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# Nanoparticle depots for controlled and sustained gene delivery

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ABSTRACT

Gene therapy is one of the most promising medical fields which holds the potential to rapidly advance the treatment of difficult ailments such as cancer as well as inherited genetic diseases. However, clinical translation is limited by several drug delivery hurdles including renal clearance, phagocytosis, enzymatic degradation, protein absorption, as well as cellular internalization barriers. Additionally, successful treatments require sustained release of drug payloads to maintain the effective therapeutic level. As such, controlled and sustained release is a significant concern as the localization and kinetics of nucleic acid therapeutics can significantly influence the therapeutic efficacy. This is an unmet need which calls for the development of controlled-release nanoparticle (NP) technologies to further improve the gene therapy efficacy by prolonging the release of nucleic acid drug payload for sustained, long-term gene expression or silencing. Herein, we present a polymeric NP system with sustained gene delivery properties, which can be synthesized using biodegradable and biocompatible polymers via self-assembly. The NP delivery system is composed of a polymeric NP which acts as a drug depot encapsulating cationic polymer/nucleic acid complexes, facilitating the enhanced retention and prolonged release of the gene payload. The NPs showed excellent cellular biocompatibility and gene delivery efficacy using the green fluorescent protein (GFP) encoded DNA plasmid (pGFP) as a reporter gene. Sustained release of the pGFP payload was shown over a period of 8 days. The physicochemical properties such as morphology, particle size, zeta potential, pGFP encapsulation efficiency and biological properties such as pGFP release profile, in vitro cytotoxicity and transfection efficacy in Hek 293 cells were characterized and evaluated. Importantly, the NP-mediated sustained release of pGFP generates enhanced GFP expression over time. We expect this NP-mediated gene delivery system to provide safe and sustained release of various nucleic acid-based therapeutics with applications in both fundamental biological studies and clinical translations.

## 1. Introduction

Nucleic acid-based gene therapy is a rapidly developing field with great potential to treat persistent and deadly disorders such as cancer and inherited genetic diseases, through rectifying the genomic errors which cause the illnesses [1]. Gene therapy broadly refers to techniques to exogenously modify genomes of cells to either block dysfunctional proteins from being formed, introduce correctly functioning sequences, or to silence transcribed mRNAs. Gene therapy shows amazing efficacy at the in vitro level, and takes many forms; from siRNA, microRNA, mRNA, plasmids to the most recently reported CRISPR genome editing complexes [1–3]. However, gene therapy presents severe challenges in delivery, which have been a barrier for translation into the clinic [4]. Naked nucleic acids are considered foreign genetic material when introduced into the body, and are rapidly cleared by the reticuloendothelial system (RES) or degraded by nucleases, rarely reaching the site of action [5]. Therefore, there is an urgent need for a delivery vector which can protect the payload during circulation in the body. The aforementioned delivery vectors can be generally classified into categories of viral and non-viral vectors, each with their benefits and drawbacks [6,7]. There were early attempts at utilizing viral vectors as a delivery method for gene delivery due to high in vivo delivery and transfection efficiency [8], however major issues with viral vectors such as the immune response of the host, and possible activation of

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oncogenes which cause malignancies [9,10] have hindered their applications.

Non-viral vectors, on the other hand, have many advantages compared with viral vectors such as significant safety advantages, reduced pathogenicity, reduced capacity for insertional mutagenesis and convenient large scale preparation [8]. Some promising non-viral vectors used in gene delivery include cationic lipids and cationic polymers due to their ability to bind negatively charged nucleic acids via electrostatic interactions. They have been extensively investigated due to their relatively high gene delivery efficiency. The cationic lipids usually share common structural similarities: the hydrophilic head bearing a positive charge binds with negatively charged nucleic acids and the hydrophobic lipid tail acts as a linker to connect them [8,11–17]. Transfection efficiency of cationic lipids depends on many factors including the geometric shape, number of charged groups per molecule, nature of lipid anchor and linker bondage [12]. However, there is a strong concern with surface charge, as it has been shown that a positive surface charge causes cellular toxicity which limits its clinical applications [8,11,12,18]. In terms of cationic polymers, in the past 2 decades, there has been much research on promising cationic polymers such as poly (ethylenimine) (pEI), poly (2-dimethylaminoethyl methacrylate) (pDMAEMA), and poly-L-lysine (pLL) due to their high efficiency for gene delivery and good transfection properties in vitro and in vivo [7,13,18–24]. These cationic polymers mix with nucleic acids to form nanosized complexes termed polyplexes which are in general more stable than the lipoplexes formed by cationic lipids [25]. However, these cationic polymers are not biodegradable, not biocompatible and present high cytotoxicity.

Poly (β-amino esters) (PBAE) are one such family of cationic polymers which solve many of these issues: they are biocompatible and hydrolytically biodegradable, while still able to condense negatively charged nucleic acids to form DNA nanocomplexes with lowered cellular toxicity and high transfection efficiency [26–30]. PBAE-447, reported by Green et al. [26] was synthesized via three monomers and demonstrated the highest transfection efficacy with low cytotoxicity in the BTIC cell line. However, this PBAE-447 DNA nanoparticle system does not show a sustained release behavior during gene transfection which is of great importance in gene therapy to maintain the therapeutic effective dose [26,31,32]. It is clear that even with the improvements PBAE brings to gene delivery vector design, improvements are necessary to generate the optimal vector.

PBAE presents an efficient vector to capture the gene payload, but also presents with fast release and difficulty in ligand modification. Sustained gene payload release from the vector is important for gene therapy as it increases the window of therapeutic effect while maintaining functionality of the therapeutic proteins and reducing the number of administrations [31,32]. Thus, developing gene delivery systems that can deliver foreign gene payloads (such as DNA, RNA, plasmid) in a sustained manner into target cells efficiently and safely is of crucial importance for successful gene therapy. Consequently, a protective, controlled-release, modifiable NP vector for the PBAE-nucleic acid complexes would be a potentially groundbreaking development.

Herein, we present our design of a novel poly(lactic acid-co-glycolic acid)-poly(ethylene glycol) (PLGA-PEG)/PBAE NP platform to deliver green fluorescent protein encoding plasmid (pGFP) as a reporter gene and explore the platform's applicability and feasibility as a non-viral vector for sustained gene delivery. The NP consists of three components: (i) an outer PEG surface, (ii) a PLGA shell, and (iii) an inner core containing PBAE/pGFP nanocomplexes. Among NP formulations, PLGA-PEG copolymers have attracted extensive attention due to their favorable properties: (1) they are biodegradable, biocompatible and FDA-approved; (2) they protect the payload from degradation; (3) capable of controlled and sustained release by both polymer degradation and payload diffusion, (4) the PEGylation modification on PLGA increases blood circulation half-life and enhances solubility in aqueous phase with low cytotoxicity and high cell permeability [33–37]. In previous publications, nucleic acids have been encapsulated into PLGA-PEG nanoparticles using the common water-oil-water (W/O/W) double emulsion/solvent evaporation method in order to achieve a better protection of plasmid and a more precise control of the release process [38,39]. However, the encapsulation efficiency of nucleic acid-based drug payload into PLGA NPs is challenging due to their extremely large size, polar character and electrostatic repulsion [36,38]. Therefore, incorporating both PLGA-PEG NPs and the cationic polymer PBAE would be a mutually beneficial design, facilitating the binding of the negatively charged gene payload inside PLGA-PEG nanoparticles. Each component plays to its strengths, with the PBAE improving gene payload encapsulation efficiency while the PLGA-PEG NP provides protection and promotes the retention of PBAE/pGFP nanocomplexes inside the particle and sustained release [33,36,40].

This new PLGA-PEG/PBAE formulation shows enhanced pGFP encapsulation efficiency and transfection efficacy compared to PLGA-PEG NP or PBAE alone. With our optimized formulation, the pGFP loaded PLGA-PEG/PBAE (hereby termed PLGA-PEG/PBAE/pGFP) NP system not only maintains NPs stability and high pGFP encapsulation, but also shows a sustained gene release behavior with high transfection efficacy and minimal cellular toxicity demonstrated on the Hek 293 cell line. More importantly, this NP-based nucleic acid depot approach can be applied to other cationic molecules, polymeric NPs and nucleic acids (siRNA, microRNA, mRNA, etc.) for screening of NP-based nucleic acid therapeutics delivery systems with prolonged drug release capability and translational potential.

## 2. Materials and methods

### 2.1. Materials

Poly(DL-lactide-*co*-glycolide) (50:50) with terminal carboxylate groups (PLGA, inherent viscosity: 0.55–0.75 dL/g in HFIP) was obtained from Lactel Absorbable Polymers (Birmingham, AL, USA). Amine PEG carboxyl, HCL salt (NH2-PEG-COOH, MW 3500) was purchased from Jenkem Technology (Beijing, China). 4-Amino-1-butanol, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCL) and 1-(3-Aminopropyl)-4-methylpiperazine were supplied from Alfa Aesar (Ward Hill, MA, USA). Poly(vinyl alcohol) 87–89% hydrolyzed (PVA, MW 13–23 kDa), N-Hydroxy-succinimide (NHS), N,N-Diisopropylethylamine (DIEA) and 1,4-Butanediol diacrylate were obtained from Sigma (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM, with 4.5 g/L D-Glucose, L-Glutamine and 110 mg/L Sodium Pyruvate), Opti-MEM reduced Serum Medium and 0.25% Trypsin-EDTA (1×) were purchased from Gibco (Paisley, UK). Lipofectamine 2000 Reagent was obtained from Invitrogen (Carlsbad, CA, USA). Tissue Culture Flasks and 12 wells Plates were supplied from VWR (Radnor, PA, USA). All reagents were analytical grade from Sigma (St. Louis, MO, USA) and used as received, unless otherwise stated.

### 2.2. Synthesis of PLGA-b-PEG

Copolymer PLGA-*b*-PEG was synthesized by the conjugation of COOH-PEG-$NH_2$ to PLGA-COOH with slight modifications as previously described [41]. In brief, PLGA-COOH (500 mg) was dissolved for 1 h in 3 mL DCM with 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCL, 23 mg, 0.12 mmol) to activate the carboxylic acid of PLGA. Excess N-hydroxysuccinimide (NHS, 13.5 mg, 0.11 mmol) was added into such solution to obtain PLGA-NHS. PLGA-NHS was precipitated with 20 mL of an ice-cold mixture of ethyl ether and methanol (1: 1, vol: vol) and repeatedly washed using the same mixture two times to remove residual EDC and NHS. After drying under vacuum, PLGA-NHS (100 mg, 0.0059 mmol) was dissolved in 3 mL chloroform followed by addition of $NH_2$-PEG-COOH (25 mg, 0.0071 mmol) and N,N-diisopropylethylamine (DIEA, 2.8 mg,

0.022 mmol). The co-polymer was precipitated with ice-cold mixture of ethyl ether and methanol (1: 1, vol: vol) after overnight reaction and washed with the same solvent two times to remove unreacted PEG. The resulting PLGA-PEG *block* co-polymer was dried under vacuum and used for NP preparation without further treatment.

### 2.3. Synthesis of a poly(β-amino ester)

The cationic polymer poly(β-amino ester) (PBAE) was synthesized using a two-step reaction as previously described [26,30]. 1,4-Butanediol diacrylate (2 g, 8.4 mmol) which acts as the biodegradable backbone was polymerized by Michael Addition with 4-Amino-1-butanol (750 mg, 8.24 mmol) side chain monomers for 24 h at 90 °C and 500 rpm stirring in the absence of solvent. For the second step of synthesis, the diacrylate-terminated backbone was dissolved in 2 mL anhydrous tetrahydrofuran (THF) and combined with 10 mL THF solution of 1-(3-Aminopropyl)-4- methylpiperazine (785 mg, 4.9 mmol) as polymer end-capping groups. The reaction was conducted at room temperature overnight with 500 rpm stirring. Polymer PBAE was then purified to remove excess monomers via precipitation in diethyl ether following centrifugation at 3000 rpm for 3 min. The supernatant was decanted to collect PBAE and PBAE was washed 2 times with 20 mL diethyl ether. The PBAE was used directly to prepare PLGA-PEG/PBAE NPs without any extra processing after drying under vacuum for 48 h.

### 2.4. Preparation of PLGA-PEG/PBAE/pGFP NPs

The PLGA-PEG/PBAE/pGFP NPs were prepared through self-assembly of polymeric and amphipathic PLGA-PEG/PBAE system using a double-emulsion solvent evaporation method with slight modifications to a previous described method [33]. Briefly, 8 mg copolymer PLGA-PEG and 2–6 mg PBAE were co-dissolved in 1 mL methylene chloride (DCM). High concentration pGFP (0.89 μg/μL) was reconstituted in UltraPure Distilled Water (DNAse and RNAse free, Invitrogen). The 0.1 μg/μL GFP solution (300 μL) was added drop-wise into 1 mL of PLGA-PEG and PBAE solution and emulsified by probe sonification (Qsonica Sonicatiors, Newtown, CT, USA) to form the first emulsion. Next, the emulsified mixture was added into 3 mL of aqueous solution containing 1.67 wt% PVA, followed by probe sonification to form the double emulsion. The final emulsion solution was added drop-wise into 7 mL of DI water and stirred for 3 h at 900 rpm to allow the DCM solvent to evaporate and the particles to harden. The remaining organic solvent DCM and unencapsulated pGFP were removed by concentrating and washing the particle solution two times using a 50 mL Amicon Ultra Centrifugal Filter (MWCO 100 kDa, Millipore) for 50 min at 1600 rpm (515 *g*) in centrifuge (Eppendorf, 5810 R) which concentrated the NPs solution to a final volume of 1 mL.

Parallel experiments were also performed to optimize the formulation by varying the amount of PBAE while keeping the amount of PLGA-PEG and pGFP constant. Five formulations were prepared and assayed for their performance, the details of the five formulations were as follows: 1) 8 mg PLGA-PEG with 6 mg PBAE was abbreviated as pp6p; 2) 8 mg PLGA-PEG with 4 mg PBAE was abbreviated as pp4p; 3) 8 mg PLGA-PEG with 2 mg PBAE was abbreviated as pp2p; 4) 8 mg PLGA-PEG without PBAE was abbreviated as pp0p; 5) 6 mg PBAE alone without PLGA-PEG was abbreviated as 6p and acted as positive control group.

### 2.5. Nanoparticle characterization

#### 2.5.1. Particle size, zeta potential

The NP size and zeta potential were measured using a Zeta Sizer dynamic light-scattering detector (15-mW laser, incident beam of 676 nm; Malvern, UK) at 25 °C and at a scattering angle of 90° at a concentration of approximately 0.1 mg NP/mL water. The intensity-weighted mean value was recorded as the average of three measurements.

#### 2.5.2. Encapsulation efficiency analysis

The encapsulation efficiency of pGFP in the NPs was determined by measuring the amount of unbound pGFP. Briefly, the amount of pGFP in the bottom liquid of the Ultra Centrifugal Filter device during the NPs suspension washing process was analyzed by using Quant-iT PicoGreen kits according to the manufacturer's protocol [36].

The fluorescence was measured by microplate reader (Infinite Pro 200, Tecan, Switzerland) at excitation and emission wavelengths of 480 and 520 nm with the gain fixed at 80. The amount of pGFP was calculated according to the linear calibration curve of DNA ($F = 53.926*C-38.235$ $R^2 = 0.9995$). The encapsulation efficiency was calculated from the following equation:

$$\text{DNA (encapsulation efficiency \%)} = \frac{(\text{total DNA content} - \text{free DNA content})}{\text{total DNA content}} * 100\%.$$

#### 2.5.3. Morphology analysis

The morphology of PLGA-PEG/PBAE/pGFP NPs was observed under transmission electron microscope (Hitachi H-7500 TEM, Japan). Samples were prepared by placing one drop of 3× dilution of concentrated NPs on TEM grids and air-dried, following negative staining with a drop of 5% uranyl acetate solution for 6 mins. The air-dried samples were then directly observed using TEM.

### 2.6. In vitro pGFP transfection

The transfection activity of PLGA-PEG/PBAE/pGFP NPs was evaluated in a Hek 293 cell line using pGFP as a reporter gene. The cells were seeded into 12-well plates at density of around $0.5 \times 10^6$ per well and maintained in 1 mL complete culture medium overnight prior to transfection. At a confluence of 80–90%, 20, 50, 70 and 100 μL of concentrated PLGA-PEG/PBAE/pGFP NPs were added into each well in serum circumstance. After 4 h culture, the transfection medium was replaced with 1 mL fresh complete culture medium and the cells were incubated sequentially for 24 h, 48 h, 72 h and 96 h post transfection. Detection of pGFP expression was carried out with fluorescent microscope at different timepoints of 24 h, 48 h, 72 h and 96 h. All transfection experiments were performed in triplicate.

A transfection optimization study was performed with three different PLGA-PEG/PBAE/pGFP NPs formulations (pp6p, pp4p and pp2p). Lipofectamine 2000 reagent and PBAE-only formulation (6p) were used to transfect Hek 293 cells according to the manufacturer's protocol and as previously reported [26] as positive control groups, respectively. Free pGFP and PLGA-PEG/PBAE NPs without pGFP loaded were examined as negative control groups.

### 2.7. In vitro pGFP release study

The in vitro pGFP release from PLGA-PEG/PBAE/pGFP NPs was measured over 10 days using separate samples for each time point according to the following procedures [36,42]. Briefly, the concentrated PLGA-PEG/PBAE/pGFP NPs were diluted by a factor of 10 using 1× PBS buffer. 200 μL of NPs solution was loaded in 1.5 mL Eppendorf tubes and then shaken horizontally at 37 °C and 300 rpm (Eppendorf Thermomixer R). At predetermined time intervals, the tubes were taken out and centrifuged at 10,000 *g* for 5 min (Eppendorf centrifuge 5418), then the supernatants were collected for analysis. The amount of pGFP released from NPs was evaluated by Quant-iT PicoGreen assay according to the manufacturer's protocol. Background readings were corrected using the centrifugation supernatants from the control group PLGA-PEG/PBAE NPs with no GFP loaded.

### 2.8. In vitro cytotoxicity

The cytotoxicity of different formulations of PLGA-PEG/PBAE/pGFP NPs (pp6p, pp4p, pp2p and pp0p) was evaluated by XTT assay kits in

Hek 293 cell line. Briefly, the cells were seeded into a 96-well plate at a density of $1 \times 10^4$ cells per well in 0.1 mL of DMEM culture medium supplemented with 10% fetal bovine serum (FBS) and antibiotics in 5% CO2 incubator at 37 °C overnight. After that, varying amounts of the concentrated NPs were proportional to that used in the previous transfection experiment (50 μL NPs/mL = 0.7 mg/mL, 70 μL NPs/mL = 1.0 mg/mL and 100 μL NPs/mL = 1.4 mg/mL) and were added into cell plate in the same manner as the transfection experiments with the untreated groups as blank control groups and PBAE-only groups (6p) as positive control. After incubation for 24 h, 50 μL of XTT stock solution in PBS was added into each well and then the cell plate was incubated at 37 °C in 5% CO2 for 18 h. Then the cell plate was read spectrophotometrically at 450 nm with reference at 650 nm by microplate reader (Infinite M200 Pro, Tecan, Switzerland). The cell viability (%) was calculated and compared with the untreated control (100%) according to the following equation:

$$\text{Cell viability (\%)} = [\text{Abs(samples)}/\text{Abs(control)}] * 100\%$$

Abs(samples) represented measurements at 450 nm minus measurements at 650 nm from the cells treated with NPs and Abs(control) represents the untreated cells.

### 2.9. Fluorescent cell imaging

Fluorescent images of pGFP transfected cells were taken by an All-in-One Fluorescence Microscope (BZ-X710, Keyence, Japan) at 24 h, 48 h, 72 h and 96 h with brightfield, fluorescent and merged pictures using 10× PanFluor lens (Nikon, Japan) and GFP-B filter (Ex 470/40, DM 495, BA 535/50, Keyence, Japan). All fluorescent images were taken under same exposure time (1 s) and analyzed using Image J software.

### 2.10. Western blot

The Western blot was prepared following a previous protocol [43]. Briefly, the Hek 293 cells after transfection using pp6p and 6p formulations were harvested at 1d, 2d, 3d and 4d, respectively and stored at −20 °C. The frozen cell pellets were ultrasonicated in 110 μL chilled lysis buffer (Boster Bio Tech, CA, USA). The cell suspension was centrifuged at 4 °C for 15 min at 1000 g and the supernatant was collected, which contained the GFP proteins in cytosol. After the concentrations of the proteins in the samples were measured using the Bio-Rad protein assay (Bio-Rad), the samples were heated at 99 °C for 5 min and loaded onto a 4–15% stacking/7.5% separating SDS-polyacrylamide gel (Bio-Rad). The proteins were then electrophoretically transferred onto a polyvinylidene difluoride membrane (Bio-Rad) in a 4 °C cool room overnight. The membrane was first blocked with 3% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature and then incubated at 4 °C overnight with the following primary antibodies: rabbit anti-GFP (1:5000; Boster Bio Tech, CA, USA), rabbit anti-GAPDH (1:2000; Boster Bio Tech, CA, USA). The membranes were submerged in Tris buffered saline Tween 20 (TBST), washed 3 times and incubated for 2 h with the peroxidase conjugated secondary antibody (1:2000; Boster Bio Tech, CA, USA) at room temperature. The proteins were visualized by western peroxide reagent and luminol/enhancer reagent (Clarity Western ECL Substrate, Bio-Rad). Exposure was done using ChemiDoc XRS and System with Image Lab software (Bio-Rad). The intensity of blots was quantified with densitometry using Image Lab software (Bio-Rad).

### 2.11. Flow cytometric analysis

The Hek 293 cells were transfected with PLGA-PEG/PBAE/pGFP NPs and then harvested at pre-set time points and resuspended in 0.5 mL PBS for flow cytometric analysis using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA) and the data were analyzed using FACSDiva software (BD Biosciences, San Jose, CA). Data were acquired using a 488 nm laser with a 530/30 BP filter for the detection of GFP positive cells under a voltage of 420 V. 10,000 events were collected for each measurement.

### 2.12. General cell culture

The Hek 293 cell line was kindly gifted from Dr. Lei Bu from NYU Langone Medical Center. The cells were cultured in DMEM (Gibco) with 10% (vol/vol) FBS and 1% penicillin/streptomycin. Cells and biological experiments were conducted at 37 °C in 5% $CO_2$.

### 2.13. Statistical analysis

Data are presented as mean ± SD. Significant differences were determined using the Student's *t*-test. *P*-values of $< 0.05$ were considered statistically significant.

## 3. Results and discussion

### 3.1. Formation of PLGA-PEG/PBAE/pGFP NPs

In this study, we have designed and evaluated a novel PLGA-PEG/cationic polymer NP system as a non-viral vector for gene delivery using PBAE as a model cationic polymer and pGFP as a model nucleic acid therapeutic. Although PLGA-PEG NPs have many advantages such as good biocompatibility, biodegradability and sustained payload release behavior, they cannot well-encapsulate or release hydrophilic and negatively charged nucleic acids in a controlled fashion due to the natural hydrophobic and neutral charge of PLGA [33,44,45]. On the other hand, the PBAE cationic polymer shows good biodegradability, minimal cytotoxicity, and excellent nucleic acid complexing ability with high in vitro transfection efficacy [26,30]. Therefore, the combination of PLGA-PEG NP and PBAE shows superior nucleic acid encapsulation, sustained release and transfection efficacy over PLGA-PEG NPs or PBAE alone. We selected a specific cationic poly(β-amino ester) termed as PBAE-447 which is a biocompatible and hydrolytically biodegradable cationic polymer, to attract negatively charged pGFP to form PBAE/pGFP nanocomplexes with less cellular toxicity as reported in previous studies [26,30] as a top performing PBAE with high transfection efficacy and good biodegradability. The NPs were prepared via a water-in-oil-in-water (W/O/W) emulsion method with PLGA-PEG and PBAE in the oil phase and pGFP in the water phase for the first emulsion. We hypothesize that the cationic PBAE and negatively charged pGFP form PBAE/pGFP nanocomplexes via electrostatic interactions during the PLGA-PEG self-assembling nanoparticle formation process. The design and characterization of PLGA-PEG/PBAE/pGFP hybrid NPs are shown in Fig. 1. The obtained NPs present an average DLS hydrodynamic diameter around 165 nm with a narrow size distribution of ~0.1 PDI. The TEM image shows the NPs have uniform compact spherical shape with a diameter around 130 nm which is in accordance with the DLS result (Fig. 1B and C). Importantly, we observed PBAE/pGFP nanocomplexes inside the PLGA-PEG NPs from the TEM image (Fig. 1C left). Notably, we did not observe this same phenomenon in PLGA-PEG NPs without PBAE incorporated (Fig. 1C middle). To further prove our hypothesis, we characterized the size and morphology of PBAE/pGFP nanocomplexes, which present with a diameter 10–20 nm using TEM (Fig. 1C right), which is consistent with the observed nanostructures in PLGA-PEG NPs.

To optimize pGFP encapsulation efficiency and transfection efficacy, we have prepared a series of PLGA-PEG/PBAE/pGFP NP formulations by fixing the amount of PLGA-PEG (8 mg) and pGFP (30 μg) and varying only the amounts of PBAE from 0 mg, 2 mg, 4 mg to 6 mg. These formulations are abbreviated as pp0p, pp2p, pp4p, and pp6p, respectively. A PBAE/pGFP formulation without PLGA-PEG was also prepared as a positive control group using the previously reported

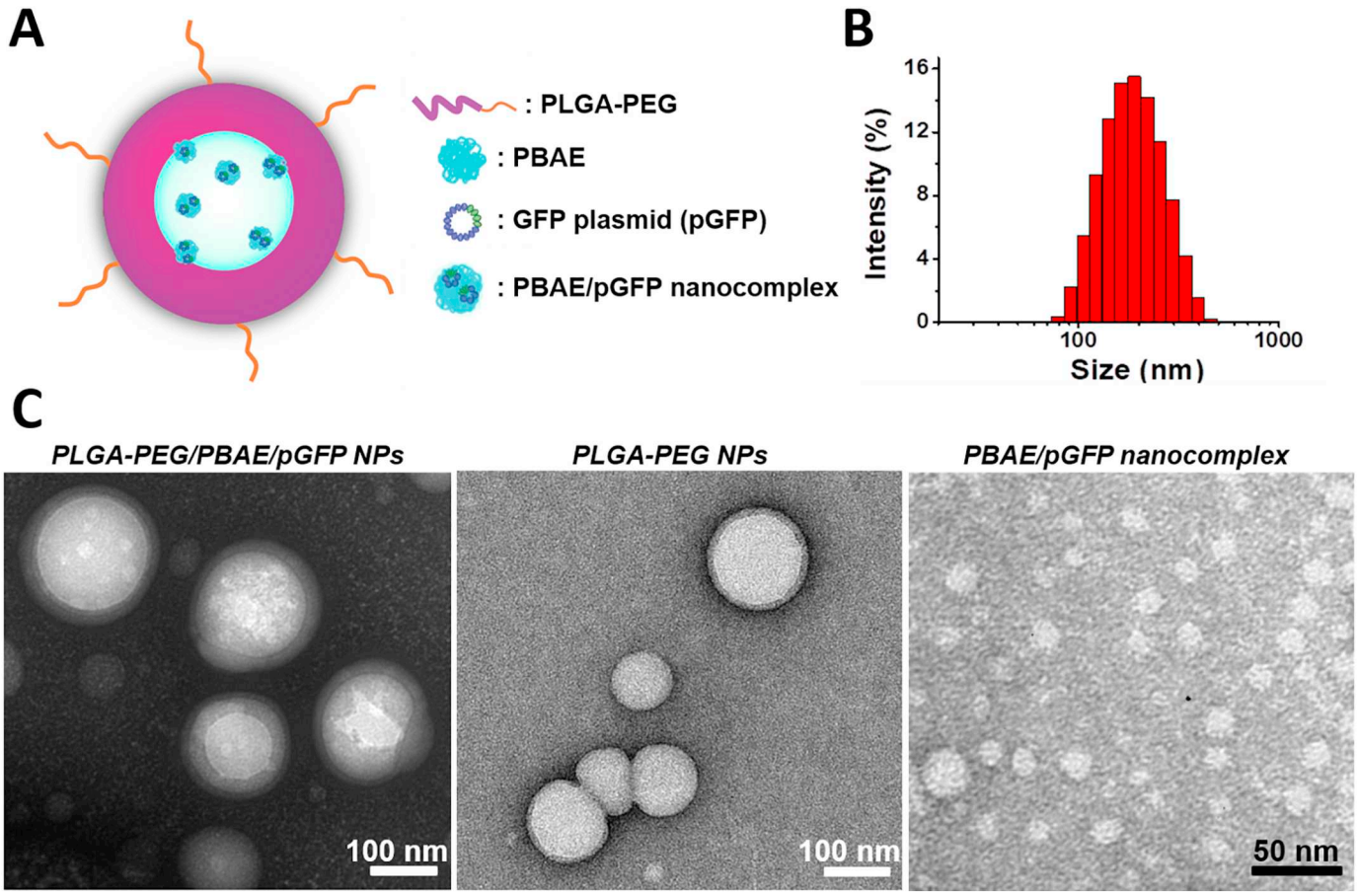


**Fig. 1.** Design and characterizations of NPs. (A) Chemical structure of PLGA-PEG/PBAE/pGFP NPs. The particle consists of three components: (i) PBAE/pGFP nanocomplexes distributed inside PLGA-PEG NPs, (ii) The PLGA layer provides sustained release and protection of encapsulated PBAE/pGFP nanocomplexes, (iii) An outer PEG surface. (B) Size distribution of the NPs determined by dynamic light scattering. (C) Representative TEM images of PLGA-PEG/PBAE/pGFP NPs (left), empty PLGA-PEG NPs (middle) and PBAE/pGFP nanocomplex (right).

**Table 1**
Characterizations of different NP formulations. Mean size (d.nm), polydispersity index (PDI) and zeta potential (mV) of different formulations of PLGA-PEG/PBAE/pGFP NPs prepared by double emulsion technology and compared with 6p.

| Formulation | Size, nm | Polydispersity | Zeta potential, mV |
|---|---|---|---|
| Pp6p | 167.8 ± 4.6 | 0.157 | +31.3 ± 3.5 |
| Pp4p | 161.2 ± 4.2 | 0.113 | +28.6 ± 2.1 |
| Pp2p | 162.3 ± 2.9 | 0.145 | +34.2 ± 2.9 |
| Pp0p | 151 ± 1.7 | 0.078 | −14.8 ± 3.8 |
| 6p | 40.8 ± 13 | 0.181 | +38.6 ± 4.6 |

optimized formulation method (6 mg PBAE with 30 μg pGFP) and this formulation is termed 6p [26,30]. All NP formulations were prepared with 30 μg of pGFP and the concentrated NPs were collected for characterization and studied after concentrating and washing, with final volume of 1 mL. The mean size and zeta potential of these different formulations are shown in Table 1. Pp6p, pp4p and pp2p have similar mean size and zeta potentials around 165 nm and + 30 mV, respectively. Pp0p (ie. PLGA-PEG) NPs present a mean size around 150 nm and negative zeta potential at around −15 mV. 6p, the NPs prepared with 6 mg PBAE without PLGA-PEG, present a smaller mean size at about 40 nm and a positive charge of about +38 mV. The increasing amount of PBAE does not significantly increase the zeta potential of PLGA-PEG/PBAE/pGFP NPs probably due to the charge shielding effect of the PEG layer.

### 3.2. Encapsulation efficiency

Encapsulation efficiency plays an important role in evaluating the NP formulations, especially in terms of their performance in transfection experiments. The encapsulation efficiency of different NP formulations was determined by measuring the unentrapped pGFP using Picogreen dsDNA quantification kits. After association with PBAE, the resulting PLGA-PEG NPs exhibited obvious entrapment of pGFP (up to 97%). In contrast, the PLGA-PEG NPs without PBAE were only able to encapsulate ~3% of the pGFP. These results demonstrate the utility of PBAE in the PLGA-PEG formulation, as it provides cationic charge and dramatically improves the entrapment of pGFP within PBAE/pGFP nanocomplexes inside PLGA-PEG NPs. It was notable that by increasing the amount of PBAE up to 6 mg, the pGFP encapsulation efficiency reached 94 ± 3%. Lowering the amount of PBAE (keeping pGFP and PLGA-PEG content constant) lowered the pGFP encapsulation efficiency. For example, the formulations of pp4p and pp2p had 62 ± 6% and 18 ± 2% encapsulation efficiency, respectively (Fig. 2A). In fact, PBAE plays a major role in complexing with pGFP by providing positive charges, thus changing the amount of PBAE will affect the complexation and encapsulation efficiency. However, the PLGA-PEG also plays a major function to improve the pGFP encapsulation efficiency. The 6p group, which was prepared by directly mixing 6 mg PBAE with pGFP without PLGA-PEG involved showed only 38 ± 6% encapsulation efficiency. Compared with pp6p and 6p, the results showed that the association of PLGA-PEG polymeric carriers also help encapsulate pGFP inside NPs and improve the encapsulation efficiency even though PLGA-PEG in general presents neutral or negative charges. A reasonable

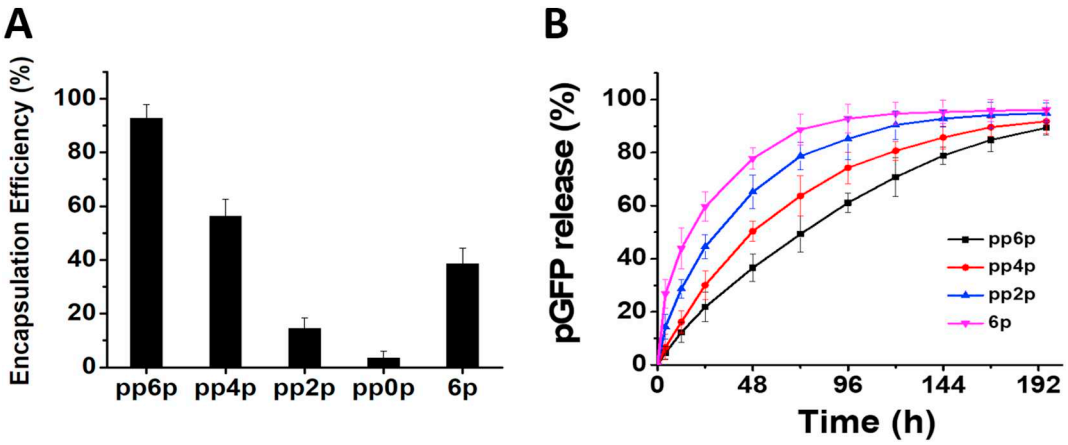


**Fig. 2.** Encapsulation efficiency and release profile of NPs. (A) The pGFP encapsulation efficiency (EE%) of different NP formulations. With the amount of PBAE increasing from 0 mg to 6 mg, the EE % increased dramatically from 5% up to 97%. (B) In vitro cumulative release profile of pGFP from different NP formulations of PLGA-PEG/PBAE/pGFP.

explanation of the improved encapsulation efficiency of pp6p over the 6p formulation is that the PLGA-PEG (which the 6p condition lacks) provides a polymeric matrix to encapsulate and maintain the PBAE/pGFP nanocomplexes inside the NPs.

### 3.3. Sustained release of pGFP from PLGA-PEG/PBAE NPs

One of the main advantages of NP delivery systems which incorporate PLGA is the sustained release of encapsulated payload, which is usually governed by diffusion and degradation processes [34]. Additionally, PBAE, apart from being a cationic biodegradable polymer not only provides efficient binding with negatively charged pGFP, but also releases pGFP during its degradation [30]. Therefore, the combination of PLGA-PEG and PBAE endows the NP delivery system with both high encapsulation efficiency and sustained gene release capability.

The amount of the pGFP released from PLGA-PEG/PBAE/pGFP NPs was determined by PicoGreen assay which is an ultrasensitive fluorescent nucleic acid stain for double-stranded DNA. The cumulative release profiles of different PLGA-PEG/PBAE/pGFP NP formulations (pp6p, pp4p and pp2p) and NPs containing only PBAE (6p) as positive control were shown in Fig. 2B. The 6p formulation very quickly released within the first 48 h and reached a maximum value of 97% over 5 days. Compared with 6p, the PLGA-PEG/PBAE/pGFP NPs have an obvious sustained release characteristic, especially the pp6p formulation. Increasing the amount of PBAE was shown to prolong the sustained release behavior. The most effective pp6p NP formulation showed that 80% of the total loaded pGFP was released at 144 h and reached a maximum value of 93% release after 10 days. These results clearly indicate that pGFP displays prolonged release from PLGA-PEG/PBAE NPs.

### 3.4. In vitro transfection efficacy

To optimize NP transfection to the cells, we selected the pp6p and 6p formulations to perform experiments. The pp6p formulation was chosen because it displayed the highest pGFP encapsulation and we compared with the 6p formulation as positive control as it does not contain PLGA-PEG. The NP transfection experiments were all performed in serum (culture medium supplied with 10% FBS) using Hek 293 cell line and adding either 0.28 mg (20 μL), 0.7 mg (50 μL), 1 mg (70 μL) or 1.4 mg (100 μL) of 1 mL concentrated pp6p NP formulation in 12-well cell culture plates. Considering pGFP encapsulation efficiency, the amount of pGFP in each well for each group mentioned above was around 0.58 μg, 1.45 μg, 2.03 μg and 2.9 μg, respectively. After adding NPs and incubating cells for 4 h, the transfection medium containing NPs was replaced by fresh complete culture medium which meant all the following transfection effects were from the cellular uptake of the pGFP loaded NPs. The 0.7 mg/mL NPs (1.45 μg pGFP in each 12-well plate) was the best performing dosage with high transfection and less cytotoxicity than the other groups. As shown in Fig. 3A, the NPs have an obvious sustained GFP expression over 4 days using GFP as the reporter gene. In the first 24 h, 16.3 ± 2.5% of the cells were transfected, which increased to 45.2 ± 4.2% after 48 h, 70.7 ± 2.6% after 72 h and 83.2 ± 4.2% after 96 h, as measured by analysis using Image J software. The GFP signal expressed by cells was generally increasing day by day and the cells presented an obvious sustained fluorescence signal with up to 87% cells transfected within 4 days after the NP uptake which shows that the pGFP was continually released out from the PLGA-PEG/PBAE/pGFP NPs to transfect cells.

In order to display the advantages of this new PLGA-PEG/PBAE/pGFP NP formulation, a positive control experiment was performed using the same amount of PBAE (6 mg) without PLGA-PEG polymeric matrix (6p). As shown in Fig. 3B, the 6p formulation still had an obvious sustained transfection between 24 h and 48 h with 5.3 ± 1.8% and 17.6 ± 2.5% cells transfected, respectively. But after 48 h, there was no obvious difference between the fluorescence transfected cells at the sequencing timepoints with 22.4 ± 2.8% transfected at 72 h and 24.3 ± 3.5% transfected at 96 h. We believe this shows that almost all the pGFP has been released from the cellular internalized PBAE/pGFP nanocomplexes within the first 48 h. As such, we did not observe much enhanced GFP expression after 48 h. Additionally, the transfection experiment results showed that differing amounts of PBAE in the NPs show differing transfection profiles due to their varying pGFP encapsulation efficiency as shown in Figs. S2 and S3 in supplementary information. Furthermore, a transfection experiment using the commercial reagent lipofectamine 2000 as control was also shown in Fig. S1, however, the lipofectamine 2000 did not show sustained release behavior. Interestingly, in our findings, the PLGA-PEG polymeric nanocarriers not only helped to enhance pGFP encapsulation over PBAE alone, but also promoted sustained release behavior of the gene payload.

### 3.5. Cytotoxicity

The in vitro cytotoxicity of different formations of PLGA-PEG/PBAE/pGFP NPs (pp6p, pp4p, pp2p and pp0p) was evaluated by XTT assay in Hek 293 cells. The cytotoxicity of NPs at various concentrations on Hek 293 cells over 4 days is shown in Fig. 4. PBAE, which was reported in previous studies [26], was evaluated as positive control (6p)

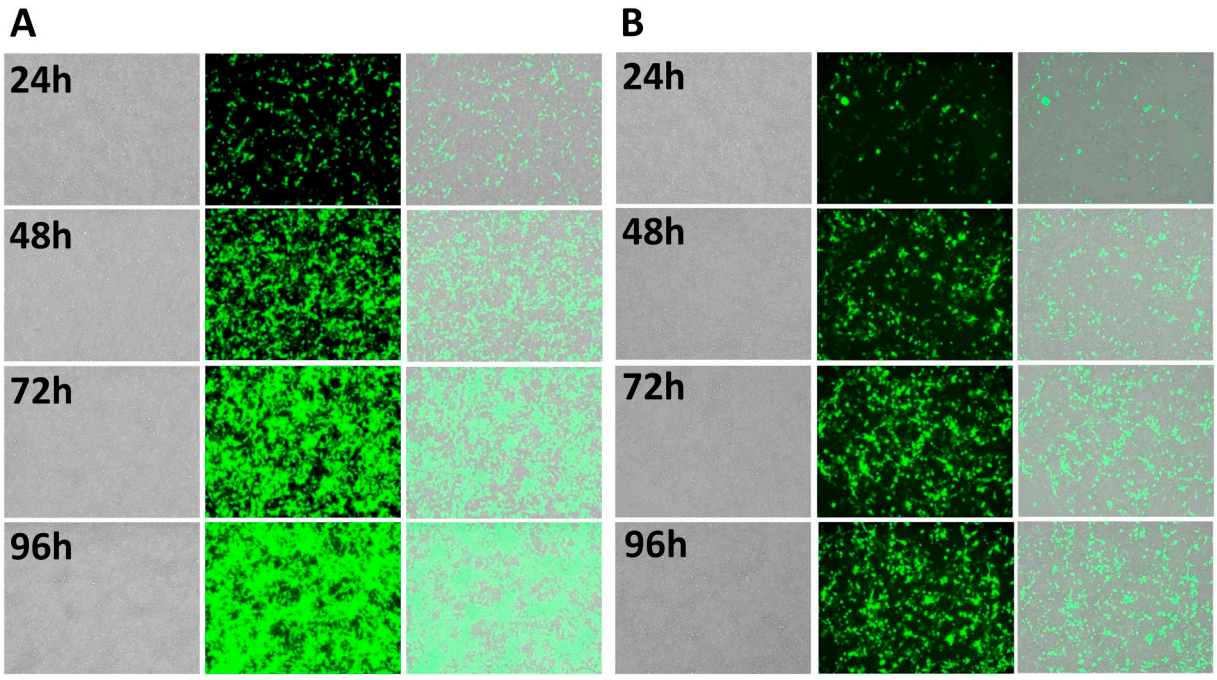


**Fig. 3.** Comparison of fluorescence and brightfield images between pGFP transfected cells. (A) Transfection with pp6p formulation and (B) transfection with 6p formulation over 96 h. Bright field (left), fluorescent (middle) and merged (right).

and the untreated group was analyzed as a blank control. As illustrated in Fig. 4, the cell viabilities decreased with the increased concentration of PLGA-PEG/PBAE/pGFP NPs while differing amounts of PBAE did not affect the cytotoxicity of NPs in lateral comparison at the studied concentrations (0.7–1.4 mg/mL NPs) over 3 days. However, at day 4, the cell viability dropped dramatically, which was likely due to the lack of nutrition in the media. It is notable that 0.7 mg/mL of NPs was the best dosage for transfection while maintaining excellent cell viability. Moreover, pp0p, which can be considered empty PLGA-PEG nanoparticle carriers and 6p representing solely the cationic polymer PBAE, both presented outstanding cell viability and indicates that the combination of PLGA-PEG and PBAE will create a promising, safe and

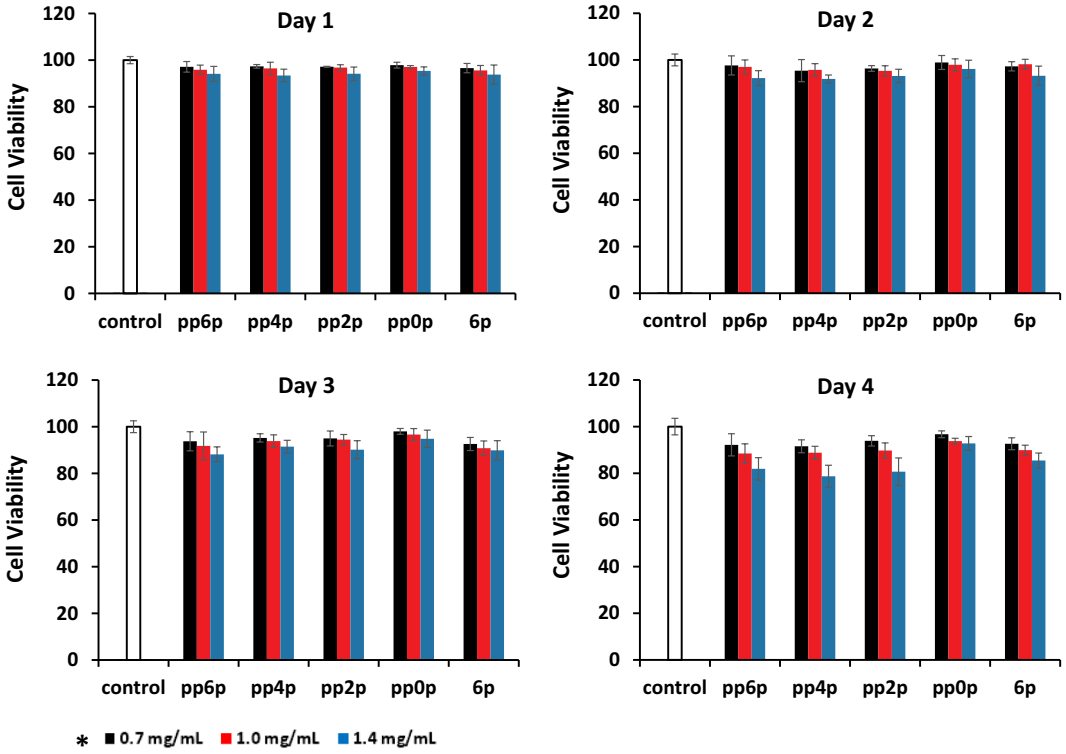


**Fig. 4.** Cell viability of PLGA-PEG/PBAE/pGFP NPs formulations on the Hek 293 cell line. Measurements were carried out by XTT assay with comparison to PBAE/pGFP nanocomplex control (6p). Values of XTT assay are given in percentages and normalized to blank control as 100%.

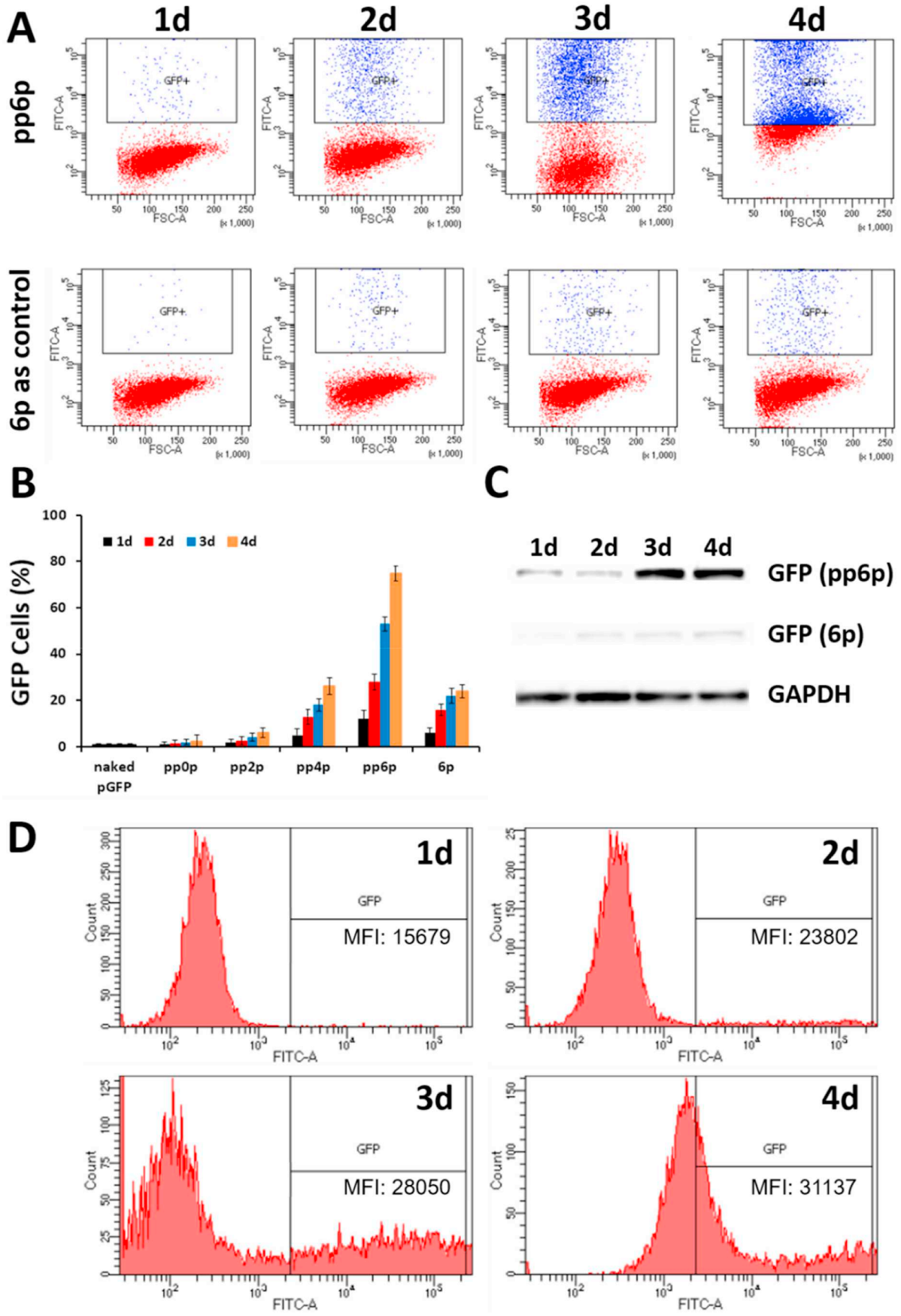


**Fig. 5.** Flow cytometry and Western blot analysis. (A) Flow cytometry of Hek 293 cells transfected with PLGA-PEG/PBAE/pGFP NPs (top row) and cells transfected with PBAE/pGFP as positive control (second row) over 4 days. (B) Quantitative summary of flow cytometry data of GFP expressing Hek 293 cells using different NP formulations over 4 days. (C) Western blot of GFP expression in Hek 293 cells transfected with pp6p and 6p formulations in comparison with GAPDH as loading control over 4 days. (D) Mean fluorescence intensity of the GFP positive cell population using pp6p formulation. (MFI: Mean Fluorescence Intensity).

efficient NP system for sustained gene delivery.

### 3.6. Flow cytometric analysis and Western blot

The transfected cells were analyzed quantitatively using flow cytometric analysis with 488 nm laser and 10,000 total events were used for detection of GFP positive transfected cells. The gate was set as "GFP+" representing the population of GFP expressing cells. Using our PLGA-PEG/PBAE/pGFP formulation in Fig. 5A (top row), it can clearly be shown that the GFP expressing cells increased over 4 days in comparison with PBAE/pGFP nanocomplex control as shown in Fig. 5A (second row). Fig. 5B shows the quantitative summary of the percentage of GFP expressing cells for each individual NP formulation measured by flow cytometry. Comparing with pp6p and 6p, our PLGA-PEG/PBAE/pGFP NPs showed efficient transfection ability and obvious sustained release behavior over the 6p control group within 4 days and other formulations such as pp4p, pp2p and pp0p. The percentage of GFP positive cells reached up to 78% using pp6p formulation in the fourth day which was similar to the data correlated with the fluorescent transfection images in Fig. 3.

Additionally, GFP expression in Hek 293 cells was also confirmed at the protein level by Western blot. Protein extracts were analyzed by SDS-PAGE and detected using anti-GFP, with anti-GAPDH antibody as a loading control. As shown in Fig. 5C, in the first two days, the GFP bands did not show clearly as the cells had not expressed GFP in sufficient levels. In contrast, at day 3 and day 4, the GFP bands were clear and distinctly increasing, indicating the cells were successfully transfected by sustained release of pGFP via PLGA-PEG/PBAE NPs. The result of the Western blot clearly demonstrates our PLGA-PEG/PBAE/pGFP NPs have sustained release of the pGFP, which in turn was expressed in high enough levels on the third and fourth day to be clearly detectable on the Western blot. This observation is consistent with our cellular transfection fluorescence imaging and flow cytometry results.

## 4. Conclusions

In summary, we reported the design of a PLGA-PEG/PBAE NP platform capable of high loading and sustained release of GFP plasmids and it proved feasible as a non-viral vector for efficient and sustained gene delivery. The PLGA-PEG NPs act as a depot for pGFP/cationic polymer nanocomplexes. Using PBAE as a model cationic polymer and GFP plasmid (pGFP) as a model nucleic acid, we have demonstrated that pGFP forms a complex with cationic PBAE through electrostatic interactions and was encapsulated in PLGA-PEG polymeric nanocarriers. Compared with PBAE alone, the PLGA-PEG/PBAE NPs showed versatility in not only sustained release of pGFP for 4 days with up to 87% cells transfected in serum medium, but also enhanced the pGFP encapsulation efficiency up to 97%, simultaneously displaying small size (165 nm) and minimal cytotoxicity. Additionally, our PLGA-PEG/PBAE NPs also show superior gene delivery and transfection efficiency over commercially available lipofectamine transfection regents. These findings suggest that our novel nano-delivery platform design is a promising non-viral gene delivery system with prolonged gene release characteristics, which may be used in gene therapy with potential clinical translation. Moreover, the PLGA-PEG component allows for further modifications (such as targeting ligands, responsive molecules) on PLGA-PEG polymeric carriers, which will make PLGA-PEG/PBAE NPs a versatile nucleic delivery system, not only with high encapsulation efficiency, transfection efficacy and sustained gene release behavior, but also the ability to target specific organs/cells with stimuli-responsive characteristics. Additionally, this combinatorial approach can be easily applied to other cationic polymers and nucleic acid-based therapeutics. For example, this platform could be further modified and deliver other gene payloads such as the gRNA-Cas9-GFP all-in-one PX458 vector plasmid for sustained gene editing. Although preliminary, the results of this work demonstrate the great potential of our PLGA-PEG/cationic polymer NPs platform to overcome the limitations of current viral and non-viral vectors and provide a promising approach for sustained gene delivery.

## Credit author statement

X.X., X.-Q.Z. and Z.L. conceived the idea and designed the research; Z.L., F.L. and Y.-J.C. performed research; X.-Q.Z. and X.B. provided material support; Z.L., W.H., X.X. and X.-Q.Z. wrote the manuscript.

## Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2020.03.021.

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