

# Polyethylenimine-based micro/nanoparticles as vaccine adjuvants

Chen Shen<sup>1</sup>Jun Li<sup>1</sup>Yi Zhang<sup>1</sup>Yuce Li<sup>2</sup>Guanxin Shen<sup>3</sup>Jintao Zhu<sup>2</sup>Juan Tao<sup>1</sup>

<sup>1</sup>Department of Dermatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; <sup>2</sup>School of Chemistry and Chemical Engineering, Huazhong University of Science and Technology, Wuhan, China;

<sup>3</sup>Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

**Abstract:** Vaccines have shown great success in treating and preventing tumors and infections, while adjuvants are always demanded to ensure potent immune responses. Polyethylenimine (PEI), as one of the well-studied cationic polymers, has been used as a transfection reagent for decades. However, increasing evidence has shown that PEI-based particles are also capable of acting as adjuvants. In this paper, we briefly review the physicochemical properties and the broad applications of PEI in different fields, and elaborate on the intracellular processes of PEI-based vaccines. In addition, we sum up the proof of their in vivo and clinical applications. We also highlight some mechanisms proposed for the intrinsic immunoactivation function of PEI, followed by the challenges and future perspectives of the applications of PEI in the vaccines, as well as some strategies to elicit the desirable immune responses.

**Keywords:** cationic polymers, APCs, immunoactivation, danger signals, anti-infection, anticancer

## Introduction

Due to their weak immunogenicity, conventional vaccines, especially the subunit vaccines, are always combined with adjuvants to ensure potent immune responses. Polyethylenimine (PEI), as a kind of cationic polymer, has been extensively applied as a nucleotide delivery reagent for decades. In recent years, the robust adjuvanticity of PEI has been continuously documented. Increasing evidence has shown that PEI-based particles are capable of improving the efficiency of conventional vaccines against infections and tumors. These efficiencies are characterized by direct indicators, such as enhanced maturation rates of antigen-presenting cells (APCs) and increased proliferation of effector cells, as well as amplified production of antigen-specific antibodies and various cytokines and chemokines.

In this review, we first introduce the physicochemical properties of PEI and its general applications in distinct areas. Then, we focus on the effects of neat PEI itself and PEI-based nanoparticles/microparticles (NPs/MPs) in antigen uptake and presentation, which is the foundation for understanding the interplay between PEI and APCs. We then extend the focus on the effects of PEI-relevant adjuvant potency from the cell level to preclinical studies or clinical trials. Subsequently, we are fascinated to figure out some possible underlying mechanisms of its intrinsic immunoactivation functions. Finally, we discuss the challenges and future perspectives of the applications of PEI in vaccines, and explore the promising approaches to optimize its immune responses while manipulating the toxicity properly.

## Synthesis and broad applications of PEI

PEI is a kind of synthesized cationic polymer with topologies of linear or branched forms, and its molecular weight ranges from 1 kDa to 1,000 kDa.<sup>1</sup> The most common

Correspondence: Juan Tao  
Department of Dermatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, No 1277 Jiefang Avenue, Wuhan 430022, China  
Tel +86 027 8572 6195  
Email tjhappy@126.com

and essential characteristic of PEI is its hydrophilic cationic polymeric structure. The strong positive-charged PEI condenses negative particles (such as the DNA, negative antigens) or plasma membranes *in vivo*. Moreover, the PEI backbone contains one nitrogen atom in every three atoms,<sup>2</sup> forming amorphous net structures to work powerfully in lysosomes, as a “proton sponge”.<sup>3,4</sup> These active amino groups, especially the primary and secondary amines, provide numerous possibilities for structural modifications, which enable them to target the agents and attenuate the potential toxicity.<sup>5</sup>

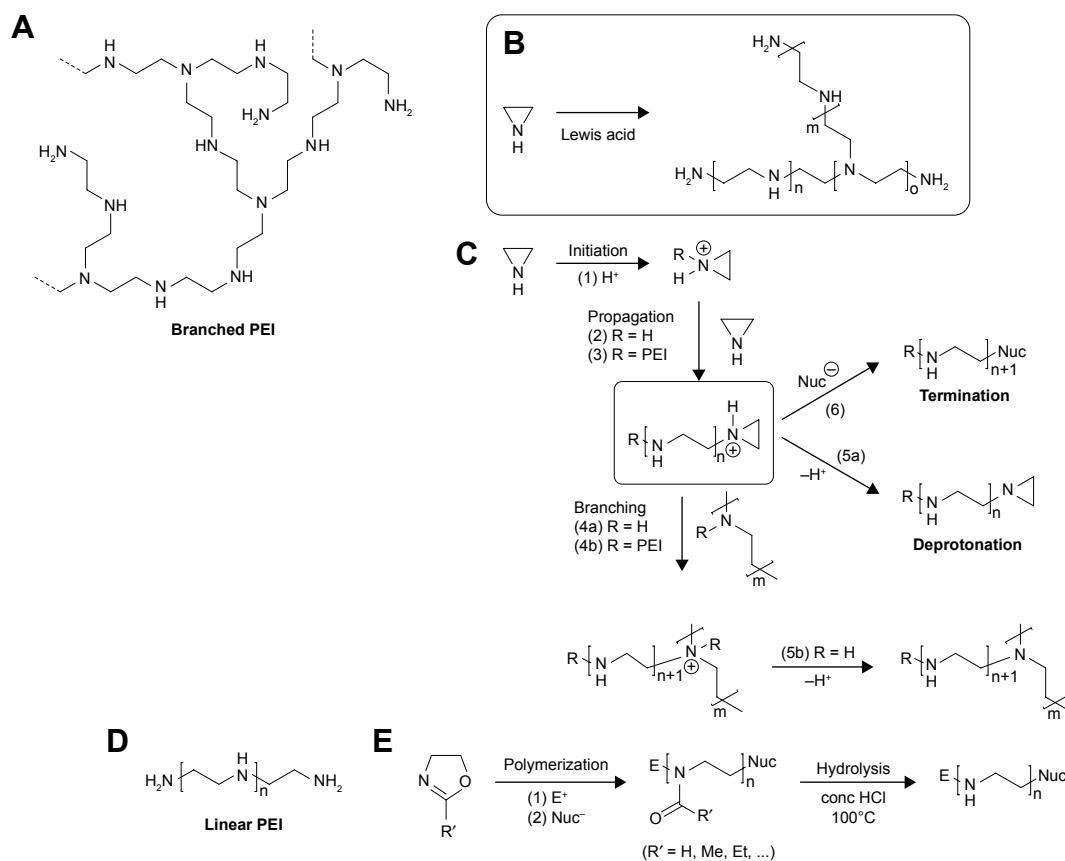
## Synthesis and physicochemical properties of PEI

In general, branched PEI, which contains primary, secondary and tertiary amine groups, can be synthesized through the cationic ring-opening polymerization of aziridine (Figure 1A–C). Linear PEI (LPEI), however, includes secondary amines only, commonly derived from acidic hydrolysis of polyoxazoline (Figure 1D and E). Generally, the synthetic processes of gaining LPEI with narrow distribution of the molecular weight could be rather challenging.<sup>5</sup> In PEI, amino groups with

different sorts possess different properties. It is evidenced that primary and secondary amines have strong abilities to bind to nucleic acids, as well as targeting agents, drugs and other functional moieties. Yet, although with less binding capacity, tertiary amines efficiently buffer the pH decline in acidic conditions.<sup>6,7</sup> In general, branched PEI is in the liquid state and water-soluble, whereas LPEI is solid at room temperature and less soluble in cold water, phenol, ethyl, ether, acetone and other solvents, and it turns more soluble in hot water, acidic aqueous solution and organic solutions (such as the methanol, ethanol or chloroform). Though PEIs of these two topologies are quite different, they both possess active amino groups, and the over-positive-charged nitrogens are always linked to the toxicity of PEI.<sup>5</sup>

## Broad applications of PEI

On the basis of the physicochemical properties, PEI is widely used in broad fields, including effluent treatments, carbon dioxide absorption, separation and purification of proteins, antibacterial operations and other procedures.<sup>8–12</sup> In 1995, Boussif et al prepared a groundbreaking type of PEI/DNA



**Figure 1** (A) Molecular structure of branched PEI. (B) Polymerization of aziridine to branched PEI. (C) Mechanism of the cationic ring-opening polymerization of aziridine. (D) Molecular structure of linear PEI. (E) Synthesis of linear PEI from substituted 2-oxazolines. Reproduced from Jäger M, Schubert S, Ochrimenko S, Fischer D, Schubert US. Branched and linear poly(ethylene imine)-based conjugates: synthetic modification, characterization, and application. *Chem Soc Rev*. 2012;41(13):4755–4767. With permission of The Royal Society of Chemistry.<sup>5</sup>

**Abbreviations:** PEI, polyethylenimine; conc, concentrate.

NPs and successfully transferred DNA into nerve stem cells.<sup>2</sup> Subsequently, PEI has drawn more attention in biomedical field, especially as drug carriers,<sup>13–15</sup> biological labels<sup>16,17</sup> and vaccine adjuvants (Table 1).<sup>18</sup>

Among all the delivered drugs, the most common ones are nucleic acids. PEI is the second acceptable nonviral nucleic acid transfer agent, besides poly-L-lysine (PLL).<sup>2</sup> Nowadays, PEI transfection reagents are commercially available, which include ExGen500®, jetPEI® and PEIpro™.<sup>19</sup> Besides nucleic acids, proteins or peptides are also included in the category.<sup>20,21</sup> PEI also works as a biological label when conjugated with imaging agents (such as the Fe<sub>3</sub>O<sub>4</sub> and fluorescent NPs), and reflects the specific cell or sub-cell information, such as migrations and other behaviors, under certain conditions.<sup>16,22,23</sup> The adjuvant effect of PEI is an emerging area. In a series of recent studies, vaccines composed of PEI as the immunostimulants are quite competent in treating infections or tumors.<sup>24,25</sup>

## Intracellular process of PEI/modified PEI as vaccine adjuvants

To better manipulate its adjuvanticity, strategies to construct the PEI-based vaccines are highly desirable. PEI could be incorporated into vaccine structures through different ways: directly binding with antigens, coating on antigen-loaded NPs/MPs, coating existing particles with antigens absorbed on the surface or co-encapsulated NPs/MPs with antigens and other constructed forms (Figure 2). During these processes, plenty of cytokines and ligands could also be added in the vaccines based on the desirable applications.

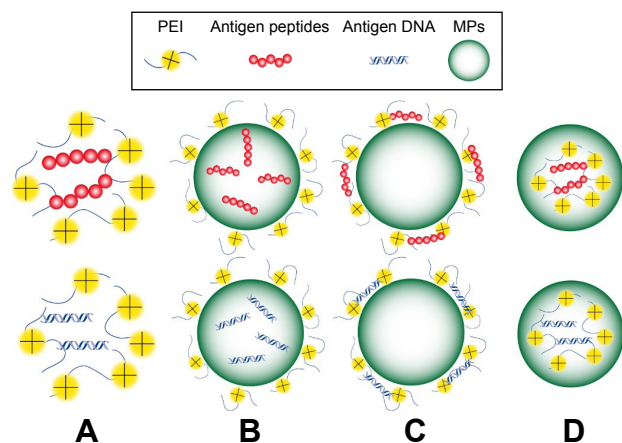
## Cell uptake of PEI-based vaccines

The first step for a vaccine to take its effect is the endocytosis process by APCs. Similar to traditional vaccines, the factors influencing the cell uptake of PEI-based vaccines include size,<sup>33</sup> shape,<sup>34</sup> charge<sup>35</sup> and surface chemistry of the particles.<sup>36,37</sup>

**Table 1** Broad applications of PEI

No	Formulations	Application	Description	References
1	Branched/linear PEI; PEI-modified biomass; thiourea-modified PEI	Effluent treatment	Cationic flocculants in industrial effluents; anionic Cr(VI) sorption and reduction in the biomass; the precious metal recovery material	8, 26, 27
2	PEI-modified mesoporous MCM-48/MCM-41 membranes	Carbon dioxide adsorption	N <sub>2</sub> /CO <sub>2</sub> selectively diminished and high capacity for CO <sub>2</sub> capture	9, 28
3	PEI-PAA bilayer-modified porous PPE membranes; silica-supported PEI	Proteins separation	PEI-PAA bilayer improving IgG-binding ability under optimized conditions; efficient separation of peptides with different isoelectric points by adjusting pH value	29, 30
4	Quaternary ammonium PEI NPs; poly(hyaluronic acid)-PEI particles	Antibacterial effects	Antibacterial activities of dental composites; antimicrobial property against G <sup>-</sup> /G <sup>+</sup> bacteria strains	10, 12, 31
5	Dishes precoated with PEI	Adhesive agents	Demonstrating strong cell adhesion which survived washing procedures	11
6	Two-photon fluorophore-conjugated PEI; commercially magnetic NPs (Gara or G100)/PEI complexes; PEI/NaYF <sub>4</sub> NPs doped with lanthanide ions	Conjugating imaging agents	Confirming the uptake and cytoplasmic localization of complexes in HeLa cells; showing a pronounced hypointense region in tumor tissues after intra-carotid administration; possessing “upconversion fluorescence” and showing potential in biological labeling	16, 17, 22
7	PEI/DNA; poly-siRNA/PEI complexes; PEI-siRNA NPs; PEI/pDTA-H19 DNA complexes	Conjugating nucleotides	Delivering oligonucleotides into embryonic neurons; RFP gene silencing efficiency of ~80% in B16F10 cells; HER-2 downregulation in breast tumors; complete tumor response of 64.1%, with 90% CI (49.7%–76.8%) in patients with superficial bladder cancer	2, 13, 14, NCT00595088
8	CNGRC/PEG/PEI/DNA vector; PEI–glutathione conjugates; PEI-coated albumin NPs for protein delivery	Conjugating peptides/proteins	When conjugating the targeting peptide CNGR protein transduction tool for GST-fused proteins; delivering BMP and promoting osteogenesis in vivo; specifically deliver β-gal genes to CD13 <sup>+</sup> cells and tissues	15, 20, 32
9	HPV16LI-BMP/mPEI/pHPV16LI NPs	Immune adjuvants	Significantly enhancing humoral immune response and the number of IFN-γ-producing CD8 <sup>+</sup> T cells	18

**Abbreviations:** Cr(VI), Chrominum(VI); PEI, polyethylenimine; PAA, polyacrylic acid; PPE, polyethylene; NPs, nanoparticles; FITC, fluorescein isothiocyanate; DTA, diphtheria toxin A; CNGRC, CD13/aminopeptidase N-binding NGR peptide; GST, glutathione S-transferase; BMP, bone morphogenetic protein; mPEI, maltosylated PEI; IFN-γ, interferon-γ.



**Figure 2** Schematic illustration of PEI-based NPs/MPs. (A) Electrostatic nanosized complexes of cationic PEI and anionic peptides or DNA. (B) PEI-coated MPs encapsulated with peptides or DNA. (C) PEI-coated MPs with peptides or DNA adsorbed on the surface by static electricity. (D) PEI MPs encapsulated with DNA or peptides.

**Abbreviations:** PEI, polyethylenimine; NPs, nanoparticles; MPs, microparticles.

## General factors influencing PEI-based polyplex uptake

With broad range of molecular weights, different topologies and plentiful modification possibilities, PEI-based polyplexes are rather versatile in size, shape and properties.<sup>5</sup> Therefore, PEI-based vaccines demonstrate broad possibilities in applications.

Also, the strong positive charge in PEI is beneficial for cellular internalization. PEI can bind to the negative proteoglycan on cell membranes and mediate the uptake process through the electrostatic interaction.<sup>33,35</sup> Generally, phagocytosis by immune cells of foreign particles is an actin-dependent process.<sup>34</sup> The loosely organized PEI in neat state will disturb the actin remodeling when initiating the internalization. Yet, PEI compresses negative substances to condense small particles, or PEI-coating strategy will result in a smooth surface.<sup>25,38</sup> These spherical particles are symmetrical and easily uptaken from any point of attachment.<sup>34</sup>

Size is one of the useful factors in the toolbox affecting the uptake of PEI-based particles, as well as other polymeric particulate carriers. Champion et al demonstrated that particle size played a role in phagocytosis only if the volume was larger than the cell size.<sup>34,39</sup> It is relatively easy to achieve small-sized PEI-based polymers. For the PEI-coated large MPs, the uptake process is restricted; however, the electrostatic forces attach the huge particles to the cell membranes, and perform as a continuous depot of the antigens.<sup>40,41</sup>

## Effect of surface properties of PEI-based polyplex on cellular uptake

In addition to the general parameters, significant attention has been paid to the chemical modifications of the surface chemistry.<sup>34</sup> Some modifications would largely influence the cellular uptake of the PEI-based polyplexes.

In APCs, phagocytosis is frequently mediated by receptors (via mannose receptor-, complement receptor-, Fcγ receptor- and scavenger receptor-mediated pathways or other pathways).<sup>34</sup> Targeted modifications can obviously improve the cellular uptake efficiency and the subsequent biological effects of the polyplexes.<sup>36,37,42</sup> For MPs or less positively charged particles, the tethering of targeting moieties on the particles' surface is even more important for endocytosis.<sup>43,44</sup> Hu et al prepared mannose-modified PEI-cell-penetrating peptide (CPP)/DNA particles and found an improved DC2.4-consuming efficiency.<sup>45</sup> It is worth noting that Toll-like receptors (TLRs) are not included in the phagocytosis-related receptors, but they initiate the phagosome-mediated APCs maturation and inflammatory secretion,<sup>46</sup> which is discussed in this review. Cho et al reported that the maltosylated PEI-mediated vaccines for cervical cancers were more effective than neat human papillomavirus antigens. Presumably, receptor-mediated endocytosis of PEI-based particles by maltose subsequently enhanced the transfection efficiency.<sup>18</sup> For PEI-coated NPs/MPs, similar patterns of receptor-mediated gene delivery have also been demonstrated. Mesoporous silica NPs coupled with PEI also demonstrated high transfection efficiency due to the more effective targeting into APCs.<sup>47</sup>

Besides the specific receptor-mediated targeting strategy, another nonspecific targeting method is the hydrophobic modification of PEI. Given the lipophilic nature of cell membranes, the lipid-like structure facilitates the interactions between the hydrophobic species and plasma membranes (eg, lysosomal membranes and nuclear membranes).<sup>7</sup> It is demonstrated that 76% and 96% of acetylated branched PEI (BPEI) enhanced the cellular uptake ability by four-fold, corresponding to the hydrophobic interaction theory.<sup>48,49</sup> When the lipid components were incorporated, though possessing a relatively decreased positive charge, the electrostatic interaction could still be maintained and aid in binding.<sup>48,49</sup> Recently, Parhiz et al reported that hexanoated-PEI vector was also more effective than the corresponding non-hydrophobic PEI in enhancing transfection rates.<sup>19</sup> Besides the transfection benefits, Wang et al proved that hydrophobic modification of alkyl chains to PEI vaccines optimized the cross-presentation of antigens and upregulated IL-2 secretion. Yet, correlation

between the properties of the synthesized hydrophobic-modified PEI and the vaccine potency still needs further investigation.<sup>50</sup>

CPPs are another valuable species that can optimize the interactions between cell membranes and the PEI-based polyplex. CPPs, characterized by the polycationic amino acid residues (ie, arginine and lysine), have been known for their specialized ability to penetrate the cellular membranes. Until now, most studies demonstrated that CPPs take effect by electrostatic binding to negatively charged cell membranes and induction of vesicle rupture by osmotic change and/or membrane lysis.<sup>19</sup> Truncated Tat peptide and penetratin are the most popular CPPs, and both are arginine-rich peptides.<sup>19</sup> Recently, Morris and Sharma constructed arginine-modified oligo-(alkylaminosiloxanes)-grafted PEI and found that the modified PEI/pDNA exhibited 98% cell viability and 150% more gene transfection efficiency than neat BPEI in human nasopharyngeal epidermoid carcinoma.<sup>51</sup> Inhibitor studies, by use of various cellular uptake inhibitors such as wortmannin and genistein, indicated the role of arginine moiety in promoting internalization of the polyplex, and the process possibly contained a combination of multiple pathways.<sup>51</sup>

More modification strategies will be developed to magnify the advantages of PEI-based vaccines in the initiation process. Yet, after certain modifications, the particle size, charge and other parameters of the polymers may vary correspondingly. Though each of these areas can be studied separately, the interplay among these parameters must be considered as a whole to identify the best performance in boosting the uptake.<sup>34,52</sup>

## Antigen presentation of PEI-based vaccines

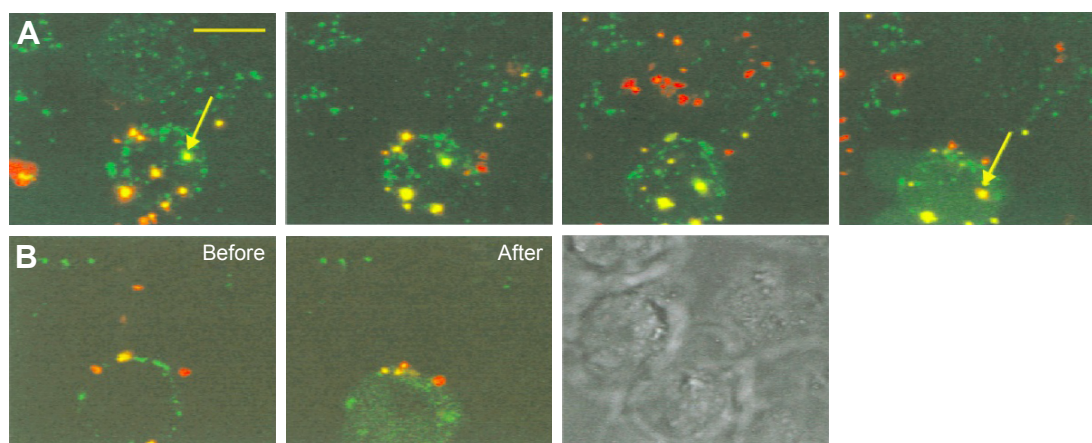
After being uptaken, antigens are processed and transported through different cell compartments and are finally presented within the peptide-binding groove of a major histocompatibility complex (MHC) molecule (MHC class I molecules or class II molecules). T cell receptors can only recognize antigens presented in this pattern. Mostly, MHC class I molecules present endogenous peptides (such as transformed or infected cell components) and elicit the cell-mediated immunity (CMI), while class II molecules acquire exogenous peptides (extracellular pathogens or vaccine peptides) and induce abundance of antibodies.<sup>53</sup> PEI-based vaccines with antigen peptides mainly follow the exogenous/class II pathway and stimulate the humoral immunity.<sup>54</sup> PEI-based DNA

vaccines provide the opportunities to synthesize multiple antigens in APCs and stimulate effective cellular immunity and long-lasting memory immunity. However, most DNA is actually delivered to bystander cells (such as myocytes and fibroblasts). In this case, newly synthesized antigen peptides are presented on or secreted out of bystander cells. They are shuttled to APCs, acting as the exogenous antigens.<sup>55,56</sup>

## The “proton sponge effect” of PEI makes cross-presentation possible

As mentioned above, MHC class I molecules present endogenous peptides synthesized in the cells, while class II molecules present the exogenous internalized antigens. Occasionally, MHC class I molecules also participate in presenting exogenous antigens, known as the cross-presentation, which is very meaningful in biological evolution. In human beings, since the virus invasion does not commonly occur in professional APCs and the CMI is not naturally generated,<sup>57</sup> virus antigens released from the bystander cells turn into exogenous antigens for APCs. The cross-presentation mechanism in APCs largely increases the efficiency of the immune system to generate CMI and eliminate all the infected cells. Yet, in application of vaccines for treating cancers and infections, which needs the CMI instead of mere humoral immunity, the cross-presentation is also of great importance.<sup>57</sup> In the processes, phagocytosed or endocytosed antigens escape from the vacuole and gain entry to the cytosol (known as the “lysosomal escape”). Then, they become qualified clients for ubiquitination and subsequent degradation by the proteasome, followed by the transporter associated with antigen processing (TAP)-mediated transfer into the endoplasmic reticulum, and presentation by MHC class I molecules.<sup>57-59</sup>

There are two representative mechanisms of the “lysosomal escape”, that are, the variation of osmotic pressure and membrane lytic activity.<sup>19</sup> Some pH-sensitive biomaterials possess the membrane-destabilizing property; thus, they are good candidates.<sup>60,61</sup> PEI has the unique “proton sponge effect”, which buffers under acidic conditions.<sup>2,62</sup> When involved in acidic conditions, the ATP-mediated pH-dependent proton pumps open, followed by passive influx of chloride ions and water molecules resulting in hyperosmolar state instantaneously, causing the vesicles to burst.<sup>2,62</sup> The “lysosomal escape” process of PEI-based polyplex was well recorded by Merdan et al (Figure 3).<sup>6,7</sup> PEI/ribozyme and PLL/ribozyme migrations in the living cells were identified under confocal laser scanning microscope, and they were found to first gather in acidic vesicles, most



**Figure 3 (A)** Living cell microscopic visualization of the “proton sponge effect” of polyethylenimine (PEI). PEI is green, while ribozyme is red in this observation. Before burst, the vesicle has a yellow core and green PEI corona, while afterwards, the faint green-yellow fluorescence can be seen evenly distributed throughout the entire cell. The remnant of the vesicles is significantly smaller and deeper red. Images were recorded 28, 31, 37, and 40 min after incubation. Yellow arrows indicate a single vesicle undergoing the process of swelling and bursting. The scale bar in the upper-left image is 10  $\mu\text{m}$  and can be applied in the other images. **(B)** The same confocal layer of 1 nm thickness before and after lysosomal burst (37 and 40 min after incubation). Clearly, fluorescence intensity increases throughout the whole cell after burst, whereas there seems to be an area where it is slightly weaker which might be the nucleus. The right image is an optical microscopy image showing the positions and numbers of the cells. Reproduced from *Pharm Res*, Intracellular processing of poly(ethylene imine)/ribozyme complexes can be observed in living cells by using confocal laser scanning microscopy and inhibitor experiments. , 2002;19(2):140–146, Merdan T, Kunath K, Fischer D, Kopecek J, Kissel T, (copyright 2002 Springer). With permission of Springer.<sup>6</sup>

probably lysosomes. Unlike the PLL-based complex, the vesicles containing the PEI-based complex met with a sudden burst while letting out the contents throughout the cytoplasm.<sup>6,7</sup> To verify the function of the pH change in the “lysosomal escape” process of PEI, they prepared the PEI/ribozyme group with bafilomycin A, a selective inhibitor of endosomal/lysosomal acidification. It showed no lysosomal rupture, suggesting the major role of acidification in the procedure.<sup>6,7</sup> The escaped antigens (peptides or DNA) in the PEI-based vaccines in the cytoplasm are then prepared to undergo a cross-presentation process. The process has recently been verified again by Song et al. They found that PEI-coated poly(lactide-co-glycolide) (PLGA) (OVA) NPs induced efficient cross-presentation of antigens on MHC class I molecules through the endosome escape and lysosomal processing.<sup>63</sup>

## Effect of surface properties of PEI-based polyplex on antigen presentation

There is a group of membrane-destabilizing peptides, which have shown the possibility of not only promoting cell uptake but also improving “lysosomal escape” and even nuclear translocation.<sup>64,65</sup> Although PEI has the proton sponge effect to carry out the “lysosomal escape” itself, sometimes it is incompetent, and addition of membrane-destabilizing peptides (such as CPPs) in the vaccines can improve the cross-presentation effect. Ogris et al have explained the conditions in their study.<sup>52</sup> The transfection efficiency was 10-fold (in B16F10 cells) to more than 100-fold (in Neuro2A cells,

K562 cells) lower in small PEI/DNA particles compared with the large ones. Introduction of the lysosomotropic drug chloroquine or the pH-specific, membrane-active peptide (INF5) really promoted a substantial increase in antigen genes expression, which confirmed the hypothesis and importance of adding CPP moieties.<sup>52</sup> Tan et al bounded two truncated peptides with penetrating properties to BPEI, resulting in higher transfection efficiency without causing cytotoxicity in CHO-K1, B16F10 and 293FT cell lines.<sup>66</sup> These results can be ascribed to the enhanced endosomal disrupting activity of CPP-bound PEI carriers than to their parent carriers.

In PEI/DNA vaccines, PEI helps with the nucleic acids protection and endosomal escape. Yet, the transfection efficiency may still be limited in these vaccines.<sup>62</sup> Particles of over 30 nm diameter or over 40 kDa molecular weight will require the active aid of the coupled nuclear localization signal (NLS) in nuclear translocation.<sup>60</sup> NLS peptides are usually short conservative sequences, which bind to cytoplasmic importins and dock to the nuclear pore complex, thus aiding the nucleic acids transcription. The most commonly used NLS contains the PKKKRKV sequence.<sup>67</sup> In addition, the well-known arginine-rich peptides, such as Tat and penetratin, also possess such nuclear transport activities.<sup>67</sup> Besides, many other viral-origin peptides and even histone H1, protamine, ribonucleoprotein A1, high-motility-group proteins and others containing the above polycationic amino acids also act as effective NLS.<sup>60</sup> Parhiz et al investigated the efficiency of PEI/DNA vaccines by attaching different arginine-rich sequences. It was found that the arginine-rich derivatives of PEI induced higher DNA and siRNA transfection efficiency

than the groups without modifications. Moreover, PEI/DNA vaccines conjugated with arginine-rich peptides and hydrophobic derivatives demonstrated the highest DNA transfection efficiency, indicating that rational designs of two or more modifications are promising, to get a better transfection efficiency (antigen DNA expression) and lower cytotoxicity.<sup>68</sup> All the efforts to elevate DNA transfection promise antigenic DNA translation and presentation. The advantages and modification strategies of PEI-based vaccines related to antigen uptake and presentation are illustrated in Figure 4.

## In vivo and clinical applications of PEI and modifiers as vaccine adjuvants

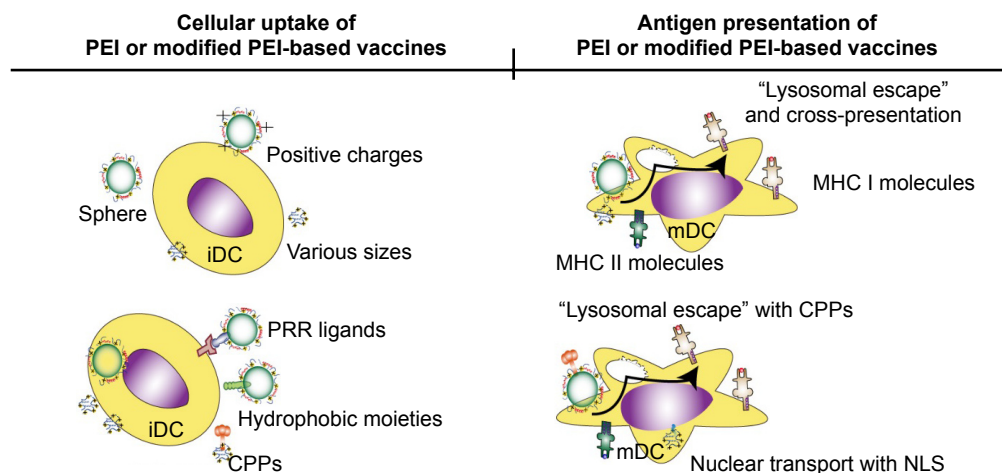
To assess the performance of the PEI-based vaccines, we must consider the following three levels: *in vitro*, *in vivo* and clinical trials. Clearly, the clinical trials of large samples are the most convincing data for evaluating the efficiency of newly developed vaccines. Although we have got much more data from animal trials supporting the superior effects of PEI-based vaccines, only a few trials of PEI-based vaccines have been processed into clinical level. We summarize the application of PEI-based vaccines in animal experiments against infections and cancers, in Tables 2 and 3, respectively.

## In vivo applications of vaccines

The *in vivo* applications of vaccines are confronted with bottlenecks sometimes, for two main reasons. One is the

safety issue, as PEI is widely known for its toxicity. The other is the complicated microenvironment *in vivo*.<sup>61,69</sup> In the microenvironment, every immune-active material has its corresponding targeting immune cells. These cells secrete various chemokines, attracting monocytes, granulocytes and other immune-promoting cells, while the attracted cells secrete more cytokines to form a positive feedback. As frequently depicted for many traditional adjuvants, the monocytes ultimately uptake the complexes, differentiate into dendritic cells (DCs) or phagocytes and then migrate to secondary lymphoid tissues.<sup>70,71</sup> Intriguingly in PEI, the local inflammation seems beneficial to the recruitment and activation of APCs.<sup>72</sup>

Though the general process is somewhat clear, the detailed signal communications of one specific vaccine in *in vivo* applications are still puzzling. For example, it was found that intramuscularly injected MF59 and aluminum adjuvants targeted different cell types, while all the recruited cells had similar potential to engulf carrier–antigen complexes. All events occurred in draining lymph nodes (DLNs) at certain time intervals, probably due to a bystander effect.<sup>70,71</sup> Further research indicated that, different from lipopolysaccharide (LPS), MF59 and aluminum took a TLR-independent mechanism to activate immunization and bias the monocytes differentiation to DCs rather than macrophages.<sup>70,71</sup> Yet, as for the PEI-based vaccines, several issues, such as the local activation of cells and the roles of PEI-based vaccines in it, the secretion of cytokines and the uptake and differentiation processes, along with the lymph drainage situations, need further investigation.



**Figure 4** Illustration showing the uptake and presentation processes of PEI-based vaccines and the modified forms. Positive charge, round shape and controllable size of PEI-based MPs and NPs are all benefits contributing to the uptake process. Besides, they could be modified with PRR ligands, hydrophobic moieties or CPPs, which facilitate the polyplex internalization by receptor-mediated routes, membrane fusions, penetration process and other unknown routes. After polyplex degradation in the endosomes and lysosomes, the released antigens are presented onto MHC molecules directly by transmission or indirectly by gene expression process. PEI itself has the “lysosomal escape” feature, while vaccines with CPPs-modified PEI possess more potent “lysosomal escape” property. They assist with the cross-presentation of delivered antigens. NLS is the specialized sequence added with nucleic acids to assist nuclear translocation of the polymers.

**Abbreviations:** PEI, polyethylenimine; MPs, microparticles; NPs, nanoparticles; PRR, pattern recognition receptor; CPPs, cell-penetrating peptides; MHC, major histocompatibility complex; NLS, nuclear localization signal; iDC, immature dendritic cells; mDC, mature dendritic cells.

Table 2 The infection applications of PEI-based vaccines in vivo

No	Particle formulations	Animal models	Routes	Antigens	Main points	Infections	References
1	25-kDa PEI/pci-S DNA NPs	Balb/c	i.n.	SARS-CoV spikes plasmid(pci-S)	Higher S-specific IgG1 in serum and mucosal IgA in lung wash than pci-S alone; higher number B220 <sup>+</sup> cells in spleen; higher CD80/CD86/MHC II expression on CD11 <sup>+</sup> DCs in cervical LNs; higher IFN- $\gamma$ /TNF- $\alpha$ /IL-2-producing T cells in lung	SARS	54
2	25-kDa BPEI/ influenza HA polymers	C57BL/6j	i.n.	Influenza HA peptide	PEI-immunized mice with enhanced protection in weight loss than CTB; an enhanced IgG ratio of native to denatured antigens than CTB; similar titers of HA-specific serum IgG1, IgG2 $\alpha$ and IgA as CTB; HA-specific IgG levels in the serum were significantly higher for PEI than for CTB after the prime vaccination but were equivalent after the booster	Influenza	25
3	PEI/pRSC-gD-IL-21 DNA NPs	Balb/c	Ocular mucosal administration	HSV glycoprotein D and IL-21 plasmid (pRSC-gD-IL-21)	Inducing higher specific sIgA in tears; IFN- $\gamma$ and IL-4 in serum; enhancing the cytotoxicity of NK cells, as well as splenocyte proliferative responses to glycoprotein D; less HSK degree than controlled group of murine ocular mucosa	HSV	81
4	25-kDa PEI/PLGA MPs/DNA	Balb/c	i.m.	DNA encoding three Ags of <i>L. monocytogenes</i>	Better survival rate of mice immunized with a sublethal dose of <i>L. monocytogenes</i> than naked DNA	<i>L. monocytogenes</i>	77
5	22-kDa, 25-kDa or 87-kDa LPEI or JetPEI/DNA NPs	Balb/c and C57BL/6j	i.m.	Recombinant plasmid <i>L. monocytogenes</i> expressing OVA	Increased in vivo DNA expression 20- to 400-fold; enhanced DNA-induced epitope-specific CD8 <sup>+</sup> T cell responses 10- to 25-fold in vivo; increased numbers of cells secreting type-1 cytokines; improved antigen-specific Th1 cell and humoral responses; eliciting memory cellular responses	<i>L. monocytogenes</i>	56
6	JetPEI/gp120 DNA polymers	Balb/c	Pulmonary administration	gp120 plasmid	Inducing 10 times greater CD8 <sup>+</sup> T cell responses; producing type-1 cytokines and higher CD4 <sup>+</sup> T cell responses; producing more IL-2 in lungs and draining LNs compared to i.m.; inducing CD8 <sup>+</sup> T cell responses in gut and vaginal mucosa, protecting mice better in a lethal recombinant virus challenge	HIV	74
7	Mannosylated-PEI/pSHIV NPs (DermaVir)	Naïve rhesus macaques	Topically on skin	SHIV plasmid(pSHIV)	Inducing antigen-specific CD8 <sup>+</sup> and CD4 <sup>+</sup> T immune responses and a similar number of transduced DC in LNs in nonhuman primates compared to ex vivo DC vaccination; in PB, a similar number of antigen-specific CD8 <sup>+</sup> CD3 <sup>+</sup> and CD8-CD3 <sup>+</sup> cells for DermaVir and ex vivo DCs; in PB, no Abs are detected; with DTH on skin	HIV	73
8	PLGA MPs containing PEI/DNA complexes	Balb/c	i.m.	HIV plasmid(Gag, Pol and Env)	Inducing significant enhanced Abs and CTL responses to HIV vaccine DNA prime/MVA boost regime	HIV	75
9	22-kDa LPEI/HIV-gp120 DNA NPs	Balb/c	i.v.	HIV-gp120 plasmid	Inducing a rapid elevation of serum level of IL-12, IFN- $\gamma$ ; a single administration eliciting a number of gp120-specific CD8 <sup>+</sup> T cells 20 times higher than DNA alone; protective responses against both systemic and mucosal challenges	HIV	76



10	LPEI or BPEI/HIV-gp 140 polyplexes	Balb/c and C57BL/6j	s.c. and i.p.	HIV-1 gp140	Enhancing antigen-specific serum IgG production; more antibodies than aluminum in mice and rabbits; recruiting neutrophils followed by monocytes to the administration site; enhancing antigen uptake by APCs; the bias was modulated by NLRP3 inflammasome toward Th2 response but global adjuvanticity unchanged; adding CpG-ODN, adjuvant potency increasing with Th1 biased responses	HIV	24
11	Mannosylated PEI/pVAX1-HIV gag DNA NPs	Balb/c	i.m.	pVAX1-HIV gag plasmid	In vitro higher transfection in DC of complexed DNA vaccines; IgG2 $\alpha$ T cell response percentage, cytokine production significantly higher even with lower DNA dose	HIV	88
12	25-kDa BPEI/WIV polymers	Balb/c and C57BL/6j	i.n.	H9N2 influenza WIV	Higher antigen-specific IgA in local nasal cavity, trachea, lung than pure WIV; increasing amount of IgG (IgG1, IgG2 $\alpha$ ) in serum	H9N2 influenza	78
13	25-kDa PEI-coated PLA NPs/HBsAg	Balb/c	i.p.	HBsAg	Generating a rapid and efficient humoral immune response and cytokine release than aluminum-absorbed or free antigens; strong stimulation to the Th1 response	HBV	82
14	PEI/ $\gamma$ -PGA NPs-coated PyTAM DNA	C57BL	i.p.	PyTAM DNA	Significantly improved survival rate from lethal <i>Plasmodium yoelii</i> challenge than naked PyTAM plasmid; enhanced antigen-specific IgG1 and IgG2 $\beta$ antibody levels, higher proportion of the IFN- $\gamma$ -producing CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells in spleen, higher level of IL-4, IFN- $\gamma$ , IL-12 and TNF- $\alpha$ levels in the sera and in the supernatants from ex vivo splenocytes	<i>P. yoelii</i> / <i>P. malariae</i>	89
15	SPIONs/25-kDa BPEI NPs/MSP1 DNA	Balb/c	i.p. and i.m.	MSP1 plasmid	The complexes induced Abs against <i>P. yoelii</i> , with higher responses induced i.p. than i.m., with IgG2 $\alpha$ subclasses as the predominance, together with cellular immunity; eliciting high levels of IFN- $\gamma$ , moderate levels of IL-4 and IL-17; inducing Th1, Th2 and Th17 cell-mediated immunity	<i>P. yoelii</i> / <i>P. malariae</i>	90

**Abbreviations:** PEI, polyethylenimine; pci-S, SARS DNA vaccine; i.n., intranasal; SARS, severe acute respiratory syndrome; *L. monocytogenes*, *Listeria monocytogenes*; CoV, coronavirus; S, spike protein; MHC II, major histocompatibility complex class II; DCs, dendritic cells; LNs, lymph nodes; IFN- $\gamma$ , interferon- $\gamma$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL, interleukin; BPEI, branched PEI; HA, hemagglutinin; CTB, cholera toxin B subunit; pRSC, mock plasmid; HSV, herpes simplex virus; sigA, serum IgA; NK, natural killer; HSK, herpes stromal keratitis; PLGA, poly(lactide-co-glycolide); MPs, microparticles; i.m., intramuscular; Ags, antigens; LPEI, linear PEI; NPs, nanoparticles; Th, T helper cells; gp, glycoprotein; pSHIV, plasmid of simian human immunodeficiency virus; PB, peripheral blood; Abs, antibodies; DTH, delayed-type hypersensitivity; CTL, cytotoxic T lymphocyte; s.c., subcutaneous; i.p., intraperitoneal; APCs, antigen-presenting cells; NLRP3, NLR family pyrin domain-containing 3; WIV, whole inactivated virus; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus;  $\gamma$ -PGA, poly( $\gamma$ -glutamic acid); *P. yoelii*, *Plasmodium yoelii*; *P. malariae*, *Plasmodium malariae* PyTAM, PyGPI8P-transamidase-related protein; SPIONs, superparamagnetic iron oxide NPs; MSP1, merozoite surface protein 1.

Table 3 The anticancer applications of PEI-based vaccines in vivo

No	Particle formulations	Animal models	Routes	Antigens	Main points	Tumors	References
1	PEI/pVAX1/C-G250 DNA complexes + C-G250 protein	Balb/c	i.m.	VAX1/C-G250 plasmid and C-G250 protein	PEI/DNA + C-G250 group exhibiting the strongest antibody titer, higher CD8 <sup>+</sup> and CD4 <sup>+</sup> T lymphocyte proliferation and cytokine production	Renal cell carcinoma	83
2	Chitosan-1,200-Da PEI/gp100 DNA complexes	C57BL/6j	i.d.	gp100 plasmid	Chitosan-PEI helping DNA enter into the cytoplasm and into the nucleus of DCs; finally improving resistance to the B16BL6 melanoma challenge	B16BL6 melanoma	91
3	2-kDa BPEI-stearic acid micelles-coated Trp2	C57BL/6j	s.c.	Trp2 peptide	Trp2-loaded PEI-stearic acid micelles promoting CD86, MHC II and CCR-7 expression in the DLNs; enhancing Trp2-specific CTL activity; inhibiting tumor growth in a B16-F10 mouse melanoma model compared to free Trp2	Melanoma	92
4	HPV16L1-MBP/maltosylated 25-kDa BPEI(mPEI)/pHPV16L1 DNA NPs	Balb/c	i.m.	HPV16L1 plasmid and HPV16L1 peptide	Protein/DNA co-delivery vaccine producing higher systemic IgG and mucosal IgA; inducing higher IFN- $\gamma$ production by splenic CD8 <sup>+</sup> T cells than protein alone and protein/DNA mixture	Cervical and other HPV-related cancers	18
5	600-Da BPEI-Tat/pE7-NT-gp96 DNA complexes + IP-10	C57BL/6j	s.c.	HPV16 E7 plasmid	Co-immunization strategy producing the highest IgG2 $\alpha$ , the highest IFN- $\gamma$ response in comparison with other groups; significantly suppressing TC-1 tumor growth with elevated levels of IFN- $\gamma$ and IL-2 production in the LNs	Cervical and other HPV-related cancers	93
6	25-kDa BPEI/pAc-neo-OVA DNA complexes	C57BL/6j	s.c.	pAc-neo-OVA DNA	PEI/DNA complexes inducing class I-restricted antigen presentation and enhancing CTL activity in vivo; exhibiting more pronounced prophylactic and therapeutic antitumor effects than DNA vaccine; PEI inducing NF- $\kappa$ B translocation	H-2b restricted murine thymoma cell line (EG7-OVA cells)	94
7	70-kDa BPEI or 25-kDa LPEI/PLGA MPs-coated MCP3-sFv20 DNA	Balb/c	i.m. and i.d.	MCP3-sFv20 DNA encoding the B cell idiotype antigen	Higher CD80/MHC II expression in RAW264.7 in vitro; improved survival rates of BPEI/PLGA MPs compared to LPEI/PLGA MPs, both longer survival rates than saline-injected controls or blank MPs	B cell lymphoma	38

**Abbreviations:** PEI, polyethylenimine; pVAX1, eukaryotic expression vector; C-G250, renal carcinoma-associated antigens; i.m., intramuscular; gp, glycoprotein; i.d., intradermal; DCs, dendritic cells; BPEI, branched PEI; Trp2, tyrosinase-related protein 2; s.c., subcutaneous; MHC II, major histocompatibility complex class II; DLNs, draining lymph nodes; CTL, cytotoxic T lymphocyte; mPEI, maltosylated PEI; HPV, human papillomavirus; NPs, nanoparticles; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; LNs, lymph nodes; LPEI, linear PEI; PLGA, poly(lactide-co-glycolide); MPs, microparticles; MCP3, monocyte chemoattractant protein 3; sFv20, heavy and light Ig chains.

## Progress of in vivo applications of PEI-based vaccines

Different types of PEI, antigens, constructed strategies and immune routes are chosen in constructing the PEI-based vaccines in vivo. Frequently researched diseases include AIDS,<sup>24,73–76</sup> *Listeria monocytogenes* infection,<sup>56,77</sup> respiratory diseases,<sup>54,78–80</sup> B cell lymphoma,<sup>38</sup> herpes simplex virus attacks,<sup>81</sup> viral B hepatitis,<sup>82</sup> renal cell carcinoma,<sup>83</sup> melanoma<sup>84</sup> and others.<sup>85</sup> The PEI-based vaccines work through almost all the known immune routes, and usually mimic the pathogenesis of the diseases themselves. For example, in respiratory infection, intranasal administration was prone to elicit mucosal secretory antibodies, as well as more serum antibodies.<sup>54</sup> A similar trend was found for the HIV vaccines which elicited more antibodies in the vagina.<sup>74</sup>

The detected markers prove the efficacy of these vaccines usually through two main aspects: (1) intermediate indicators, such as antigen DNA expression, APCs maturation rates, humoral and cellular immunity responses in serum, lymphoid organs or tissues, the T helper type 1 (Th1)/T helper type 2 (Th2) response bias, the memory T and B cells production and secretion levels of the related cytokines;<sup>56,76,86</sup> (2) comprehensive effects, such as higher animal survival rates, less weight loss and less tumor metastasis rates.<sup>38,54,77</sup> The vaccines have demonstrated positive results in a series of in vivo experiments, while only a few clinical trials have been performed.<sup>40</sup> The only satisfactory example was the DermaVir (mannosylated-PEI/plasmid Simian HIV DNA). Lisziewicz et al performed the ex vivo DC-based vaccination of DermaVir and found that DermaVir-transduced autologous monocyte-derived DCs induced HIV-specific T cells in rhesus macaques.<sup>87</sup> Moreover, the same group improved the function of the ex vivo DC vaccination strategy with topical administration. Compared with the previous ex vivo DC-based vaccinations, the vaccination strategy proposed by Lisziewicz et al resulted in a similar number of transduced DCs in the lymph node and induced a similar quantity and quality of HIV-specific Th1-type T cell responses. The DermaVir Patch has been tested in Phase I/II clinical trials at present, and is the new promising agent in clinical applications.<sup>73</sup>

## Intrinsic immune-activating function of PEI

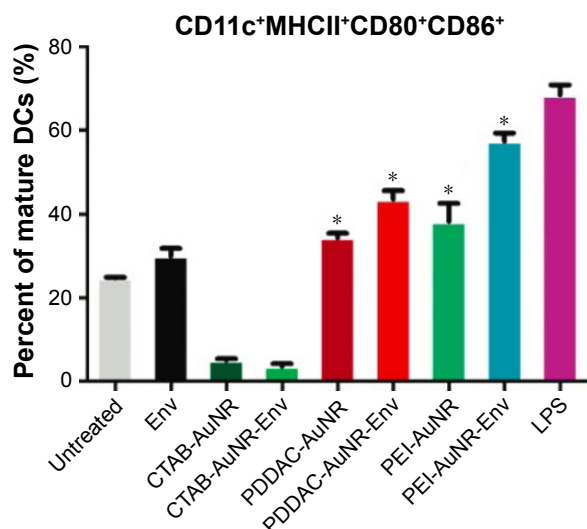
Though many studies showed the performance of PEI-based vaccines in vitro and in vivo, the immune-activating properties of PEI still need to be revisited. It is not clear from these studies whether the improved immune efficiency was solely

due to antigen protection (vaccine delivery vehicle) or due to the intrinsic adjuvant property (immunostimulator) of PEI.<sup>25,56,78,95</sup> Moreover, in the case of PEI-coated polyplex, PEI together with the NPs/MPs should be regarded as a whole to take effects. When encapsulated inside, it was even more difficult to prove the adjuvanticity of PEI. Thus, the observed immunoactivation effects of PEI need further confirmation, and the revelation of the possible molecular pathways of the PEI-based vaccines would help us in understanding and developing new PEI-based vaccines.

Notably, some biomaterials have been regarded as well-known immunostimulants. Such immunologically active materials have been continuously discovered, and the underlying mechanisms were partially explained.<sup>35,96</sup> Among them, the clinically approved adjuvants (aluminum and MF59) are good examples.<sup>97</sup> Besides, 2,000-kDa poly( $\gamma$ -glutamic acid) ( $\gamma$ -PGA) NPs were proved to activate DCs through TLR4 pathway, similar to  $\gamma$ -PGA-Phenol NPs.<sup>98–100</sup> The activation by polyanhydride NPs and fullerene NPs was mediated by multiple TLRs on DCs.<sup>101–104</sup> Inspiring in vitro and in vivo results have also suggested the immunoactivating functions of PEI, especially in DNA vaccines. With the understanding of the above-stated characteristics of PEI and PEI-based polyplex, it is necessary to provide an overview of the adjuvants' properties and their underlying mechanisms.

## Effect of immune activation of PEI

A well-established mode of an adjuvant taking effects is the direct activation of APCs, resulting in costimulatory molecules expression and cytokines secretion in vitro.<sup>63</sup> Compared to bare Au nanorods (AuNRs), PEI-coated AuNRs induced a higher level of phenotypic maturation of DCs (Figure 5) and cytokines release. The immune-promoting effects almost matched LPS to some extent, while the subsequent activation of specific immunization and the interferon (IFN)- $\gamma$  production were strictly Env-dependent.<sup>105</sup> Similarly, when conjugating hepatitis B surface antigen (HBsAg), cationic chitosan and PEI-coated poly(lactic acid) (PLA) MPs also had a stronger capability than PLA MPs or bare antigens to promote macrophages internalization and maturation, followed by stronger humoral immune and Th1 responses in vivo.<sup>82</sup> The anti-B cell lymphoma vaccines made of 70-kDa BPEI and 25-kDa LPEI-functionalized PLGA MPs also resulted in higher CD80 and MHC II expression in RAW264.7 cells in vitro than bare MPs.<sup>38</sup> Moreover, the immunoactivating effects of PEI should also be assessed to understand if better in vivo effect can be achieved without adding PEI in polyplex compared to control groups.



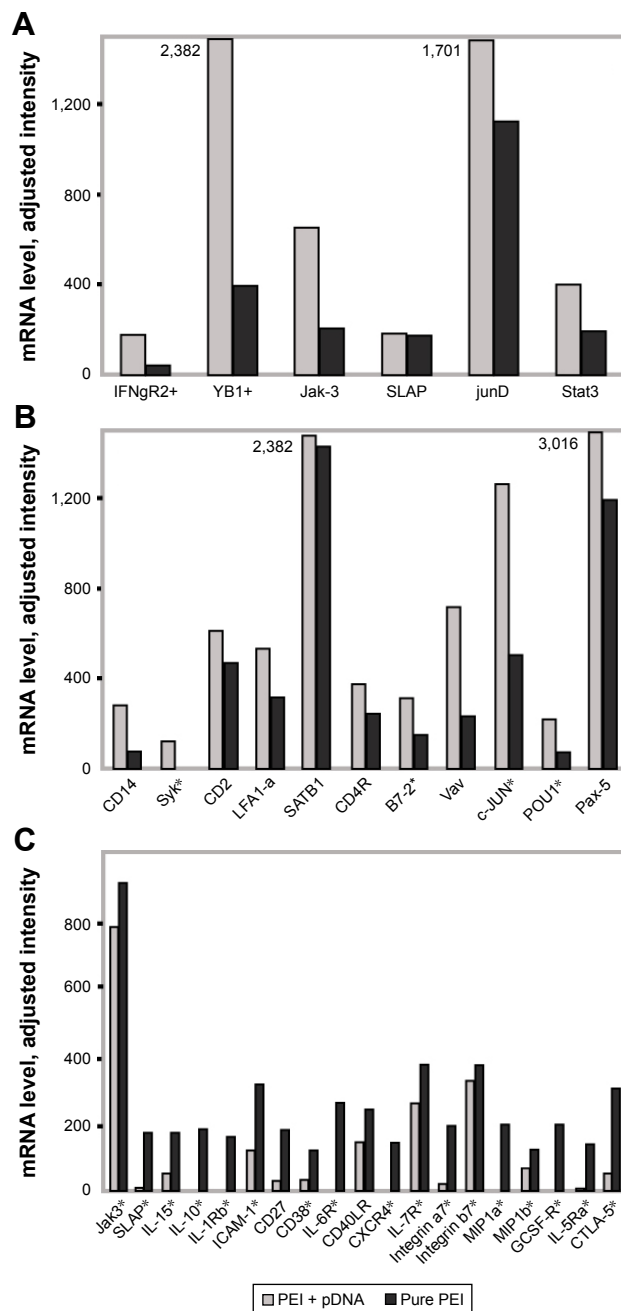
**Figure 5** Significant increase in the percentage of mature DCs (CD11c<sup>+</sup>MHCII<sup>+</sup>CD80<sup>+</sup>CD86<sup>+</sup>DCs) when DCs were treated with PEI-coated AuNRs polyplex. \* $P < 0.05$ . Reprinted with permission from Xu L, Liu Y, Chen Z, et al. Surface-engineered gold nanorods: promising DNA vaccine adjuvant for HIV-1 treatment. *Nano Lett.* 2012;12(4):2003–2012. Copyright 2012 American Chemical Society.<sup>105</sup>  
**Abbreviations:** DCs, dendritic cells; PEI, polyethylenimine; AuNRs, Au nanorods; CTAB, cetyl trimethyl ammonium bromide; PDDAC, poly(diallyldimethylammonium chloride); LPS, lipopolysaccharide.

## Immune-activating mechanisms of PEI

To reveal the immunoactivating property of PEI, gene expression profiles of in vivo immunization of mice with PEI were investigated.<sup>86</sup> Compared with the control group, immuno-related genes of two injected groups were activated at considerably high levels regardless of the antigens (Figure 6). Based on the results and relations analyzed by PubGene, Regnström et al speculated that PEI has important immune-activating effects, presumably through the granulocyte colony-stimulating factor, and the next step will be the exploration of their interplay.<sup>86</sup>

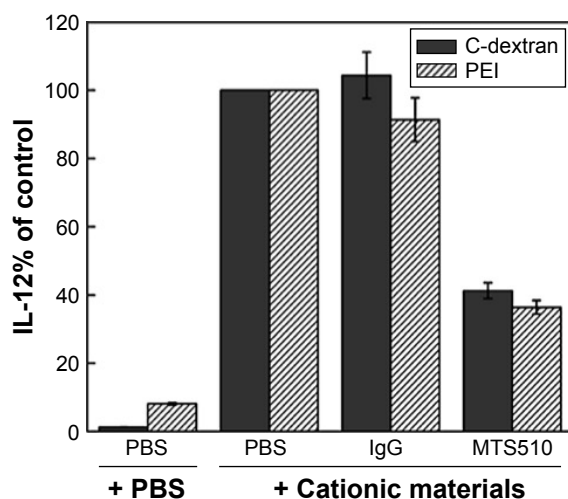
## TLR pathways

With many immune-related genes being upregulated in the treatment of PEI, it is interesting to reveal the precise molecular pathways behind this upregulation. The TLRs and their downstream myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF) pathways are the most classical immuno-activation routes, which stimulate the nuclear transcription factors, nuclear factor- $\kappa$ B (NF- $\kappa$ B), to activate the expression of a series of cytokines and maturation markers (such as B7 molecules).<sup>57</sup> Shokouhi et al studied a series of biomaterials and found that many of them modulated the maturation and cytokine secretion of DCs to different extents in vitro. The TLRs cascade effects may play an important role in sensing the existence of these materials, and the hydrophobic domains



**Figure 6** Immune-related gene expressions in spleen cells of bare polyethylenimine (PEI)-treated mice as compared to reporter plasmid-conjugated PEI-immunized mice. The mRNA levels are shown as adjusted intensities for each gene. (A) Upregulated markers for the Th1 and Th2 responses. (B) Genes of the adaptive and innate immune responses. (C) Upregulated genes involved in immunogenicity and immunostimulation. Adapted by permission from Macmillan Publishers Ltd: *Gene Ther.* Regnström K, Ragnarsson EG, Köping-Höggård M, Torstensson E, Nyblom H, Artursson P. PEI – a potent, but not harmless, mucosal immuno-stimulator of mixed T-helper cell response and FasL-mediated cell death in mice. *Gene Ther.* 2003;10(18):1575–1583. copyright 2003.<sup>86</sup>

may be the “danger signals” which start the APCs activation, which are similar to “pattern antigens”.<sup>106</sup> Huang et al and Chen et al found that cationic polymers (such as cationic dextrans and PEI) promoted the maturation of macrophages via TLR4, and secreted type-I cytokines (Figure 7). Surprisingly, further



**Figure 7** PEI-induced IL-12 secretion of macrophages was inhibited by TLR4 antibody. In contrast, the addition of isotype control antibody did not inhibit such IL-12 production significantly. Reprinted from *Biomaterials*, 2010;31(32), Chen H, Li P, Yin Y, et al, The promotion of type I T helper cell responses to cationic polymers in vivo via toll-like receptor-4 mediated IL-12 secretion. 8172–8180, Copyright (2010), with permission from Elsevier.<sup>108</sup>

**Abbreviations:** PEI, polyethylenimine; IL-12, interleukin-12; TLR4, Toll-like receptor 4.

investigation revealed that PEI reversed the differentiation of tumor-associated macrophages and modulated the activity of natural killers (NKs) to kill the tumor cells.<sup>107,108</sup> Similarly, cationic dextran and PEI have also been found to repolarize myeloid-derived suppressor cells (MDSCs) into the antitumor phenotype. Knock-out mice experiments further proved the requirement of TLR4 signaling.<sup>109</sup> Other results found that in in vitro investigation, LPEI produced the TLR5-inducible cytokines in wild-type mice instead of TLR5<sup>-/-</sup> littermates. Therefore, LPEI was considered as a TLR5 agonist, and it is speculated that LPEI structurally resembled the flagellin.<sup>110</sup> Ma et al found that the PEI/DNA vaccines were much more effective than naked DNA vaccines in antitumor trial. The co-cultivation of PEI with BMDCs in vitro revealed the activation and expression of important nuclear translocation factors (such as NF- $\kappa$ B p50 and p65), which were important nuclear transcription molecules of TLRs. Therefore, the enhanced type I-mediated cytotoxic T lymphocyte activation, type II-mediated Th1/Th2 response and interferon- $\gamma$ -activated NKs might relate to the NF- $\kappa$ B-dependent inflammation and apoptosis induced by PEI.<sup>94</sup>

### NLR family pyrin domain-containing 3 (NLRP3) inflammasome pathways

Wegmann et al also supported the adjuvanticity of PEI, which promoted DCs trafficking to DLNs and led to cytokines secretion.<sup>25</sup> They proved the immunoactivation mechanisms of PEI related to the interferon regulatory factor 3 (IRF3)-dependent signaling and NLRP3 inflammasome activation,

especially the in vivo process.<sup>25</sup> The NLRP3 inflammasome is among the nod-like receptors (NLRs), a kind of pattern recognition receptors found on APCs. The NLRP3 inflammasome was known to be activated by various stresses, including K<sup>+</sup> efflux, reactive oxygen species (ROS) generation and lysosome rupture.<sup>111</sup> Thus, the NLRP3 inflammasome activation by PEI-antigen NPs in the experiment is potentially through the lysosomal-destabilizing activity or other damage-associated molecular patterns (DAMPs) caused by the toxicity of PEI. Interestingly, NLRP3 inflammasome function only biased adaptive immunity toward a Th2 response, instead of affecting the overall PEI adjuvant activity.<sup>25</sup>

### ROS

ROS generation is always related to tissue or cell damage, which is a kind of “danger signal”. The ROS and innate-mediated pro-inflammatory cytokines (such as the tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$ ) were signals in arming the adaptive immunity, sufficiently demonstrating the adjuvant effects of PEI.<sup>112</sup> Mulens-Arias coated superparamagnetic iron oxide NPs (SPIONs) with PEI and used them to stimulate macrophages. They found that the TLR4 and ROS signaling were involved. Intriguingly, though PEI-coated SPIONs and LPS both induced macrophages activation and M1 phenotype genes upregulation, the gene expression profiles were different.<sup>113</sup> It was also reported that the OVA-loaded PLGA MPs with a PEI-DS polyelectrolyte multilayer induced ROS in attachment with APCs. More positive charges on the outermost layer and higher molecular weight resulted in higher ROS production.<sup>114</sup> Many nonpathogenic adjuvants, including PEI, are toxic to tissues, under which circumstances ROS is generated. Although ROS are of extensive interest in the exploration of PEI-based vaccine mechanisms, the specific role and relationship with other immunity-related molecules still merit further investigations.

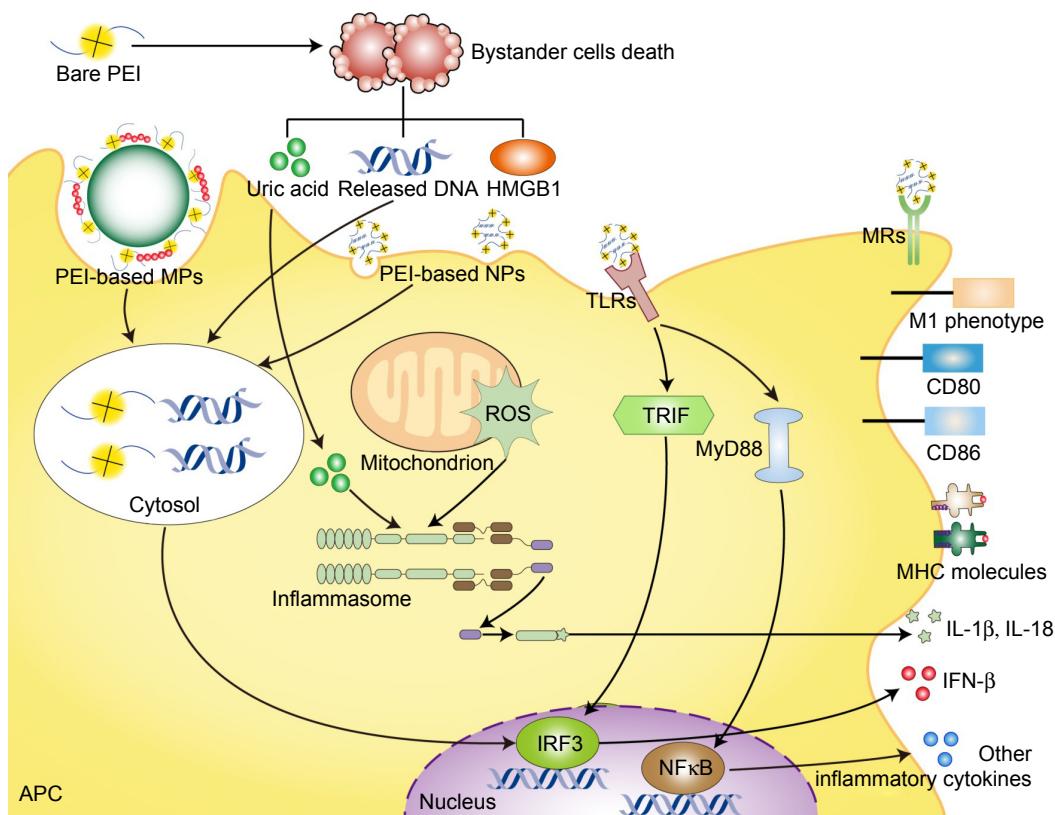
### Other factors in the “danger signals”

The “danger signals” elicited from PEI seem to be the main cause of its adjuvant effects. Regnström et al explored the gene expression profiles of PEI/DNA bronchial vaccines and found that bare PEI upregulated the expressions of genes involved in cell cycle regulation, oncogenesis and differentiation. These findings indicated the cytotoxicity and risks involved with PEI-based vaccines.<sup>86</sup> The hypothesis went along with the previous explanations of aluminum adjuvants and that the immunoactivation potency may be caused by the physiological reaction toward the sensed danger signals.<sup>115</sup> Meanwhile, the toxicity from adjuvants

would affect the cell states to exhibit various activities, which will impair the outcome of overall effects of PEI. Recently, Palumbo et al constructed an in vitro co-culture system and demonstrated the feasibility to transfect the PEI/DNA vaccine via fibroblasts. The vaccination surprisingly resulted in the cross-presentation of antigens and DC maturation. By comparing the high- and less-toxic PEI/DNA compositions and corresponding DC maturation states, as well as MHC I-restricted OVA presentation, the results highlighted that polymer-induced cytotoxicity probably benefited the immune activation.<sup>116</sup> Many endogenous antigens from the apoptotic or necrotic cells (such as the dsDNA, HMGB1, heat shock proteins and uric acids) have long been known to recruit and activate immune systems.<sup>116</sup> In Wegmann et al's report, they proved the strong dependence of double-stranded DNA (dsDNA)-mediated IRF3-triggered adjuvant effect of PEI. The free cellular dsDNA was released from the apoptotic or dead cells.<sup>25</sup> It is a good representative of the DAMPs signal to investigate an immune response.

## Summary of the immunoactivation mechanisms of PEI

Generally, PEI or PEI-based particles can stimulate the immune system, accompanied by the induction of various sorts of cell stress and immune response-related transcription factors.<sup>117</sup> Yet, it is notoriously difficult to elucidate the mechanisms clearly. The evidence for adjuvant effect of PEI or PEI-based particles in different research is generalized and drawn out by our own understanding (Figure 8). Most current evidence supports the “danger signals” or “damage-associated molecular patterns” theory. Both TLRs and NLRs are receptors in APCs that recognize the danger signals. PEI-associated cytotoxicity may be one manifested pattern of such danger signals.<sup>24,25</sup> Some nontoxic biomaterials also have profound effects on immune cells.<sup>106</sup> In some experiments, PEI was also found to be nontoxic in vivo, but still possessed the adjuvanticity.<sup>25</sup> Thus, other mechanisms may exist to stimulate immunity in addition to the direct toxicity of PEI. Some products reflecting the danger or damage signals have



**Figure 8** Intrinsic immunoactivation properties of PEI-based vaccines. PEI-based particles bind the PRRs and trigger the NF- $\kappa$ B and IRF3 factors to elicit a series of APCs maturation manifestation. They are also observed to elicit ROS in APCs, generating the “danger signal” by various routes. Besides, PEI-based particles cause the bystander cells death, and release a series of products, including uric acid, released DNA, HMGB1 and others. These materials activate IRF3, inflammasome and other pathways to generate the “danger signals” and APCs maturation.

**Abbreviations:** PEI, polyethylenimine; PRRs, pattern recognition receptors; NF- $\kappa$ B, nuclear factor- $\kappa$ B; IRF3, interferon regulatory factor 3; APCs, antigen-presenting cells; ROS, reactive oxygen species; MPs, microparticles; NPs, nanoparticles; TLRs, Toll-like receptors; MRs, mannose receptors; TRIF, TIR-domain-containing adapter-inducing interferon- $\beta$ ; MyD88, myeloid differentiation primary response gene 88; MHC, major histocompatibility complex; IL, interleukin; IFN, interferon.

been tested continuously, such as the ROS and the uric acids, while the roles involved have not been clarified.<sup>97,116,118</sup> Moreover, all the above-explained factors are actually not independent but work as a network that affects mutually. For example, the activation of NLRP3 inflammasome may be caused by the uric acids, which formed under necrosis or ROS.<sup>25,97</sup> Besides, ROS can be induced by the primary generation, or the product from cell stress, while NF- $\kappa$ B is the key suppressor of ROS-induced apoptosis.<sup>117</sup> Other intermediates (such as the dectin-1) intermediate bioactive cytokines and chemokines (such as the CCL family), and accessory immune cells (such as neutrophils and invariant NK T cells) are also involved in the network.<sup>119</sup>

## Conclusion and future perspectives

Although adjuvants are capable of increasing the efficacy of clinical vaccines and have been extensively studied, the pace of their development is relatively slow. Till now, only five adjuvants have been licensed for use in clinical applications by the US Food and Drug Administration and European Medicines Agency, including aluminum (1926), MF59 (1997), virosome (2000), AS04 (2005) and AS03 (2009). Among them, even aluminum, the most widely used adjuvant in vaccination for almost one century with many advantages such as good safety profile, antigen stabilization and augmentation of high-titer antibody production, does not have the ability to elicit CMI. Furthermore, only a few adjuvants have been licensed for cancer immunotherapy, indicating a need for novel adjuvants that can induce robust antitumor CMI. Therefore, it is desirable to develop new immunostimulant adjuvants in vaccine formulations to induce robust immune responses including both humoral immunity and CMI.

Interestingly, PEI confers the ability to induce the “danger signals” of immune cells and the production of pro-inflammatory cytokines, which forms the basis of its adjuvanticity. Given limited research and publications related to the effect of PEI on immunity, an in-depth understanding of its immune mechanism of action, especially the relationship with the cytotoxicity, is of great significance. Moreover, the biodistribution and biodegradation of PEI-based vaccines in vivo, with different sizes and shapes, different administration routes and different modification strategies, still need further investigation.<sup>85,120,121</sup> With breakthrough advances in multidisciplinary fields including chemistry, materials science, immunology and nanotechnology, it is believed that PEI-based vaccines, as well as other new adjuvants that improve the potency of the vaccine with precise modulations, will appear on the list of licensed adjuvants in the near future.

## Acknowledgments

The authors gratefully acknowledge funding for this work provided by the National Natural Science Foundation of China (81573047 and 81502367) and Clinical Research Physician Program of Tongji Medical College, HUST (5001530014).

## Disclosure

The authors report no conflicts of interest in this work.

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