Polyphosphazenes for the delivery of biopharmaceuticals

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Abbreviation:

Polyphosphazene (PPZ), Poly(dichlorophosphazene) (PDCP)

Poly[di(carboxylatophenoxy)-phosphazene] (PCPP), Poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP)

Polylactide (PLA), Poly(lactide-co-glycolide) (PLGA), Polycaprolactone (PCL), Polyethylenimine (PEI)

Diisopropyl ethylenediamine (DPA), 2-dimethylaminoethanol (DMAE), 2-dimethylaminoethylamine (DMAEA), N,N-diisopropylethylenediamine (DPA), Carboxylatoethylphenoxy (CEP)

Lower critical solution temperature behavior (LCST)

Human growth hormone (hGH)

Aluminum-based mineral salts (Alum), Hepatitis B surface antigen (HBsAg)

Type 1 T helper (Th1), Type 2 T helper (Th2), Dendritic cells (DCs)

Abstract

Polyphosphazenes (PPZs) are a relatively new family of polymers based on a nitrogen-phosphorous backbone where organic side-groups can be grafted. The synthetic route to PPZs is highly versatile such that it is possible to add many different functionalities that change completely the physicochemical and biological properties of the polymers. For instance, PPZs can be designed with a variety of organic side groups that render these materials biodegradable and highly biocompatible. Based on these positive features, PPZs have been explored for many biomedical applications including the design of numerous advanced drug delivery systems. In this area, PPZs have been particularly investigated as materials for the formulation of biopharmaceuticals of high added value. These include protein- and polynucleotide-based medicines, applications where PPZ carriers have obtained very positive results in pre-clinical models. A further area of major interest for PPZs has been vaccination, where these materials have obtained excellent results in vivo as polymer adjuvants and have advanced to clinical evaluation.

Part I. Background and fundamental properties of PPZ

New biodegradable materials are required in many biomedical applications, and polymers continue to be critical for this field. In comparison to natural polymers, synthetic biodegradable polymers are generally better defined and are easier to modulate regarding their mechanical and degradation properties. Polyesters such as polylactide (PLA), poly(lactide-co-glycolide) (PLGA), or polycaprolactone (PCL) are some of the most used synthetic polymers, but other polymer families such as polyphosphazenes (PPZs) are also of interest since they bring advantages of tunable biodegradability, polymer elasticity and chemical versatility. Due to these characteristics, polyphosphazenes are being investigated in a range of biomedical applications such as controlled drug delivery and tissue engineering[1,2].

Polyphosphazenes (PPZs) are a class of polymers having an inorganic backbone made of repeating units of phosphorus and nitrogen in an alternating sequence, and which can be configured in cyclic or linear conformations. The repeating unit of PPZs is $N{=}PR_1R_2$ (**Figure 1a**), a structure built by phosphorus covalently linked to nitrogen via alternating σ-σ bond and σ-π bonds. Each phosphorus atom offers five electrons and each nitrogen provides another five electrons, forming sequential saturated and unsaturated bonds on the backbones. Within this structure two electrons of the phosphorus are used for side chain conjugation, and two electrons of nitrogen remained as a lone-pair (**Figure 1b**). Although the linear backbone contains unsaturated bonds, the $d\pi$(P)−$p\pi$(N) bond is expected to have flexible rotation because several *3d* orbitals of phosphorus can hybridise with the p_z orbital of nitrogen once the π bond undergoes torsions (**Figure 1c**) [3,4]. As theoretical calculations report, the bond energy of inherent torsional barrier in the phosphazene backbone is as low as 100 cal per bond [4,5]. Nevertheless, the linear PPZ conformation is significantly influenced by their side groups (i.e. Cl, OCH_2CF_3, etc.), since the phosphazene skeleton is easily distorted and lies preferably in a cis-trans rather than in a trans-trans planar conformation to minimize internal repulsions (**Figure 1d**) [3,6]. Because of the flexible $d\pi$(P)−$p\pi$(N) bond, most PPZ are colorless, have insulating properties and lack microcrystallinity. This is more apparent in PPZs having two or more different substituting side groups [4].

The most commonly prepared PPZ is poly(dichlorophosphazene) (PDCP), which is synthesized as a precursor for its derivation to a wide range of polymers by nucleophilic substitution [7].

Many PDCP derivatives have been synthesized and formulated to various products in biomedical applications. For example, amino acid ester PPZs have been widely studied and applied to prepare fibers or scaffolds by electrospinning for bone tissue engineering [8,9]; amphiphilic PPZs have been used for the preparation of nanoscale polymeric carriers in controlled drug delivery [10,11]. For vaccine delivery the most common prototypes are based on PPZs with carboxylic acids side chains, such as poly[di(carboxylatophenoxy)-phosphazene] (PCPP). The versatility of PPZ chemistry is also important from scale-up and manufacture perspectives [12-14]. For many industrial applications, it is desirable to have 'platform' materials that can be manufactured in bulk and adapted or modified for specific purposes. For pharmaceutical applications, a single precursor polymer that can be tailored for different therapies would be highly advantageous. In this review we focus on linear PDCP derivatives and their bio-applications in gene delivery, vaccine delivery and protein drug delivery.

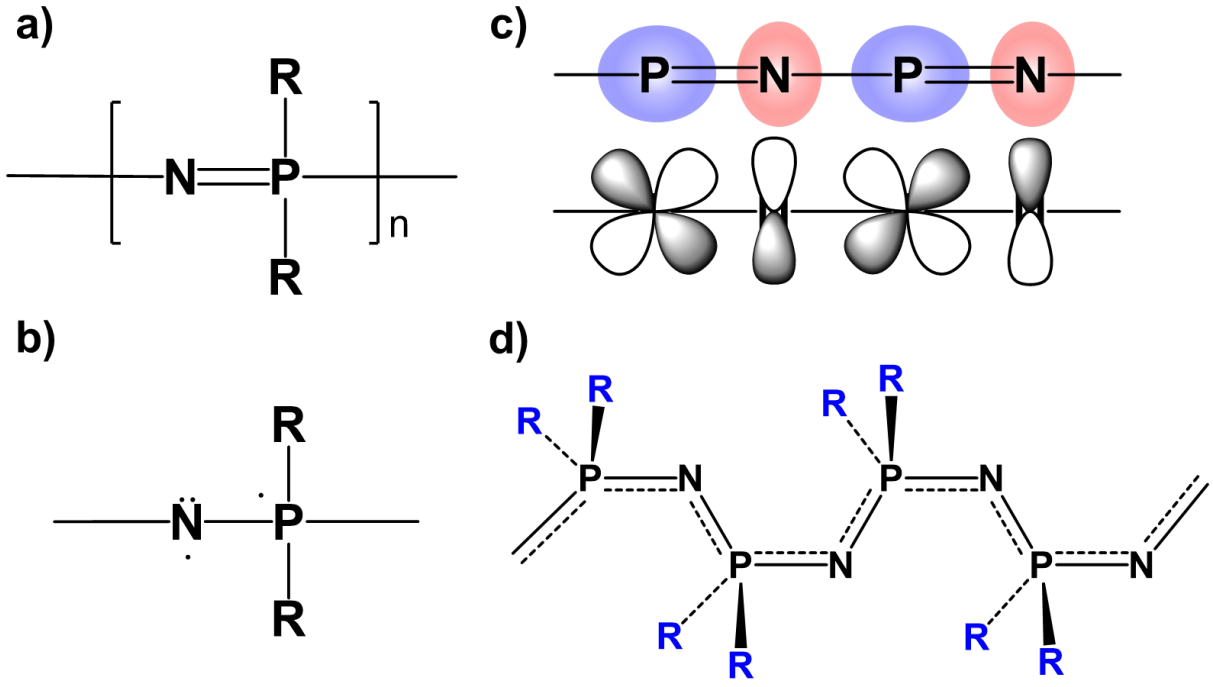

Figure 1. General structures of PPZ (**a**), electron arrangement in the phosphazene bond (**b**) and its orbital hybridization (**c**), in which phosphorus provides d_{xz} to hybrid with p_z of nitrogen. (**d**) Cis-trans planar conformation of repeating phosphazene backbones.

Synthesis of polyphosphazene and functional additions

The first synthesis of PPZ was reported by Stokes as early as 1897 in a high-temperature polymerization reaction that led into an insoluble elastomer known as inorganic rubber. The first stable synthesis of PPZ was reported by Allcock and co-workers [15], a work that resulted in increased interest for these kind of materials for biomedical and other applications. The scheme developed by Allcock is still the basis of the most general procedure for linear PPZ synthesis: the precursor poly(dichlorophosphazene) (PDCP) is prepared in the first step, and then the final polymer is formed by nucleophilic substitution of the desired side chains [1,16,17].

Based on the raw monomers used to prepare linear PDCP, synthetic methods can generally be classified based on those starting from cyclic trimers (i.e. hexachlorocyclotriphosphazene, $(NPCl_2)_3$) [15,18], and those starting from non-cyclic monomers (i.e. dichlorophosphinoyl-iminotrichloro phosphorene, $Cl_3P{=}N\text{-}P(O)Cl_2$, or trichloro(trimethylsilyl)phosphoranimine, $Cl_3P{=}NSiMe_3$) [19-22]. In the former case, the polymerization is typically thermo-initiated and the ring-opening reaction propagates towards linear PDCP (**Figure 2a**). Many aspects of this general PDCP synthesis procedure have been improved, including catalyzed polymerization [23,24] and solvent-mediated stabilization[25]. Although the mechanism of this ring-opening polymerization is still open to debate, the most broadly accepted mechanism is that one phosphorus-chloride bond of the cyclic trimer is cleaved by heating above 250 - 260 ºC, triggering the opening of the next cyclotriphosphazene and starting the chain propagation reaction. In the terminal step, the reactive head of the PPZ chain ($\sim N{=}PCl_2^+$) can recapture the chloride anion as the reaction temperature decreases. This reaction is extremely sensitive to contamination by water and other nucleophiles, which can cause unwanted cross-linking, and precipitation of an insoluble material (i.e. Stokes' "inorganic rubber") as well as limiting the reproducibility of the synthesis. For avoiding water contamination, a solvent-free melt polymerization reaction can be used, but this reaction is difficult to control and forms ultra-high molecular weight PDCP.

The use of efficient catalysts is a critical factor to achieve high-yield, controllable polymerization of PDCP and to obtain a product with sufficient purity to proceed for side change substitution reactions without further purification [23,24]. Boron trichloride (BCl_3) and aluminum chloride ($AlCl_3$) are strong Lewis acids that facilitate the extraction of the chloride

on the trimers and act as catalysts for cyclic trimer activation. BCl_3 has the additional advantage of reducing the possibility of crosslinking by eliminating trace amounts of water in the polymerization by forming $B(OH)_3$ [23]. Sohn *et al.* also reported that $AlCl_3$ can be used as a catalyst in PPZ polymerization to yield PDCP with a mass average molecular weight (M_w) range between 10kDa and 100k Da [24], which is preferred for many biomedical applications, especially drug delivery and tissue engineering [26,27].

Besides these methods relying on ring-opening polymerization starting from hexachlorocyclotriphosphazene, PDCP can also be synthesized through phosphorus pentachloride (PCl_5) initiated polymerization starting from non-cyclic monomers, such as trichloro(trimethylsilyl)phosphoranimine, $Cl_3P{=}N\text{-}Si(CH_3)_3$ **(Figure 2b)** [19,20,22]. This method has several advantages such as room-temperature synthesis and controllable molecular weights with narrow polydispersity. Most interestingly, this polymerization method can be used to prepare further block copolymers, such as PLA-co-PDCP, PEO-co-PDCP and polystyrene-co-PDCP [28-30]. The first section of polymer with terminal primary amines can conjugate with $(CF_3CH_2O)_2BrP{=}NSiMe_3$ by nucleophilic substitution and then react with PCl_5 to initiate the polymerization of the PDCP block. In light of this, PCl_5-induced living cationic polymerization provides broader possibilities for PPZ platforms, such as the preparation of amphiphilic polymers or multi-arm branch polymers, which could be used in medical applications.

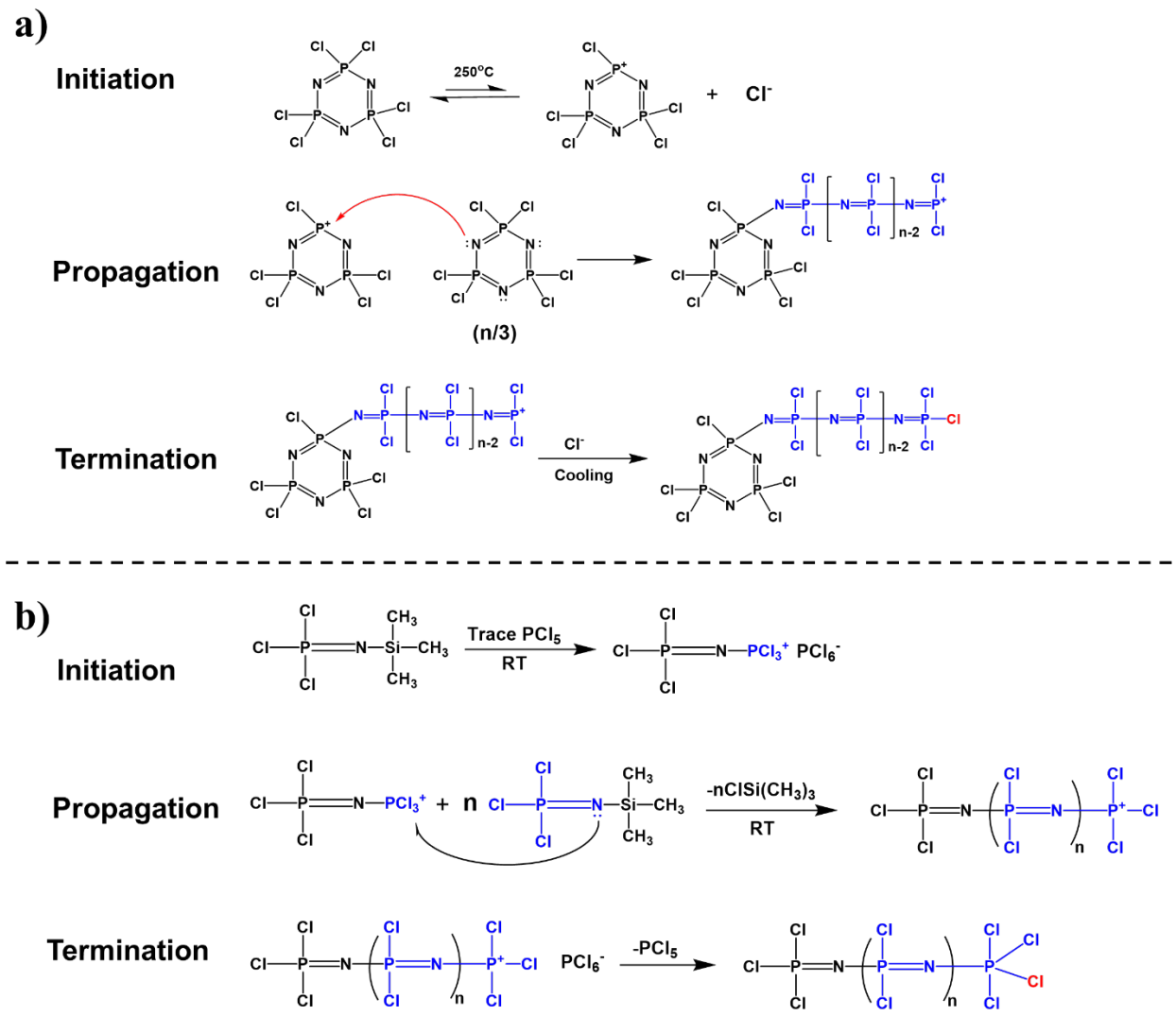

Figure 2. Potential mechanisms for PPZ polymerization. (a) Ring-opening polymerization of cyclotriphosphazene and (b) living cationic polymerization of trichloro(trimethylsilyl)phosphoranimine to yield linear poly(dichlorophosphazene)s (PDCP).

Despite all the work on improving its synthesis, PDCP has few applications itself, but rather acts as a precursor polymer that, to date, has been modified into over 700 different derivatives [4,7]. Indeed, PDCP provides an easy-coupling platform for conjugation with side-chain candidates, in which nucleophile terminals can substitute the chlorines of this polymer, effectively conjugating to the PPZ backbone. Despite the flexibility of this reaction, the nucleophilic substitution is limited to functional groups having only one nucleophilic center. If

groups having more than one nucleophilic center are used, a crosslinking reaction and the precipitation of the polymer intermediate will result. Therefore, many functional groups of biological interest (amines, acids, or saccharide molecules etc.) need to go through protection/deprotection procedures for their nucleophilic substitution on PDCP. Fortunately, an extensive library of PPZs has been developed, including derivatives substituted with alkene, alkyne, or vinyl groups, for example, allylamine [14,31], 2-aminoethyl methacrylate [32], allyl glycinate [33], methacrylic acid [34,35] and propargylamine [36] derivatives. These PPZs with alkene and alkyne side chains can be used as secondary precursors for free radical polymerization [32,33,35,37]. Furthermore, recent works have shown the possibility to use these groups in thiol-ene, thiol-yne and azide/alkyne click reactions as simple pathways for PPZ derivatization with biomolecules [14,36,38,39]. These reactions have also been applied as simple schemes to add ionic side chains on the PPZ backbone for the design of gene therapy polymers [40]. Overall, free-radical polymerization and click reactions bring additional versatility to PPZs and allow for the introduction of potential biomolecules or therapeutic compounds as side chains.

Biocompatibility and Biodegradability of polyphosphazenes

Poly(organophosphazenes) is a term for PPZs having organic side groups. These polymers cover a broad range of materials with tunable cytocompatibility and biodegradability [7,41], and thus the following discussion is centered on these PPZs. A considerable number of organic side chains have been introduced in PPZs, including amino acid esters [7,42-44], peptides [33,42,45], saccharides [14,36,46], arylcarboxylates [47], ethylene oxide/PEG [11,27,48], and other biomolecules (purine and pyrimidine bases, vitamins, etc.) [49,50]. For biomedical applications, the safety of these materials is a key consideration. As with other properties of polyphosphazenes, the cytocompatibility of the final materials is dictated by the side groups used. For instance, polyphosphazenes substituted with tertiary amines can exert certain toxicity even at moderate concentrations [51]. On the other hand, other polyphosphazenes used in bone tissue engineering show excellent safety profiles [9]. For instance, poly[(ethyl glycinato) phosphazene] has been compared to PLGA in a cytocompatibility test performed in primary rat osteoblasts, where this PPZ showed no negative effect on cell proliferation [52]. This study also inspired further tests of alanine-based PPZs regarding osteocompatibility. This material showed low toxicity and good

capacity to support cell adhesion, proliferation, and maintenance of cellular phenotype [53,54]. Other types of PEGylated PPZs also show excellent biosafety profiles that combine with their antifouling properties [55-57]. The biocompatibility of the PPZ backbone has also been supported by the FDA-approval of Cobra PzF stents (CeloNova Biosciences Inc.) in 2017, which are coronary stents coated with nanostructured PPZs for thrombo-resistant properties [58].

A critical characteristic of poly(organophosphazenes) is their capacity to biodegrade, which is beneficial in many medical applications. The mechanism for hydrolysis of these PPZs is still controversial and three possible mechanisms have been suggested [41,59,60]. In mechanisms 1 and 2 (see **Figure 3**), the side groups can react with the phosphazene backbone and accelerate polymer hydrolysis. In the other mechanism, the side chains of PPZs may be eliminated from their backbone first, and finally the backbone can be hydrolyzed. In all cases, the result of the hydrolysis is the formation of degradation products from the side groups and a self-neutralizing buffer of ammonium phosphate derived from the backbone [7,43]. The neutrality of these degradation products is another advantage over other biodegradable polymers that form acid residues that can harm delicate biomolecules [61,62].

The degradation rate of PPZs is also dependent on the substituting side groups and their ratios when there is more than one type [63]. Within this regard, important characteristics of the side groups are: hydrophobicity, steric hindrance, and the degree of crosslinking of the side chains. The lack of systematic studies has made it difficult to compare a broad range of side chains under standardized conditions [7]. However, some amino-acid ester PPZs have been studied in detail by the group of Allcock, where they calculated their degradation half-life ($T_{1/2}$) in PBS 37 °C as follows: ethyl glycinate ($T_{1/2}$ ~3 months) < alanine ($T_{1/2}$ ~6 months) < valine ($T_{1/2}$ ~ 1 year) ≦ phenylalanine ethyl ester ($T_{1/2}$ ~1 year) [43,44,64]. Moreover, when analyzing two water-soluble PPZs with the same terminal functional side group (i.e. a tertiary amine), but linked to the PPZ backbone by a different nucleophile group, –N=P(–**O**$CH_2CH_2N(CH_3)_2)_2$ vs. –N=P(–**NH**$CH_2CH_2N(CH_3)_2)_2$, the authors found that the alkoxide-substituted PPZ degraded faster ($T_{1/2}$ ~7 days) than the amine-substituted PPZ ($T_{1/2}$ ~24 days) [51].

In conclusion, the good cytocompatibility profile of poly(organophosphazene)s together with their biodegradation kinetics that can be tuned by selecting appropriate side groups is another

positive characteristics that indicates the interest of these materials for tissue engineering and drug delivery [9,41,65,66].

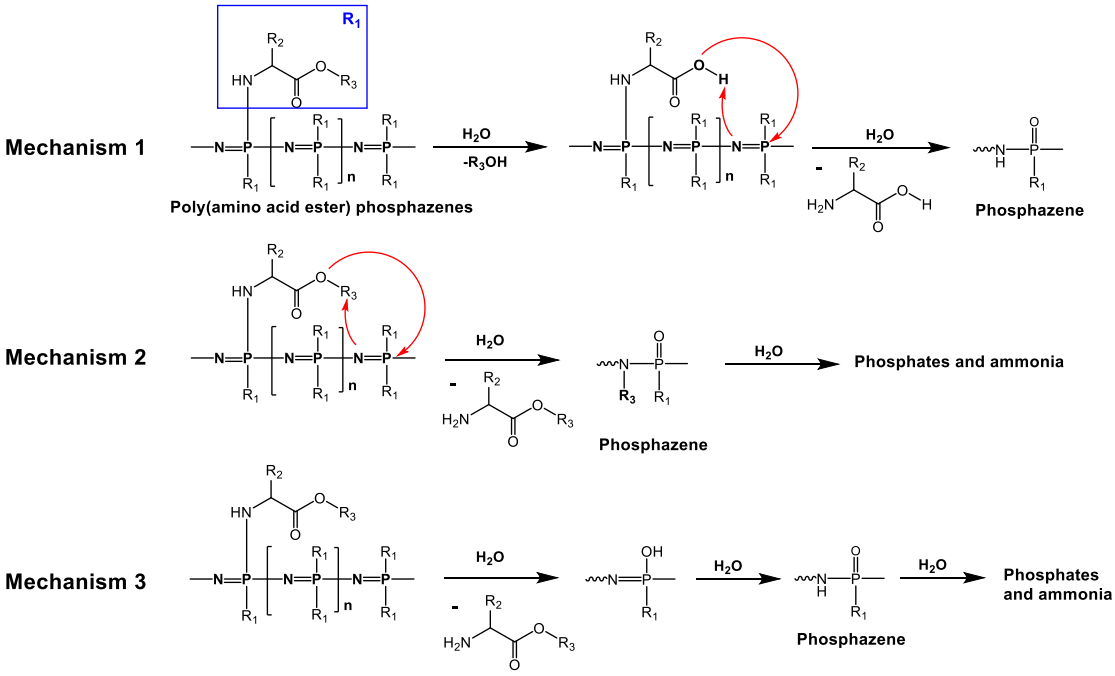

Figure 3. The three common hypotheses of poly(amino-acid ester)phosphazenes for degradation mechanisms. [Adapted from Allcock, H. R. et al. 2012, Polymer Chemistry [7], with permission].

Part II. Use of PPZs for the delivery of biomacromolecules

Macromolecular drugs (polysaccharides, protein, nucleotide, etc.) are increasingly important in the clinical arsenal and are likely to be the main class of therapeutics in many future disease treatments. However, most biomacromolecules have low stability in the body and lack the capacity to cross biological barriers. New carriers to improve the delivery of macromolecules are thus urgently needed. PPZ-based platforms combine chemical flexibility, biodegradability and biocompatibility, suitable characteristics that suggest their use for macromolecule carrier design. Here we review the use of PPZ platforms for gene and protein delivery; afterwards, we cover specifically the use of PPZs for protein and gene delivery in vaccination, since this application has particular technical requirements.

Gene delivery

Gene therapies are some of the most promising advanced treatments under investigation today, and they have already resulted in some new medicines translated to the clinic. The major bottleneck towards the successful use of DNA and RNA is their delivery to the target tissues as well as to their target cell compartments. This delivery problem results in poor efficacy/toxicity ratios and has slowed the development of gene therapy for decades [67-69]. In polymer-mediated gene delivery, the most widely-investigated material is polyethylenimine (PEI), whose positively charged amine groups can condense polynucleotides leading to the formation of nanocomplexes. PEI-nucleotide polyelectrolyte complexes can be taken up by cells and the buffering capacity of the PEI backbone amines can facilitate their escape from endosomal compartments, effectively improving the intracellular delivery of the gene medicines [70]. However, PEI has important limitations regarding its medical use: it is non-biodegradable and has detrimental effects related to mitochondrial dysfunction [71-73], thus better alternative polymers are sought as gene delivery carriers.

The first PPZ employed for the purpose of gene delivery was reported by Hennink's group [51], who compared the degradation ratios of two terminal tertiary amines as PPZ side chains, 2-dimethylaminoethanol (DMAE) and 2-dimethylaminoethylamine (DMAEA). The

corresponding cationic polymers were used to complex plasmid DNA for gene delivery (**Figure 4**). The studies indicated that DMAEA-PPZ complexing DNA has higher gene transfer efficiency than DMAE-PPZ in the COS-7 cell-line, especially at low N/P ratios ≤ 10 (the molar ratio of pronated amine group to negative phosphate of nucleotide). However, DMAEA-PPZ has higher toxicity than DMAE-PPZ at high N/P ratios (≥ 20), likely due to the short half-life of DMEA-PPZ. Besides, these authors also investigated the effect of DMAEA-PPZs molecular weight on DNA delivery efficiency. The lower molecular-weight polymers (M_w range of 130-950k Da) resulted in acceptable toxicity both *in vitro* and *in vivo* [74]. Further improvements in these early prototypes were achieved by introducing imidazole groups in DMAEA-PPZs as added functionalities to improve endosomal escape and enhance transfection efficiency [75,76]. Other chemical modifications of PPZ included grafting with galactose [77] and PEG [78] to improve biocompatibility and stability of the drug carriers.

These initial designs were mostly limited to grafting PPZs with groups having only one nucleophilic center due to synthetic limitations related to uncontrolled polymer crosslinking that could only be avoided by cumbersome protection/de-protection procedures [79-81]. For addressing this issue, Hsu et al. used a click-addition extension starting from a PPZ backbone (**Figure 4**) and applied this synthetic strategy to the design of a small library of materials for gene delivery that included previously unexplored primary amine PPZs and carboxylic acid PPZs of medium/low molecular weight ranges (10 kDa to 50 kDa) [40]. Primary amine PPZs were found to be more efficient for gene delivery than tertiary amine PPZs due to their higher binding capacity for DNA. Additionally, the authors identified a carboxylic acid functional grafting group that could be added to the gene nanocarriers as a delivery enhancer. This material not only reduced the toxicity on the nanocarriers, but improved gene transfection in several 2D and 3D cell models, as well as *in vivo*. In perspective, cationic PPZ-based gene complexes show comparable gene delivery efficacy to commercial standards (PEI or Lipofectamine), but they tend to show lower cytotoxicity and have the additional advantage of being biodegradable [40,57,76,78,82].

Standard polyelectrolyte complexes are not the only type of structures that have been tested for gene delivery. For instance, thermosensitive, injectable PPZ gels have been used for the controlled release of chitosan-graft PEI/DNA complexes or PEI-grafted PPZ/DNA complexes

[83]. This type of system can be used for local administration to achieve sustained release of the polymer/gene complexes [84,85]. Other types of structures of interest are polymersomes and polymeric micelles. Qiu's group developed PEG-PPZ block copolymers where PPZs were substituted with hydrophobic N,N-diisopropylethylenediamine (DPA) groups. The copolymers were amphiphilic and could be formulated as polymersomes. When these polymersomes were loaded with microRNA (miR-200c), the nanomedicine was capable of inhibiting tumor progression in xenografted mice with a drug-resistant lung cancer model [57]. Polymersomes were also used to deliver plasmids coding for recombinant IL-12, a protein that can activate cytotoxic T lymphocytes and natural killer cells in antitumor immunotherapy, but which can also present severe side effects via standard intravenous administration [86,87]. The polymersome was able to deliver the plasmid to CT-26 tumors in mice upon intravenous administration. This delivery system prolonged the half-life of IL-12 in the tumor region and in serum, while they minimized the therapy adverse effects [88]. After the polymersome treatment, immune effector cells (CD8+ T cells, NK cells and NKT cells) were recruited in the tumor environment, and increased concentrations of IFN-γ shortly after the treatment.

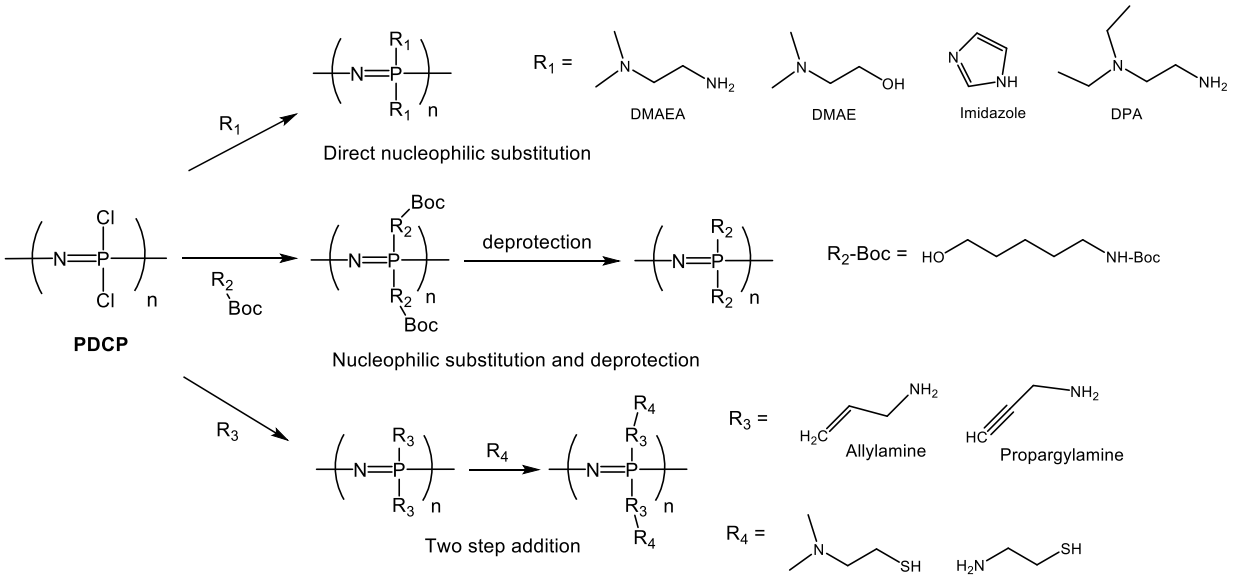

Figure 4. The common techniques to introduce cationic side chains on polyphosphazene (PPZ) backbone for gene delivery application.

Protein drug delivery

The possibility of generating materials with tailored degradation kinetics and their amenability to easily integrate other functionalities make poly(organophosphazene)s an attractive choice for protein delivery. Based on their general structure, we highlight two main strategies that have been followed in PPZ-based protein formulation: (i) nanoparticles and (ii) injectable gels.

Nanoparticles can be designed to provide protein stabilization and improved intracellular trafficking. For instance, the group of Andrianov presented an ionic polyphosphazene for "smart" protein delivery [89]. In this work, poly(carboxylatoethylphenoxy)-co-(3-(2-oxo-1-pyrrolidinyl)-propylamino)phosphazene, PPA was designed with degradable/pH-sensitive functions. The optimized composition of PPA can induce hemolysis at endosomal pH, facilitating endosomal escape after internalization in cancer cells. Carboxylatoethylphenoxy (CEP) side chains are well-known for their use in complexation with proteins, but they are also sensitive to changes in pH within the physiological range. Complexation of L-Asparaginase with both negatively-charged CEP-grafted and positively-charged tertiary amine-grafted PEG-PPZ derivatives can form non-covalent PEGylated nanoassemblies for protein delivery; these nanocarriers promoted the stability of this enzyme and reduced their undesirable antigenicity [55].

Since the short *in vivo* half-life of proteins is one of their most frequent shortcomings, injectable controlled release formulations are in great need. Non-ionic, thermosensitive PPZ polymers that gel at body temperature and provide sustained delivery of proteins are very promising in this regard. For instance, this type of hydrogel can be generated with PPZs grafted with three side chains corresponding to different functions: the hydrophobic L-isoleucine ethyl ester (IleOEt), the hydrophilic α-amino-ω-methoxy-poly(ethylene glycol) (AMPEG), and the hydrolysis-sensitive ethyl-2-(O-glycyl)lactate (GlyLacOEt). In this design, the ratio between IleOEt and AMPEG side groups define the lower critical solution temperature behavior (LCST), and thus the body temperature where the polymer will gel. GlyLacOEt moieties facilitate acid-catalyzed degradation and controlled protein drug release [27,90]. A composition of this polymer family capable of gelling at body temperature upon injection was used to formulate human growth hormone (hGH). This formulation formed a depot with sustained release properties that could address clinical issues associated with hGH treatments, such as renal toxicity and short

half-life, which otherwise require multiple-injections leading to poor patient compliance [91]. Unfortunately, direct loading of hGH in this PPZ hydrogel still results in a large burst and fast release rates, which result in total cargo release in 1 week or less [92,93]. To improve this result, hGH was complexed with poly-L-arginine, and these biodegradable nanocomplexes then were loaded in the thermosensitive PPZ hydrogel. This multi-stage formulation could significantly prolong the release phase for several weeks. In another work, PPZs were designed to have dual interactions with proteins in order to generate more stable release profiles. The PPZs were grafted with hydrophilic PEG, hydrophobic isoleucine ethyl ester (IleOEt) and carboxylic acid moieties that could complex BMP-2 and form stable nanocomplexes. Compared to non-anionic amphiphilic PPZs complexes, anionic BMP-2 nanocarriers were able to duplicate the duration of BMP-2 release and avoid the burst effect. This formulation achieved continuous BMP-2 stimulation *in vivo* and resulted in osteocalcin secretion even two weeks after a single-dose injection of this nanocomplex. The treatment demonstrated new bone generation on mouse ectopic and orthotopic sites after 8 weeks [94].

Vaccine delivery

Vaccination is currently the most cost-effective method for protection against infectious diseases. Generally, vaccines are derived from either live attenuated pathogens, killed antigens or their sub-units [95]. Vaccines based on whole pathogens are usually highly immunogenic but present safety concerns which often limit their use in high-risk populations (eg. pregnant women, elderly etc.). Recently developed vaccine (i.e. subunits, recombinant antigens, nucleotide, surface saccharides, etc.) are safer but typically generate weak immune responses. In light of this, such antigens generally require the use of adjuvants, which could improve both the delivery of the antigen and its immunostimulatory properties [96]. This concept of immune adjuvants was originally proposed by Gaston Ramon [97], as "substances used in combination with a specific antigen that produce a more robust immune response than the antigen alone." The most common adjuvants are aluminum-based mineral salts ("alum") which have been FDA-approved for several decades [98]. Over the last decades, a few lipid-based adjuvants have been approved by regulatory agencies for seasonal and pandemic flu vaccines. Highly-

functional polymeric adjuvants are also being investigated and this is an area of application where PPZs have shown particular promise.

Already in 1998, a polyanionic PPZ derivative, poly[di(carboxylatophenoxy)-phosphazene] (PCPP), was identified as a promising adjuvant for commercial influenza vaccines since it could significantly improve the immunogenic responses in mice around ten-fold in comparison with the antigen alone [99]. It was found that the molecular weight of PCPP was also an important factor for boosting the immunogenic response. The longer PPZ (Mw 1500 kDa), when used as adjuvant for triple influenza vaccine, had 5 times higher hemagglutination inhibition antibodies than the same formulation prepared with the shorter PPZ (Mw 58 kDa).

The mechanism behind the immunostimulatory properties of PCPP is still unclear, but several explanations have been suggested [100]. First, PCPP does not seem to form a "depot" like alum [99], but rather moves out of the injection site as dispersed hydrophilic complexes carrying the antigen. Second, PCPP forms non-covalent complexes with antigens, and the resulting nanoparticle structure seems to promote protein stability and more efficient antigen presentation to immune cells. This molecular complex formation process is likely affected by the molecular weight of PCPP, since larger polymers result in larger nanocomplexes and those induce higher amount of serum IgG titers in mice [99]. In addition, the carboxylic acid grafted groups on the PPZ backbone have inherent immunostimulatory activity, which could be mediated by TLRs and Mannose Receptors [101,102].

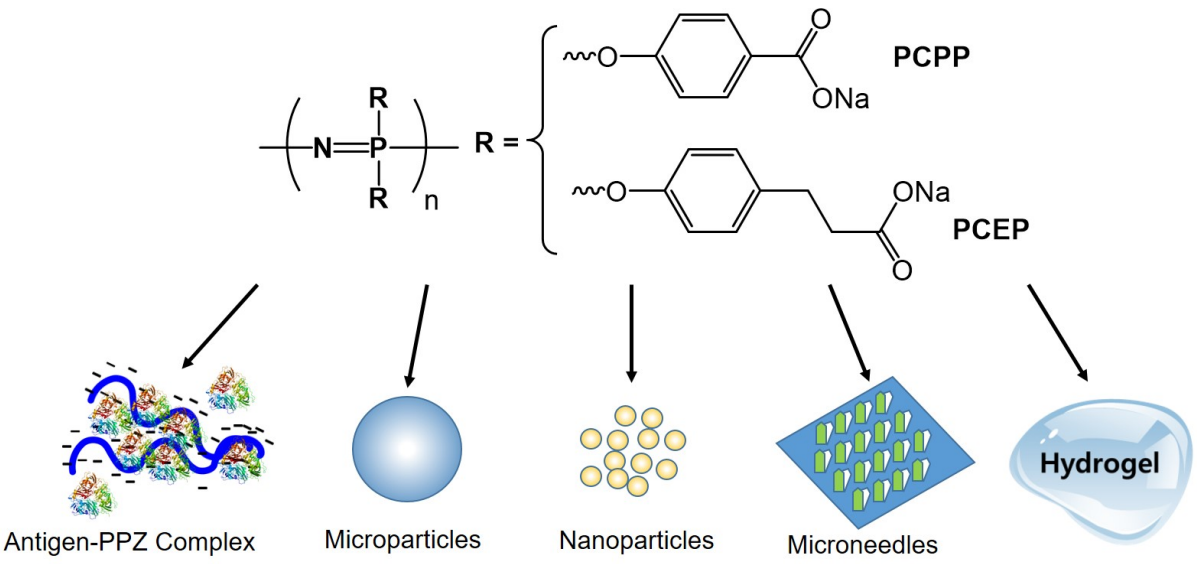

Figure 5. The chemical structure of PCPP and PCEP, two PPZ-based adjuvant materials, and the different types of vaccine formulations derived from them.

The use of PPZs as adjuvants is not only restricted to PCPP. Another PPZ with similar structure, poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP), can also boost immune responses in vaccination [103,104]. PCEP has been tested as adjuvant with different antigens: BSA, X-31 influenza, and hepatitis B surface antigen (HBsAg). When compared to PCPP, it was observed that PCEP trigger higher total IgG titers *in vivo* after several weeks. More specifically, PCEP used as adjuvant with relative low doses of X-31 stimulated high IgG titers and induced higher interferon-γ production than alum or PCPP [104]. Either alum or PCPP mixing with X31 has a relatively high IgG1/IgG2a ratio of 1.3, indicating a predominantly Th2 type immune response in mice. In contrast, PCEP-adjuvant X31 has a, IgG1/IgG2a ratio of 0.9, presenting a balanced Th1/Th2 response with more Th1 isotype IgG2a. The reason behind this effect seems to be related to the different immunomodulatory effects of these adjuvants. While PCEP can contribute to stimulate both Type 1 T helper (Th1) and Type 2 T helper (Th2) responses, alum is mostly associated to Th2 responses. In fact, one of the main limitations of alum is its poor capacity to elicit Th1 responses, which is a main immune defense against intracellular pathogens.

Besides vaccine delivery by direct complexation with antigens, other PZZ systems have also been prepared, including nanocarriers (particles, liposomes) [105], microparticles [106-110], hydrogels [111], and microneedle arrays [112] (**Figure 5**). A particularly promising approach is the use of viscous PCPP-coated microneedle arrays that can penetrate the skin and degrade once in the body, releasing the antigen and the adjuvant. Andrianov and co-workers developed a single dose vaccine of HBsAg based on PCPP-coated microneedles to be used via transdermal delivery. In studies performed in pigs, this delivery system produced around 10 times higher IgG titers than the same formulation administered by intramuscular injection [112]. In summary, this study showed synergistic effects between the physical delivery exerted by the small needles and PPZs and demonstrated the potential of these material for minimally-invasive transdermal vaccination strategies.

The first clinical trial on a PPZ-based adjuvant used PCPP co-administered with influenza antigens of three stains (A/Johannesburg/33/94 (H3N2)) in young adults and elderly [113]. This phase I study confirmed no serious adverse effects for this formulation and achieved better immune responses in young subjects, although elderly subjects still benefited from higher seroconversion rate in the PCPP-adjuvant group than in the standard vaccine group.

In addition to influenza, PCPP could be combined with HIV antigens to form macromolecular complexes. *In vivo*, PCPP-HIV Gag complexes activate more efficiently human dendritic cells (DCs) from adults and newborns and generate further cytokine production than the same antigen formulated with alum [114]. PCPP complexed with HIV vaccine formulations (ALVAC-HIV (vCP1521) primed with oligomeric gp160 (92TH023/LAI-DID) or Bivalent gp120 (CM235/SF2) boost) were evaluated for immune responses and safety in clinical studies. The results of these studies indicated the safety of the vaccines and their capacity to induce cell-mediated immunity [115,116]. However, the protective effect of ALVAC-HIV (vCP1521) formulation was found to decay over time in these studies [117]. Another prime-boost Phase I study reported the combinations of ALVAC-HIV (vCP205) with oligomeric glycoprotein 160 (ogp160) and either PCPP or alum as adjuvants [118]. The ogp160 subjects receiving the PCPP adjuvant had higher endpoint responses, including geometric mean antibody titers and T-Cell lympho-proliferation, than the subjects receiving the alum adjuvant (ClinicalTrials.gov Identifier NCT00004579).

In summary, for the moment the clinical evaluation has indicated that PPZ adjuvants are safe and immunostimulatory in humans, and that they can be translated to a variety of platforms including complexes, microparticles, gels, microneedles, etc. Further clinical studies are required to validate their utility for both influenza and HIV vaccination, particularly in comparison with other better-known adjuvant systems.

Conclusion

Compared to other polymer families, stable PPZs have been synthesized relatively recently, and because of this and their anomalous inorganic backbone, they have not been explored as much as other biomaterials. Still, their many advantages have spurred increasing interest particularly concentrated in some areas like tissue engineering, gene delivery, protein delivery and vaccination. Particularly critical to this interest, is the biodegradability of most poly(organophosphazenes) and the chemical flexibility of these materials. Although other types of polymers can also be chemically diverse, engineers find appealing the special synthesis pathway of PPZs where a precursor is modified to tailor the material's structure and meet the requirements of specific applications. Such synthesis route simplifies adapting existing technologies to new areas of interest.

A pending challenge for the use of PPZs in pharmaceutics and medical devices is the optimization of this synthetic route to make it more cost-effective and eco-friendlier. Indeed, current synthetic pathways either rely on costly monomers and/or the use of high temperatures, organic solvents, and organic reactions proceeding under strict conditions. Cost considerations might be acceptable if the PPZ is integrated in large added-value systems where low amounts of material are needed (e.g. gene delivery systems) but can block further development in other applications.

Regulatory considerations have also been a concern for the development of PPZ-based systems since these materials do not have a long history of medical record comparable to other polymers. In this regard, good biocompatibility data observed in several animal models suggests that many PPZs can be considered for clinical translation. This good biocompatibility profile has been confirmed by the first clinical trials conducted with poly[di(carboxylatophenoxy)-phosphazene]. It is expected that all this new data on safety and bioactivity will encourage new laboratories to consider PPZ as important materials for the future design of advanced drug delivery systems.

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