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# The use of PEGylated poly [2-(N,N-dimethylamino) ethyl methacrylate] as a mucosal DNA delivery vector and the activation of innate immunity and improvement of HIV-1-specific immune responses

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ABSTRACT

To minimize the cytotoxicity of poly (2-(dimethylamino) ethyl methacrylate) (PDMAEMA) as a gene delivery vector, we synthesized PEGylated PDMAEMA by atom transfer radical polymerization (ATRP). Here we report its effects on transfection efficiency *in vitro* delivered with a GFP expression plasmid and immunogenicity *in vivo* after complexed with a HIV *gag* gene DNA vaccine. $mPEG_{113}$-*b*-$PDMAEMA_{94}$ was efficient in condensing DNA and formed polyplexes with an average diameter of about 150 nm. The *in vitro* transfection experiments demonstrated that PEGylation dramatically decreased the cytotoxicity at the *N*/*P* ratios above 30, although the transfection efficiency *in vitro* was reduced. Interestingly, mice *in vivo* vaccination study clearly showed that PEGylated PDMAEMA used as DNA delivery vector significantly improved the prime effect of DNA vaccine through intranasal administration. Importantly, PEGylated PDMAEMA was further proved its ability to induce cytokines production by murine macrophages. Overall, mPEG-*b*-PDMAEMA can be used as an efficient DNA vaccine vector which enhances adaptive immune responses by activating innate immunity.

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## 1. Introduction

Development of safe and efficient vectors, including both viral and nonviral ones, is crucial for gene therapy and vaccine delivery. Currently, viral vectors are the most efficient vectors used for gene delivery that depend on virus physiological binding to host cells and deliver their genetic material into host cells as part of their life cycle. However, several considerations, such as safety concern, high immunogenicity against viral vector itself, limited size for inserts and narrowed tropism for host cells hinder the extensive use of viral vectors in gene therapy and vaccination. A major barrier for viral vector based vaccine is the pre-existing immunity to vectors that might cause the vaccine to be ousted before antigen-specific response develops. In contrast to viral vectors, DNA vaccine has highly limited vector-related immunogenicity and can be used for multiple times, but its low levels of transfection and expression of the inserted gene *in vivo* limit its capacity of inducing strong immune responses. Therefore, a safe and efficient gene delivery system is desirable for gene therapy and vaccine delivery [1–3].

With the recent advances in nonviral vectors researchers believe that this category of carriers is one of the most promising gene delivery systems [4]. Cationic polymers are one major class of nonviral vectors that have received considerable attention by its high *in vitro* transfection efficiency [5,6], and have been promoted as a possible subrogate deliver system for viral carriers [7]. In previous study, we observed that intranasally priming with DNA plasmid formulated with polyethylenimine (PEI) could elicit strikingly stronger HIV-specific T-cell and humoral immune responses than priming with naked DNA alone [8]. PDMAEMA, like PEI, is a synthetic polycation, of which tertiary amine groups can be protonated at physiological pH. DNA can be condensed and protected by the interaction between positively charged PDMAEMA and negatively charged DNA [9,10]. Two major determinants for transfection efficiency are size and zeta potential of polymerized particles depending on the polymer/DNA molar ratio [11]. As a promising transfection reagent, PDMAEMA have potential applications in gene delivery

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systems. However, the high cytotoxicity is the major drawback to restrain its advances into clinical evaluation [12–16]. Many groups have tried to improve the biocompatibility of PDMAEMA by different approaches [17–21]. Polyethylene glycol (PEG) is one of the most widely used biocompatible polymers in drug delivery systems [22]. PEGylation of cationic polymers used for gene delivery in order to reduce cytotoxicity have been investigated [23–27] because of the prominent characteristics of PEG such as biocompatibility, water-solubility, charge shielding effect which can reduce nonspecific interaction between polyplexes and blood component, etc. [28]. The properties of PEGylated cationic polymers could be influenced by the molecular weight of PEG and the degree of PEGylation [29–31]. We chose $PEG_{113}$ ($M_n = 5000$) in the present study to improve PDMAEMA's biocompatibility.

In this study, we characterized the properties of polyplexes formed by plasmid DNA and PEGylated PDMAEMA by employing dynamic light scattering (DLS), zeta potential, transmission electron microscopy (TEM) and gel retardation assay, and subsequently tested its transfection efficiency *in vitro* and immunogenicity *in vivo* in a well-developed immunization system.

## 2. Experiment section

### 2.1. Materials

PDMAEMA and methoxy poly (ethylene glycol) ($mPEG_{113}$, $M_n = 5000$) were purchased from Sigma–Aldrich (Milwaukee, WI). 2-bromoisobutyryl bromide (BIBB) was kindly provided by Yancheng Creator Chemical Co. Ltd (Yancheng, China) and 2,2′-bipyridine (bpy) was purchased from Beijing Shiying Reagent Manufactory (Beijing, China). CuBr was prepared in our laboratory. Dichloromethane (anhydrous, Jiangtian, Tianjin, China), ethyl ether (anhydrous, Damao, Tianjin, China) and tetrahydrofuran (anhydrous, Jiangtian, Tianjin, China) were all analytical grade and used as received.

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate-buffered saline (PBS, pH = 7.2) were purchased from Invitrogen Corporation (Grand island, N.Y.). PEI ($M_n = 60{,}000$), Ethidium bromide, Dimethyl Sulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (Milwaukee, WI). Agarose was purchased from GEN TECH (Shanghai, China).

### 2.2. Synthesis and characterization of mPEG-b-PDMAEMA

In accordance with the approach in the reference [32], mPEG-*b*-PDMAEMA copolymers were synthesized by ATRP in water/2-propanol mixtures at 25 °C using mPEG-Br as macroinitiators, CuBr as catalyst and bpy as ligand. The structure of mPEG-*b*-PDMAEMA macromolecule is shown in Fig. 1. We characterized the copolymer by both $^1$H nuclear magnetic resonance ($^1$H NMR, Varian Unity-Plus INOVA 500) and gel permeation chromatography (GPC, Agilent 1100), and the detailed data were listed in Table 1.

### 2.3. Preparation of plasmid

The plasmid pEGFP used for transfection *in vitro* was generated by our laboratory. The gene encoding for enhanced green fluorescence protein (EGFP) was inserted into the plasmid vector pDRVI SV1.0 (kindly provided by Drs. Yiming Shao and Yong Liu at China CDC). The plasmid was amplified in *Escherichia coli* of DH5a and purified by column chromatography which can remove endotoxins (QIAGEN-Giga kit, The Netherlands). The purity and quantity of the plasmid were determined by Biophotometer (Eppendorf, Hamburg, Germany).

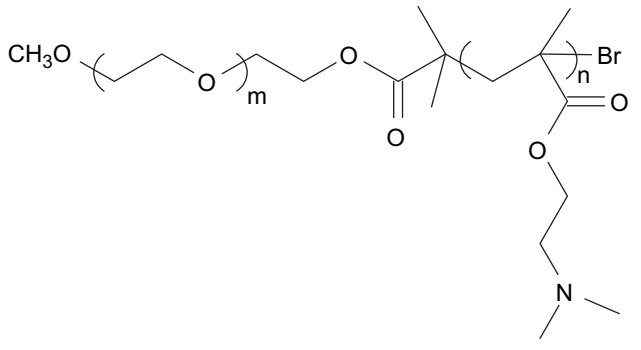


**Fig. 1.** Structure of mPEG-*b*-PDMAEMA.

**Table 1**
Polymers used in this paper and their characteristics.

| Molecular formula | $M_n$[a] ($10^3$ g/mol) | $M_n$[b] ($10^3$ g/mol) | Polydispersity[b] ($M_w/M_n$) |
|---|---|---|---|
| $mPEG_{113}$-*b*-$PDMAEMA_{94}$ | 19.96 | 14.73 | 1.40 |
| $PDMAEMA_{94}$ | / | 11.02 | 1.36 |

[a] $M_n$ is calculated according to the results of $^1$H NMR.
[b] $M_n$ and Polydispersity are both obtained by GPC.

### 2.4. Cell culture

293 T cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cultured cells were grown at 37 °C in 5% $CO_2$ humidified atmosphere.

### 2.5. Preparation of polymer/DNA polyplexes

Proper amounts of copolymers were dissolved in PBS buffer (pH = 7.2) to prepare aqueous stock solutions, and then all pH values were adjusted to 7.2. All solutions were filtered through 0.22 μm Millipore membranes and stored at 4 °C. 1 μg DNA (containing 3 nmol phosphate group) and appropriate amount of polymer solutions were diluted to 50 μl with PBS buffer, respectively and incubated for about 10 min at room temperature. Then the diluted DNA solutions were combined with diluted polymer solutions to form polyplexes with the *N*/*P* ratio (which is defined as the ratio between polymeric nitrogen residues and DNA phosphate groups [33]) ranging from 0.5 to 90, respectively. The resulting mixtures were vortexed for 5 s and incubated for 30 min at room temperature before further operation.

### 2.6. Characterization of polymer/DNA polyplexes

#### 2.6.1. Particle size and zeta potential

The measurements of particle size and zeta potential of mPEG-*b*-PDMAEMA/DNA polyplexes were performed on a BI 90 Plus/Zetaplus instrument of Brookhaven Corporation at 25 °C, employing a laser source (618 nm) at an angle of 90°.

#### 2.6.2. Transmission electron microscopy (TEM)

The polyplexes of mPEG-*b*-PDMAEMA/DNA were observed under a Philips EM400 ST transmission electron microscope. Samples were prepared by dipping a Formvar coated copper grid into the sample solution. And then, a drop of 0.1 wt% phosphotungstic acid aqueous solution was deposited onto the surface of the sample loaded grid and the excess solution was removed by filter paper after absorption for about 3 min.

#### 2.6.3. Agarose gel electrophoresis

The agarose gel retardation assay was performed as follows: 5 μl well incubated polyplex solution was mixed with 1 μl 6 × loading buffer (Takara Biotechnology, Dalian, China), and then the mixture was loaded onto 1% agarose gel containing ethidium bromide (0.5 μg/ml). Electrophoresis was carried out at a voltage of 5 V/cm for 30 min in 1 × TAE running buffer. Finally, the results were recorded at UV light wave-length 254 nm with image master VDS thermal imaging system (Bio-Rad, Hercules, CA).

### 2.7. Fluorescent labeling of pDNA and cell uptake of polyplexes

We employed a nucleic acid stain propidium iodide (PI, BD Company) to label pDNA. PI solution (1 mg/ml) was slowly added into pDNA solution, and the resulting mixture was incubated in dark at room temperature for 1 h. After that, the PI-labeled pDNA was purified by using DNA purification kit (Beyotime, Shenzhen, China) for several times until there was no absorption at 535 nm could be observed in the filtrate.

The PI-labeled pDNA was complexed with proper amount of polymer solution (*N*/*P* = 45 for both PDMAEMA and mPEG-b-PDMAEMA) as described above, and then the polyplex was added into cell seeded wells. After incubation for 1 h at 37 °C in 5% $CO_2$, the cells were stained with Hoechst 33342 (final concentration 5 μg/ml), incubated for another 30 min at 37 °C and observed with fluorescent microscopy.

### 2.8. Transfection and cytotoxicity

293 T cells were seeded in 24-well plates at $5 \times 10^5$ cells per well and incubated in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin for 24 h before transfection. Then the medium were removed and replaced by serum-

free DMEM. Subsequently 100 μl polyplex containing 1 μg plasmid DNA was added into each well. All transfections were conducted in triplicate. After 4 h incubation at 37 °C in 5% $CO_2$ humidified atmosphere, the transfection solutions were aspirated and substituted by DMEM containing 2% FBS. After additional 48 h incubation, the transfection efficiencies were determined by Flow Cytometry (BD FACSAria, BD Biosciences, San Jose, CA).

The cytotoxicity was determined by the viability of transfected cells with MTT assay. The cells were seeded in 96-well plates at $1 \times 10^4$ cells per well and subsequently transfected using similar protocol as described above. After 48 h incubation, 20 μl MTT solutions (5 mg/ml in PBS) were added to each well and incubated at 37 °C in 5% $CO_2$ for 4 h. Then solution in the wells was aspirated gently and 150 μl DMSO was added into each well to dissolve the formazan crystals. After shaking for 10 min, the absorbance of each well at 490 nm was measured by 1420 Multilabel Counter (PerkinElmer, Waltham, MA). The results were expressed as the mean percentage of cell viability relative to untreated cells.

### 2.9. Animal immunization

In this study, specific pathogen free (SPF) female BALB/c (H-2d) mice, aged 6–8 weeks with 18–22 g, were randomized into 4 groups (Table 2). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Shanghai Public Health Clinical Center and were performed in accordance with relevant guidelines and regulations.

The plasmid DNA pSV-CN54gag and recombinant TianTan vaccinia rTTV-CN54gag for animal vaccination were kindly provided by National Center for AIDS/STD control and prevention, China CDC. Both vaccines expressed the full length of HIV-1 CN54 Gag protein. The immunization regimen was scheduled as in Table 2. Briefly, Mice were immunized intranasally with naked DNA, $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA polyplexes or PEI/DNA polyplexes at weeks 0, 2 and 4, and intramuscularly with rTTV-CN54gag at week 6.

### 2.10. Enzyme-linked immunosorbent assay (ELISA)

Mice sera were collected for determining antibody responses by ELISA. The detailed protocols were described in our previous study [8,34]. For ELISA assay, microplates were coated with 5 μg/ml of p24 protein (Naikai Company, Hangzhou,-China) at 4 °C overnight. After two washes with PBS, the wells were blocked by PBS, containing 2% BSA and 0.05% Tween 20 for 2 h at room temperature. Mice sera were diluted in PBS, 2% BSA and 0.05% Tween 20 (dilution buffer). Diluted solution was added to the wells and incubated at room temperature for 4 h. After five washes with PBS containing 0.05% Tween 20, the wells were incubated with goat anti-mouse IgG-horseradish peroxidase (Zhongshan company, Beijing, China) for 1 h at 37 °C. Plates were washed five times, and 100 μl 3,3′,5,5′-Tetramethylbenzidine peroxidase substrate was added to each well. The reaction was terminated after 15 min incubation by adding 50 μl of 2 M $H_2SO_4$. The plates were then read by an ELISA reader (Multiskan Ascent, Thermo) at an optical density of 450 nm.

### 2.11. IFN-γ based enzyme-linked immunosorbent spot (ELISPOT) assay

6 weeks after the final inoculation of rTTV vaccines, mice were sacrificed and splenocytes were harvested. IFN-γ Elispot kits (Cat. No. 551083, BD Pharmingen, CA) were used to determine vaccine-elicited IFN-γ responses in BALB/c mice. 96-well plates were coated with purified anti-mouse IFN-γ monoclonal antibodies at the concentration of 5 μg/ml in 100 μl/well, and incubated at 4 °C overnight, then washed once with 200 μl/well R10 (RPMI-1640 containing 10% fetus bovine serum and 1% Penicillin–Streptomycin-L-glutamine) and blocked with 200 μl/well R10 for 2 h at room temperature. Mice splenocytes were isolated and red blood cells (RBC) were lysed by RBC Lysis Buffer (139.6 mmol/l $NH_4Cl$, 16.96 mmol/l Tris, pH 7.2). Cells were then washed 2 times with R10 and re-suspended in R10 complete culture medium. After cell counting, splenocytes were then adjusted to the concentration of $1 \times 10^7$ cells/ml and plated into 96-well Elispot plate at 50 μl/well ($5 \times 10^5$ cells per well) with addition of 50 μl HIV-1 Gag peptide (GHQAAMQILKDTINE) the final concentration for each peptide was 50 μg/ml. 96-well Elispot plates were incubated for about 20 h at 37 °C and 5% $CO_2$. After incubation, the Elispot plates were developed according to the kit instruction. Finally, plates were air-dried and the resulting spots were counted with ChampSpot III Elispot Reader (Beijing SAGE Creation Science, Beijing, China). Peptide specific IFN-γ Elispot responses were considered as positive only when the responses were 4-fold above negative control with no peptide stimulation.

### 2.12. Measurement of cytokine production

Murine RAW 264.7 macrophages (ATCC TIB71) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin. Suspensions of $0.5 \times 10^6$ cells per well were seeded into 24-well plates. After 18 h, the medium was replaced with fresh DMEM supplemented with 10% FBS and polymers alone, or polyplexes containing 2.0 μg of pSV-EGFP. Culture medium containing 100 ng/ml LPS was used as positive control. Supernatants were then collected at indicated time-points and stored at −80 °C. Concentrations of TNF-α, IL-6 or IL-10 in the supernatants were determined by ELISA (Dakewe, Shenzhen, China).

## 3. Results and discussion

### 3.1. Characterization of mPEG-b-PDMAEMA/DNA polyplexes

The mPEG-*b*-PDMAEMA/DNA polyplexes were prepared as described in experiment section. Agarose gel electrophoresis was conducted to determine the binding capability of mPEG-*b*-PDMAEMA to DNA. The results of this experiment were shown in Fig. 2a. Complete DNA retardation occurred when the *N*/*P* ratio was equal to or higher than 2 for PDMAEMA homopolymers, whereas the similar phenomenon was observed when *N*/*P* reached 5 for PEGylated copolymer [17]. This was probably due to the influence of PEG segments, which partially shielded the effective positive charges on PDMAEMA when nanoparticles with PDMAEMA/DNA cores and PEG shells were formed. Thus neutralization of negative charges on DNA needed more amounts of PEGylated polymers than homopolymers as previously reported [31,35].

Next, dynamic light scattering (DLS), zeta potential and TEM were also employed to further characterize the properties of the PEGylated PDMAEMA/DNA polyplexes. As shown in Fig. 2b, the particle size of $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA polyplex sharply decreased when *N*/*P* ratio increased from 0.5 to 2, further increase of *N*/*P* ratio above 2 only slightly changed the particle size, the mean diameter for polyplexes remained ~150 nm when *N*/*P* ratio was >5 (Fig. 2b and c), which indicated that stable polyplexes were formed. The zeta potential switched from negative to positive at the *N*/*P* ratio between 2 and 5 and finally reached the plateau at approximate 3 mV. The low potential may also reflect the shielding effect of PEG segments. In addition, we further characterized $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA polyplex by TEM. TEM images of $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA polyplexes at *N*/*P* ratios of 30 and 60 showed that the polyplexes was evenly dispersed with a diameter of ~150 nm (Fig. 2c), which was in accordance with DLS data above.

### 3.2. Internalization of polyplexes into cells

In order to show the internalization of polyplexes and to determine the influences of PEGylation on cell uptake efficacy, we

**Table 2**
Immunization regimens and schedule.

| Groups | DNA vaccine intranasal priming | | | rTTV vaccine intramuscular boosting | | |
|---|---|---|---|---|---|---|
| | DNA construct | Dose | Timing/week | Construct | Dose/PFU | Timing/week |
| Naked DNA ($n = 8$) | pSV1.0_CN54gag | 10 μg | 0, 2, 4 | rTTV-CN54gag | $5 \times 10^6$/mice | 6 |
| | pSV1.0 | 10 μg | | | | |
| $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA | pSV1.0_CN54gag | 10 μg | 0, 2, 4 | rTTV-CN54gag | $5 \times 10^6$/mice | 6 |
| ($N/P = 45$, $n = 8$) | pSV1.0 | 10 μg | | | | |
| PEI/DNA ($N/P = 10$, $n = 10$) | pSV1.0_CN54gag | 10 μg | 0, 2, 4 | rTTV-CN54gag | $5 \times 10^6$/mice | 6 |
| | pSV1.0 | 10 μg | | | | |
| Mock ($n = 6$) | pSV1.0 | 20 μg | 0, 2, 4 | rTTV | $5 \times 10^6$/mice | 6 |

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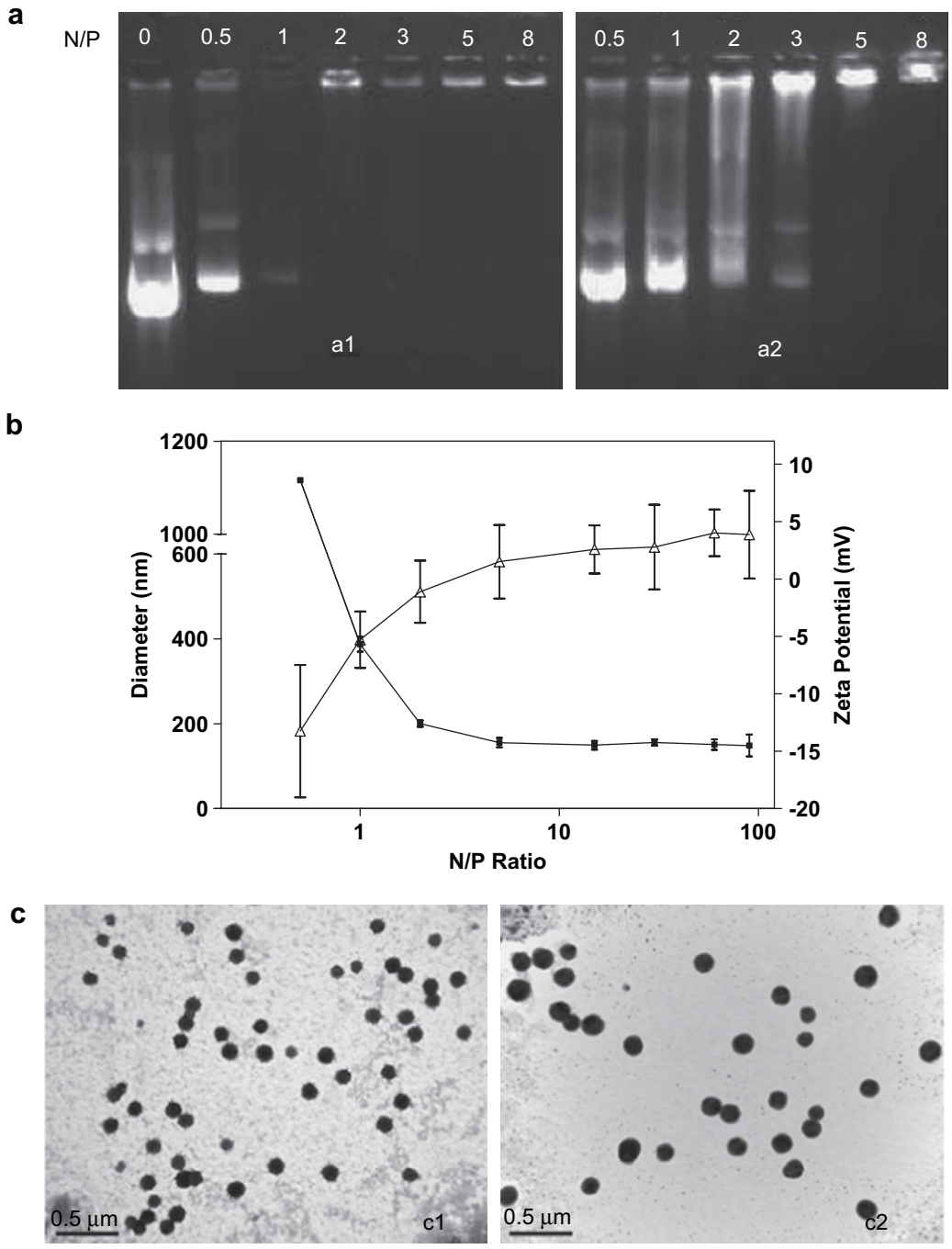


**Fig. 2.** Characterization of mPEG-*b*-PDMAEMA/DNA polyplexes. a) Agarose gel electrophoresis of DNA complexed with homopolymer $PDMAEMA_{94}$ (a1) or copolymer $mPEG_{113}$-*b*-$PDMAEMA_{94}$ (a2) at different *N*/*P* ratios. b) Particle size (■) and zeta potential (△) of $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA polyplex with the increase of *N*/*P* ratios. c) TEM images of $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA polyplexes at different *N*/*P* ratios. c1, c2 showed the particles at *N*/*P* = 30 and 60 respectively. All measurements were performed after the complexes were incubated for 30 min at room temperature.

labeled pDNA with PI, and then incubated it with 293 T cells for 1 h. After additional 30 min staining with a nuclear staining dye Hoechst 33342, internalization of polyplexes was examined by fluorescent microscopy. As shown in Fig. 3b and c, there was no red fluorescent signal for the naked PI-pDNA incubated cells, which meant that the naked DNA is unable to be internalized. In contrast, bright red fluorescent signals could be observed in PDMAEMA/PI-pDNA (Fig. 3e, f and j) or PEG-*b*-PDMAEMA/PI-pDNA (Fig. 3h, i and l) polyplexes treated cells. These data demonstrated that polyplexes could be efficiently internalized by cells. It should be noticed that the uptake of PEG-*b*-PDMAEMA/pDNA polyplexes was much lower than that of PDMAEMA/pDNA polyplexes as shown in Fig. 3e and h, indicating the charge shielding effects by PEG segments may greatly reduced the binding efficacy between polyplexes and negatively charged cells.

### 3.3. Cytotoxicity and transfection efficiency in vitro

To estimate the effects of PEGylation on cytotoxicity of polyplexes, the viabilities of cells transfected with different polyplexes were determined by MTT assay. As shown in Fig. 4a, all polyplexes presented low cytotoxicity when *N*/*P* ratio was lower than 30,

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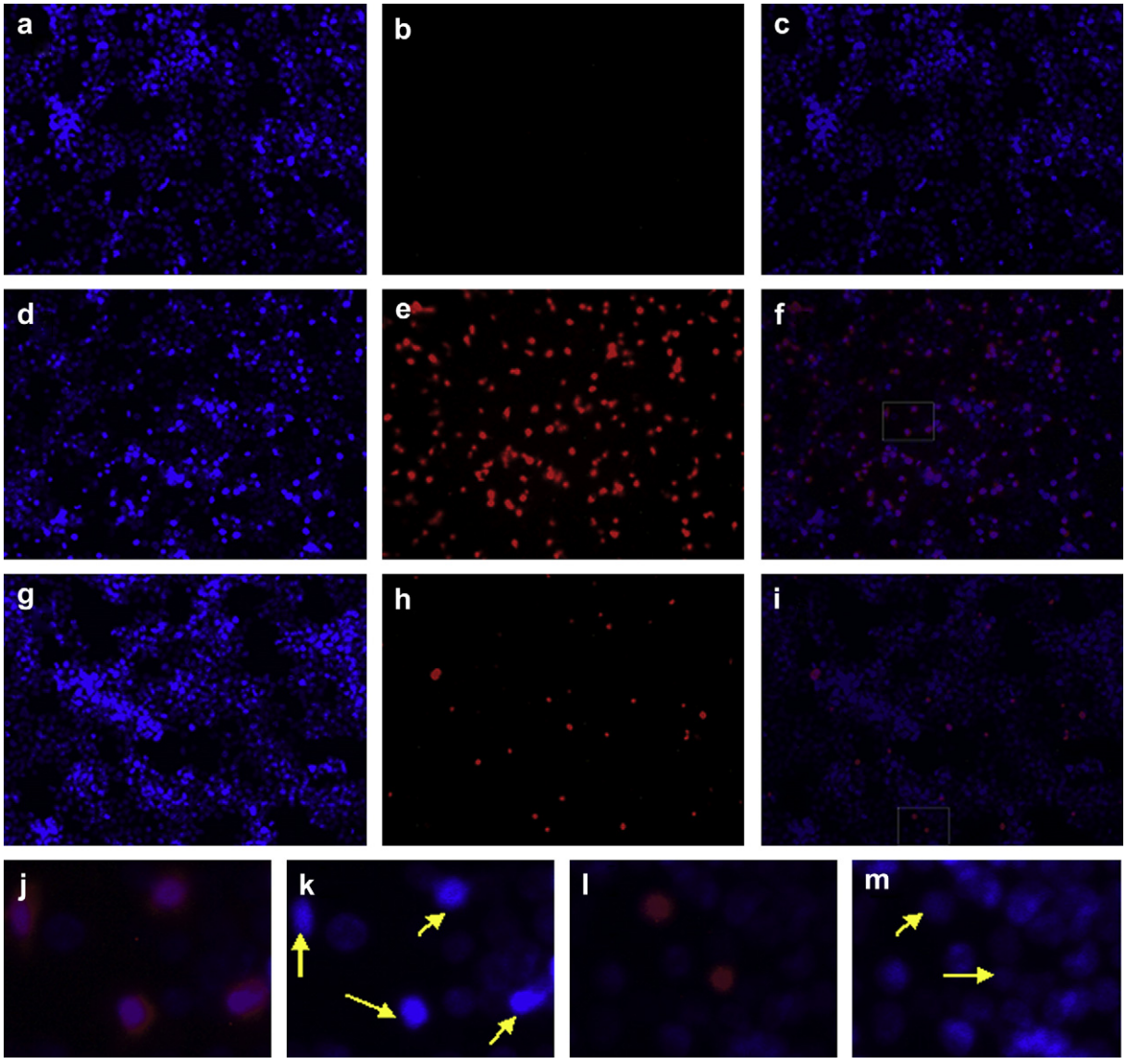


**Fig. 3.** The fluorescent microscopic images of cells. The cells were incubated with PI-pDNA (a, b and c), PDMAEMA/PI-pDNA polyplex (d, e and f) and mPEG-*b*-PDMAEMA/PI-pDNA polyplex (g, h and i) for 1 h. After staining with hochest 33342 (5 μg/ml) and incubation for another 30 min, the cells were observed with fluorescent microscopy. Images a, d and g are the fluorescence blue channel of hochest, images b, e and h are the red channel of PI, images c, f and i are the overlays of fluorescence red and blue channels. Photos j and l are the megascopic version of pictures in white rectangular area of prints f and i. Photos k and m are the fluorescent blue channel corresponding to pictures f and i, respectively. The yellow arrows in prints k and m point to the nuclei of polyplexes internalized cells.

further increases of *N*/*P* ratio for $PDMAEMA_{94}$/DNA polyplex could cause remarkable decline of cell viabilities, which was only evident for $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA polyplex at *N*/*P* ratio above 60. These results indicated that the biocompatibility of PDMAEMA was dramatically improved by $mPEG_{113}$. This may be attributed to the fact that $mPEG_{113}$-*b*-$PDMAEMA_{94}$ could sufficiently condense DNA to form compact spherical particle as described above; under this circumstance, the PDMAEMA/DNA polyplexes was tightly enwrapped by PEG and thereby greatly reduced their cytotoxicity [36].

We next evaluated the transfection efficacies of both PDMAEMA and PEGylated copolymer, as shown in Fig. 4b, pEGFP plasmid was used in this study as a reporter able to express green fluorescence protein that allowed quantification of the transfection efficiency by flow cytometry (Fig. 4c). It was observed that the PEGylation caused the reduction of the transfection efficiency *in vitro*. For example, the maximal transfection efficiency was 11% for homopolymer $PDMAEMA_{94}$ but approximate only 1% for PEGylated polymers, because PEGylation of polymer can lead to the reduction of internalization of polyplexes as demonstrated in Fig. 3, and probably also related to the decreased capacity of escaping from endosomal degradation [37–39]. Furthermore, the *N*/*P* ratio was influential for transfection efficiency with both PDMAEMA and PEGylated PDMAEMA. The transfection efficiencies of $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA polyplex peaked at *N*/*P* ratio of 45.

### 3.4. $mPEG_{113}$-b-$PDMAEMA_{94}$ as a carrier for DNA vaccine

Since the $mPEG_{113}$-*b*-$PDMAEMA_{94}$ at *N*/*P* ratio 45 could achieve considerable transfection efficacy with less cytotoxicity *in vitro*, we employed this formulation for vaccination *in vivo*. To test the $mPEG_{113}$-*b*-$PDMAEMA_{94}$ as a mucosal vector for DNA vaccine, we intranasally vaccinated mice with $mPEG_{113}$-*b*-$PDMAEMA_{94}$ formulated plasmid expressing HIV-1 CN54 Gag and intramuscularly boosted with recombinant Tiantan vaccinia (rTTV) vector expressing the identical immunogen. As we previously reported [8], PEI formulated DNA was able to induce vigorous antigen-

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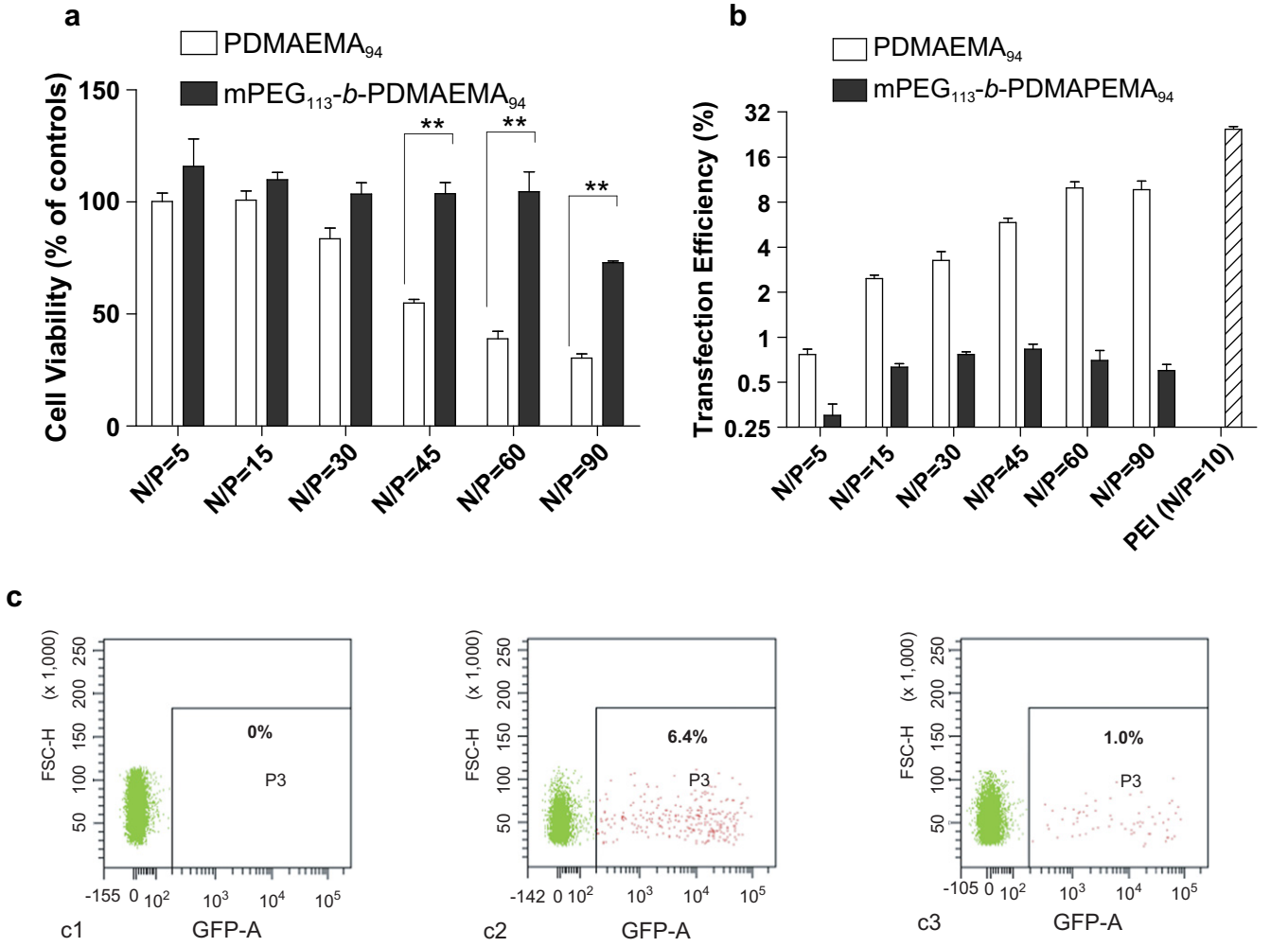


**Fig. 4.** Cytotoxicity and transfection efficiency *in vitro*. a) Cell viabilities of 293 T cells treated by polymers complexed with pEGFP determined by MTT assay ($^{*}p < 0.05$, $^{**}p < 0.001$). Experiments were performed in triplicate; values represent the relative viability compared to untreated cells as means ± SEM of one representative experiment ($n = 3$). b) The transfection efficiencies of polymers complexed with pEGFP as reporter gene in 293 T cells determined by flow cytometry. Experiments were performed in triplicate; values represent the EGFP-positive cells as means ± SEM of one representative experiment ($n = 3$). c) Flow cytometry measurement of transfection efficiency ($N/P = 45$), c1, c2, c3 correspond to naked DNA, $PDMAEMA_{94}$/DNA and $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA, respectively.

specific T-cell and antibody responses through a mucosal prime-systemic boost approach, thus we compared the immune responses elicited by $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA polyplexes to PEI/DNA polyplexes. Naked DNA was also included as a control. Immunization regimens and schedule are listed in Table 2. Splenocytes and sera were collected 6 weeks post-rTVV-CN54gag boosting, Gag-specific IFN-γ responses to the stimulation by HIV Gag peptide were quantified by ELISPOT assay, and antibody responses were measured by ELISA.

It has to be noticed that DNA was fully condensed and no free DNA was left in the PEG-*b*-PDMAEMA/DNA polyplex solution used for vaccination at $N/P = 45$ (data not shown). However, we found that there still existed uncomplexed polymer in polyplex solution, as much as 89.2% (Supplementary Fig. S3).

The ELISPOT results showed that intranasally priming with $PEG_{113}$-*b*-$PDMAEMA_{94}$/DNA polyplexes and intramuscularly boosting with rTTV-CN54gag elicited considerable HIV-1 Gag epitope (GHQAAMQILKDTINE) specific spot forming cells (SFCs) ($163 \pm 35$ SFCs/$10^6$ splenocytes, Mean ± SEM) (Fig. 5a), which were significantly higher than that in PEI/DNA group ($99 \pm 14$ SFCs/$10^6$ splenocytes, Mean ± SEM) ($p = 0.0441$) and that in naked DNA group ($56 \pm 5$ SFCs/$10^6$ splenocytes, Mean ± SEM) ($p = 0.0052$). The humoral responses were quantified with an HIV-specific ELISA. The antigen used was purified p24. HIV-p24-specific IgG antibodies in three different groups were compared in Fig. 5b. The antibody titer of mice primed with $mPEG_{113}$-*b*-$PDMAEMA_{94}$/DNA polyplex ($525 \pm 84$, Mean ± SEM) were significantly higher than that induced by naked DNA ($271 \pm 94$, Mean ± SEM) ($p = 0.0341$) and also above titers elicited in PEI/pDNA group ($440 \pm 25$, Mean - ± SEM) though no statistic significance was reached ($p = 0.4286$).

We noticed that the optimal *in vitro* transfection efficiency by mPEG-*b*-PDMAEMA/DNA polyplexes was 25-folds lower than that by PEI/DNA polyplexes (Fig. 4b). However, it is interesting that the *in vivo* results of T-cell and antibody responses were higher than or at least comparable to that of PEI/DNA polyplexes. These data suggested that transfection efficacy of DNA polyplexes *in vitro* in non-antigen presenting cells (APC) does not predict its immunogenicity *in vivo*.

### 3.5. PEG-b-PDMAEMA induced cytokine production by murine macrophages

Since result of *in vivo* immunization was contrast to reduced transfection efficiency observed in transfection assays, we

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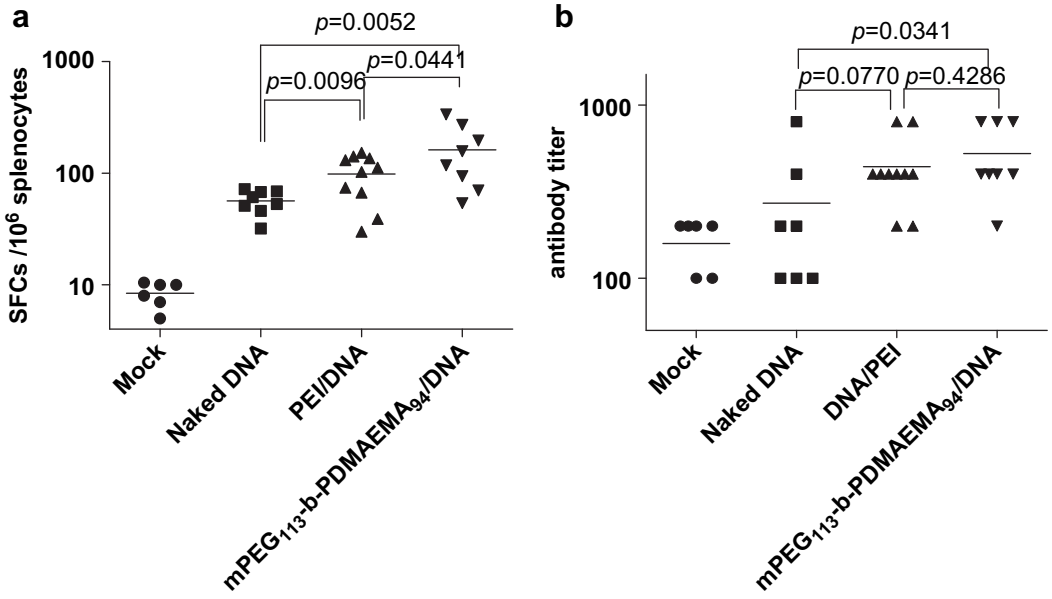


**Fig. 5.** Immunogenicity of polyplexes. a) Cellular immune response elicited by different immunization regimens. BALB/c mice were immunized as described in Table 2. The gag-specific IFN-γ-secreting cells were quantified by ELISPOT assays. Results are presented as the average number of spots. b) Humoral immune response after immunization with different regimens. Gag-specific IgG antibody titer in serum was determined by ELISA with optical density at 450 nm.

proposed that the free PEG-*b*-PDMAEMA or the PEG-*b*-PDMAEMA/DNA polyplexes may act as an adjuvant which can enhance the antigen-specific adaptive immune responses [3]. In order to validate this hypothesis, we determined the cytokines secreted by murine macrophage cell line (RAW 264.7) *in vitro* after stimulation by either polymers or polyplexes.

Two pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) and another anti-inflammatory cytokine interleukin 10 (IL-10) were selected as the indicators of activation of macrophages. RAW 264.7 cells were treated by free polymers or corresponding DNA formulated polyplexes at the optimal *N*/*P* ratios for 6 h or 24 h. As shown in Fig. 6,

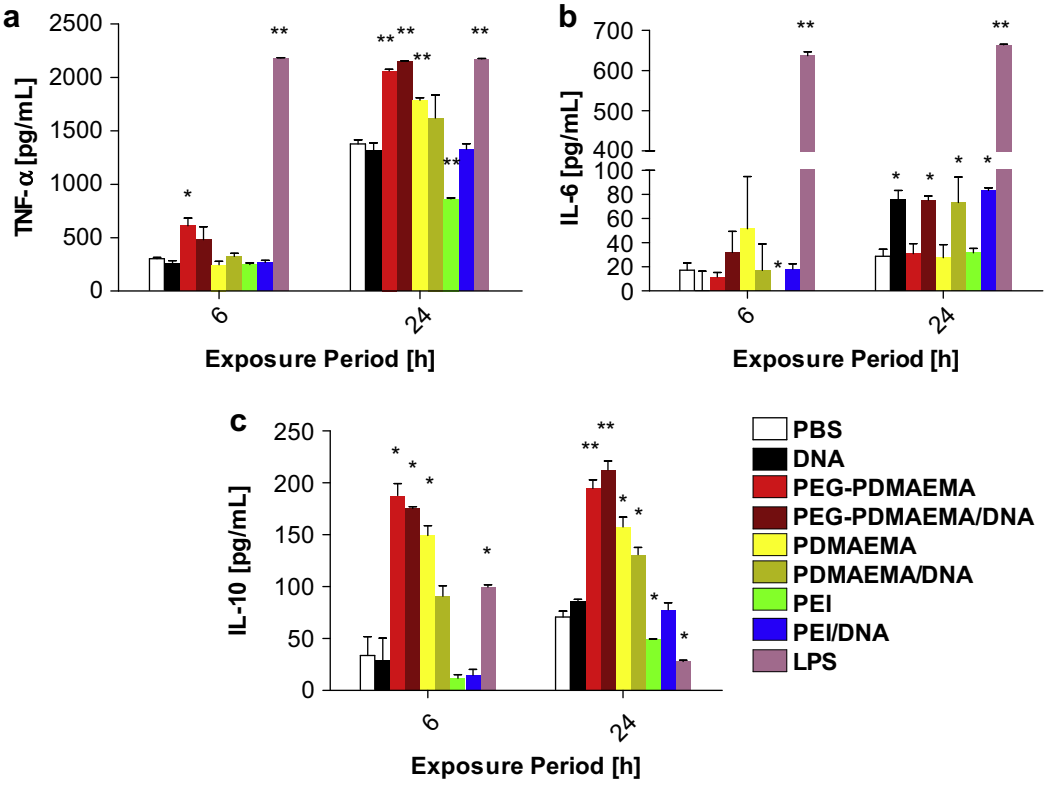


**Fig. 6.** Immune stimulatory activity of polymers and polyplexes. Cytokines secreted by RAW 264.7 cells, as induced by different polymers alone or complexed with plasmid DNA (2 μg) at the optimal *N*/*P* ratio respectively (45 for PEG-PDMAEMA and PDMAEMA, 10 for PEI) for 6 h or 24 h were detected. PBS and LPS were used as negative control and positive control (100 ng/ml) respectively. Concentrations of (a) TNF-α, (b) IL-6 and (c) IL-10 in the medium were measured by ELISA. Each value represents mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.001$, significant difference compared with negative control (PBS).

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the concentrations of TNF-α in culture supernatants treated with PEG-*b*-PDMAEMA/DNA polyplexes (480.2 ± 210.08 pg/ml for 6 h, 2149.5 ± 10.32 pg/ml for 24 h) or free PEG-*b*-PDMAEMA (611.5 ± 124.15 pg/ml for 6 h, 2062.2 ± 25.60 pg/ml for 24 h) were significantly higher than those treated with PEI/DNA polyplexes (268.5 ± 34.65 pg/ml for 6 h, 1327.9 ± 87.87 pg/ml for 24 h) and PEI (249.0 ± 26.31 pg/ml for 6 h, 860.7 ± 20.34 pg/ml for 24 h) both at 6 h and 24 h (Fig. 6a). A similar trend was observed for IL-10 secretion (Fig. 6c). PEG-*b*-PDMAEMA and its polyplexes induced much more IL-10 production than PEI and PEI/DNA. In our model, PEI alone or PEI/DNA polyplexes could not effectively induce cytokines secretion (Fig. 6a and c) and this is consistent with previous reports and related to its lower induction of NF-κB activation [40]. The homopolymer PDMAEMA also induced high level of cytokines production (Fig. 6a) in both free and plasmid DNA complexed forms. However, PDMAEMA had high cytotoxicity in both 293 T (Fig. 4a) and RAW 264.7 (data not shown), and the pro-inflammatory responses might be mediated by heat shock proteins (Hsps) released from necrotic cells induced by the high cytotoxicity of PDMAEMA [41]. It should be noticed that PDMAEMA or PEGylated PDMAEMA in the absence of DNA could stimulated significant amount of IL-10 and TNF-α, thus, the free PEG-*b*-PDMAEMA in our vaccine formulation as determined in supporting data Fig. S3 can enhance the immunogenicity of DNA vaccine (data not shown). Interestingly, it seems that IL-6 production depended on DNA because DNA alone or complexed with different polymers generated similar amount of IL-6 (Fig. 6b).

## 4. Conclusion

Overall, $mPEG_{113}$-*b*-$PDMAEMA_{94}$ could condense DNA into smaller particles with reduced cytotoxicity comparing to homopolymer $PDMAEMA_{94}$, indicating that PEGylation could decrease the cytotoxicity. In addition, PEGylated PDMAEMA used as DNA delivery vector could significantly improve the priming effect and thereby increase the immunogenicity of DNA vaccine through intranasal administration. Transfection efficacy *in vitro* in non-APC does not correlate to the immunogenicity *in vivo* for polymer vectors. Furthermore, we proved that the adjuvant effect of mPEG-b-PDMAEMA polyplexes was greatly enhanced compared to naked plasmid DNA *in vitro* through immune stimulatory activity assay. Through installation of specific ligand on PEG or introducing cytokine genes, we may further improve the efficacy of PEGylated PDMAEMA/DNA vaccines.

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## Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular Figures 3, 4 and 6, may be difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.09.032.

## Appendix. Supplementary data

Supporting information associated with this article could be found on-line, at doi:10.1016/j.biomaterials.2009.09.032.

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