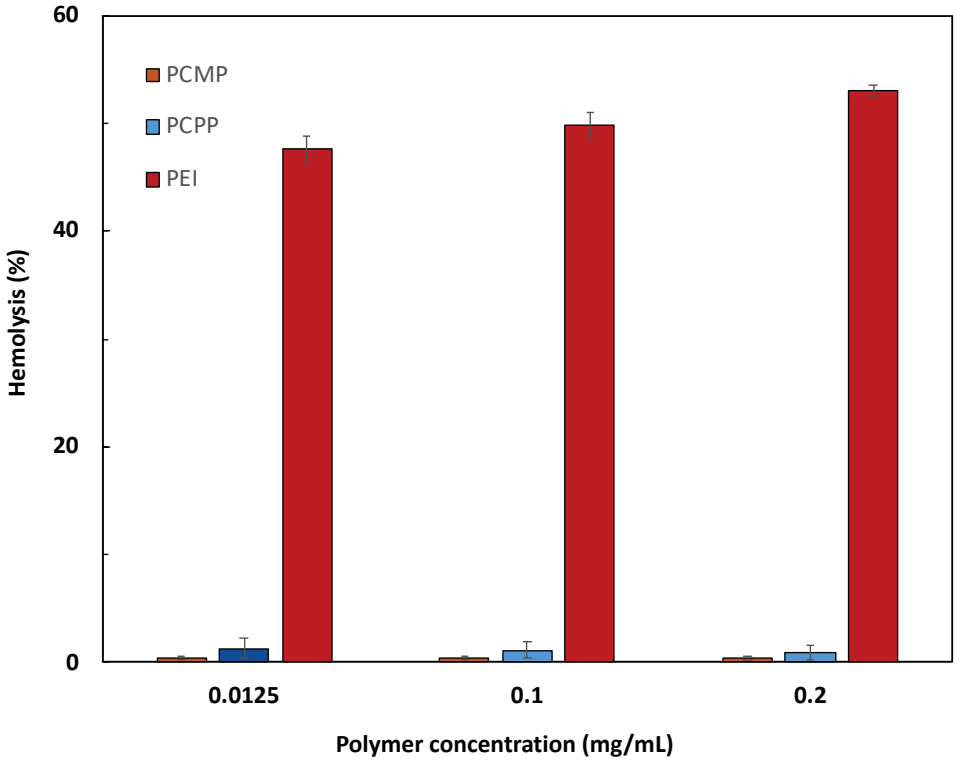

Figure S4. Hemolytic activity of PCMP and PCPP at various concentrations (RBCs, PBS, pH 7.4, 1h, 37°C, cationic polymer - polyethyleneimine was used for comparative purposes)

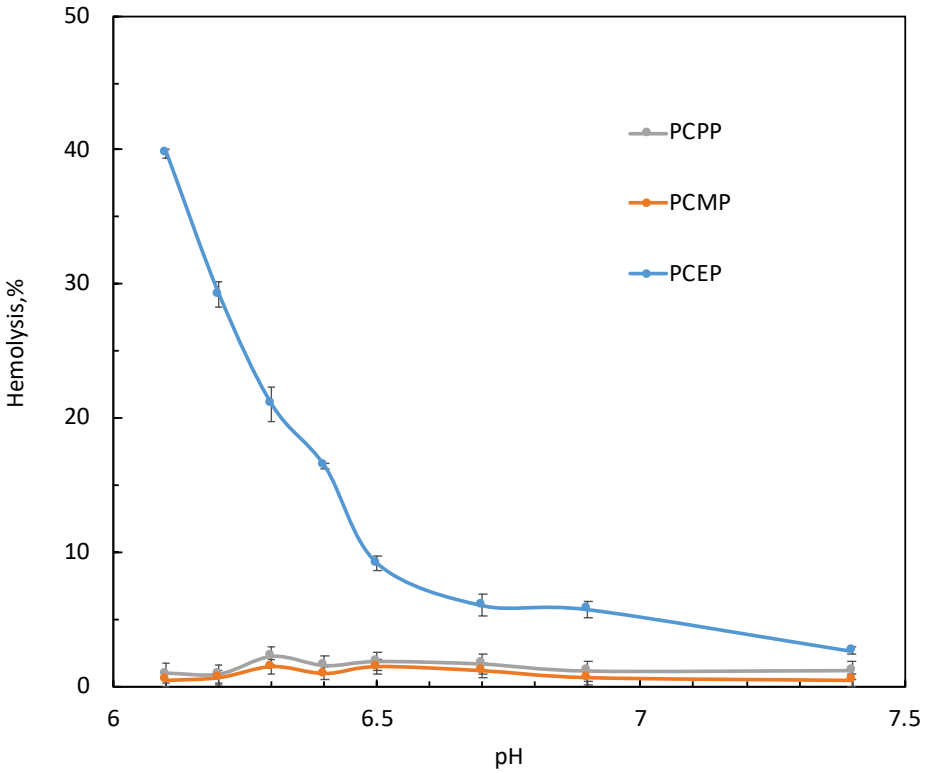

Figure S5. Hemolytic activity of PCPP and PCMP as a function of pH (10 mM phosphate buffer, 0.9 % of sodium chloride). In contrast with PCEP, PCMP and PCPP do not show pH dependent membrane disruptive potential, which is typically correlated with endosomolytic activity. Differences in endosomolytic activity of PCPP and PCEP and how they can be potentially linked to in vivo behavior (Th1/Th2) of the adjuvant are discussed elsewhere.[3]

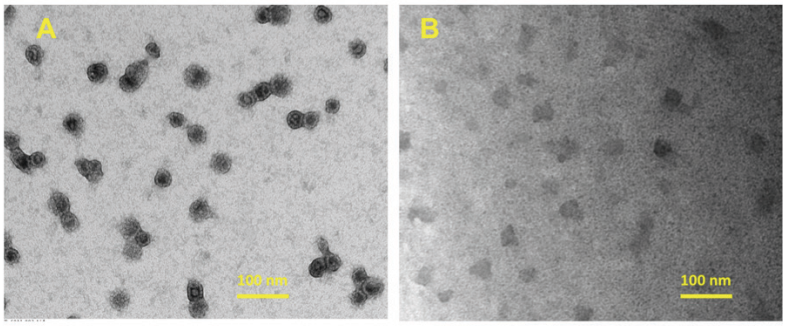

Figure S6. Electron microscopy images of (A) VLPs and (B) PCMP:VLPs formulation at 2:1 (w/w) ratio.

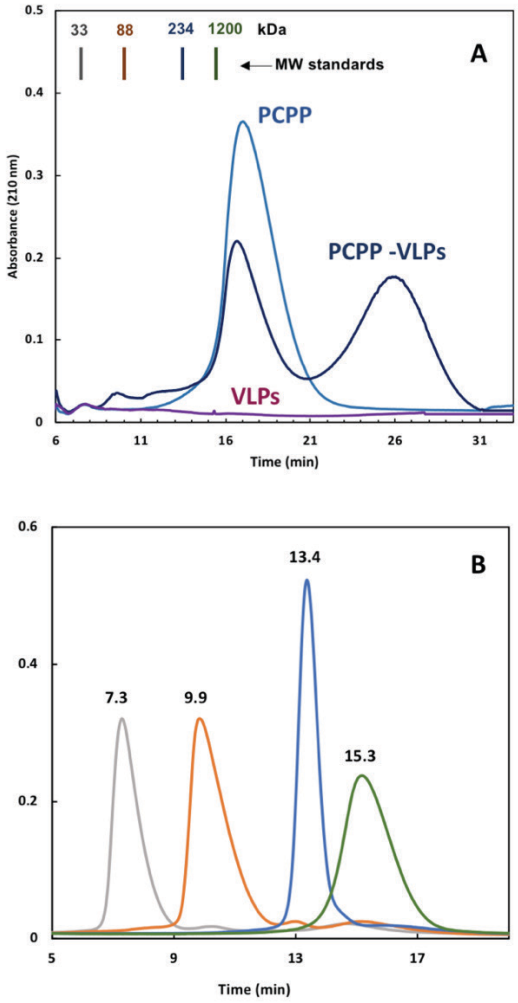

Figure S7. AF4 fractograms of (A) PCPP, VLP, and their formulation and (B) of molecular weight standards (poly(styrene sulfonic acid) standards: 33 kDa (gray); 88 kDa (orange); 234 kDa (blue), 1,200 kDa (green), elution times are shown).

High variability of HPV39 neutralization titers

HPV16 and HPV18 share more sequence homology in the RG1 epitope than HPV16 and HPV39. Therefore, neutralizing antibodies generated against the HPV16-L2 RG1 epitope may have higher affinity for the HPV18-L2 RG1 epitope and thus be more neutralizing. In contrast, neutralization of HPV39 through its RG1 epitope may be more challenging. There may also be multiple B cell epitopes within the 20 amino acid RG1 epitope leading to diversified polyclonal responses that can still differ within syngeneic mice.

Batch-to-batch reproducibility for two synthetic lots of PCMP

(data for first lot/data for second lot)

M_w, GPC (456/400 Kg/mol);

Đ, GPC (3/3);

z-average diameter, DLS (75/73);

polydispersity index, DLS (0.45/0.48).

References

1. Andrianov, A. K.; Chen, J.; LeGolvan, M. P., Poly(dichlorophosphazene) as a precursor for biologically active polyphosphazenes: Synthesis, characterization, and stabilization. *Macromolecules* **2004,** 37, (2), 414-420.

2. Andrianov, A. K.; Svirkin, Y. Y.; LeGolvan, M. P., Synthesis and biologically relevant properties of polyphosphazene polyacids. *Biomacromolecules* **2004,** 5, (5), 1999-2006.

3. Andrianov, A. K.; Marin, A.; Fuerst, T. R., Molecular-Level Interactions of Polyphosphazene Immunoadjuvants and Their Potential Role in Antigen Presentation and Cell Stimulation. *Biomacromolecules* **2016,** 17, (11), 3732-3742.