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Degradable poly(amino ester) based on poly(ethylene glycol) dimethacrylate and polyethylenimine as a gene carrier: molecular weight of PEI affects transfection efficiency

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Abstract The aim of the research is to study the effect of polyethylenimine (PEI) molecular weight on the gene transfection efficiency of degradable poly(amino ester) based on poly(ethylene glycol) dimethacrylate (PEGDMA) and polyethylenimine (PEG-*cr*-PEI) as a gene carrier. Various low molecular weight (LMW) branched PEI based PEG-*cr*-PEI was synthesized via Michael addition. The degradation half-life of PEG-*cr*-PEI was longer at pH 5.6 than that at pH 7.4. The plasmid condensation and protection ability of the PEG-*cr*-PEI were confirmed by agarose gel electrophoresis assay. PEG-*cr*-PEI/DNA nanoparticles showed high positive zeta potential (>+20 mV), narrow size distribution, and spherical shapes with size below 250 nm when N/P ratios of PEG-*cr*-PEI to DNA were above 10, suggesting that they have endocytosis potential. The cytotoxicity of PEG-*cr*-PEI/DNA complexes was lower than that of PEI 25K/DNA complexes, and the transfections mediated by PEG-*cr*-PEI were checked in 293T, HeLa and HepG2 cell lines. The report gene expression was increased with increasing the molecular weight of LMW PEI. The "proton sponge effect" was proposed as the mechanism of PEG-*cr*-PEI mediated gene transfection.

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1 Introduction

Gene therapy shows a promising approach to treat the intractable diseases and has been gained increasing consideration to treat human diseases directly in recent years [1]. However, the considerable difficulty for gene therapy is gene delivery since gene can be easily degraded by nuclear enzyme, and difficult to be internalized by cells due to its polyanionic nature. Therefore, it is a prerequisite to deliver therapy gene efficiently and safely to target positions. In this aspect, gene carrier system is one of most important parameters in gene therapy [2].

As gene carriers, viruses have been widely investigated due to their high gene transduction potential. Viral carriers have also been used in most of clinical gene therapy in recent years [3]. In China, recombinant adenoviruses-p53 anticancer injection has already been approved by government to treat head and neck squamous cell carcinoma in clinic [4]. However, the clinical security of viral carriers is always being oppugned due to their immune response against repeated administration, oncogenicity, and random DNA insertion [5, 6]. Therefore, the use of polymer-based gene carriers in gene therapy has been increasingly proposed as safer alternatives to viral carriers because of potential advantages such as low immune response, easy of synthesis and unrestricted plasmid size [7–12]. Both

natural and synthetic polycations, such as chitosan [13], methoxy poly(ethylene glycol)-modified galactosylated chitosan [14], poly(amido amine) dendrimer [15, 16], poly(L-lysine) (PLL) [17, 18], and polyethylenimine (PEI) [19–24], have been widely used as gene carriers. The nucleic acid materials can be delivered, and protected from enzymatic degradation in the form of nanoparticles [14], facilitating the cell uptake and endolysosomal escape [25].

Of all the natural and synthetic polycations, PEI is one of the most effective gene carriers in vitro and in vivo because of its enhanced "proton sponge effect" in endolysosome [23, 24]. However, non-degradability and high charge density of PEI result in high toxicity, limiting its clinical application in gene therapy. It is also generally believed that high molecular weight PEI shows relatively high gene transfection efficiency along with high toxicity [26, 27]. To improve the gene transfection efficiency while maintaining the proton sponge capacity and low toxic nature, various degradable polycations based on LMW PEI have been investigated as non-viral gene carriers [28–32]. Pack group synthesized degradable PEI derivative through Michael addition of PEI 0.8K to small molecular diol diacrylate. The PEI derivative showed 2- to 16-fold greater gene expression than that of PEI 25K [28]. By using the same strategy, Park et al. synthesized degradable polycation based on PEI 423 and poly(ethylene glycol) diacrylate (PEGDA). Results showed that the transfection was decreased with increasing PEG molecular weight [29]. In correlation to that, our approach is to screen degradable gene carriers based on PEG dimethacrylate (PEGDMA550, MW: 550) because PEG dimethacrylate is more hydrophobic than PEG diacrylate and PEIs with different molecular weights (MW: 0.6, 1.2 and 1.8 K). Herein, PEG-*cr*-PEI was synthesized *via* Michael addition reaction. The physiochemical properties of PEG-*cr*-PEI as a gene carrier were characterized. Especially, the molecular weights of parent PEI in PEG-*cr*-PEI on the gene transfection efficiency were detailed.

2 Materials and methods

2.1 Materials

PEG dimethacrylate (PEGDMA, MW: 550), branched PEIs (MW: 0.6, 1.2, 1.8 and 25 K), anhydrous dichloromethane, dimethyl sulfoxide (DMSO), agarose, ethidium bromide (EtBr), bafilomycin A1 and calf thymus DNA were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from GIBCOBRL-Life Technologies (Paris, France). Cell Titer 96 Aqueous One Solution Cell Proliferation Kit (MTS) for cell viability, Luciferase Reporter 1000 Assay System for in vitro transfection assay and pGL3-control vector with SV-40 promoter and enhancer encoding firefly (*Photinus pyralis*) luciferase were obtained from Promega (Madison, WI, USA). Plasmids were amplified with a competent *Escherichia coli* bacterial strain DH5α and their purification was performed using a QIAGEN (Chatsworth, CA, USA) kit. The concentration and purity of plasmid were determined by measuring UV absorbance at 260 and 280 nm, respectively. All samples showing an A_{260}/A_{280} ratio of 1.9–2.0 were stored at −20°C before use.

2.2 Cell lines and culture

HeLa (human cervix epithelial carcinoma cells), 293T (human kidney cells), and HepG2 (human hepatoblastoma cells) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Paris, France), supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, Utah), streptomycin at 100 μg/ml, and penicillin at 100 U/ml. All cells were incubated at 37°C in humidified 5% CO_2 atmosphere. Cells were split by using trypsin/EDTA solution when almost confluent.

2.3 Preparation of PEG-*cr*-PEI

PEG-*cr*-PEI was synthesized by Michael addition reaction [33, 34]. In a typical reaction procedure, branched PEIs (MW: 0.6, 1.2 and 1.8 K) and PEGDMA (MW: 550) were separately dissolved in anhydrous dichloromethane (10 ml) in three different molar ratios as 1:1, 2:1 and 4:1 of PEI to PEGDMA, then mixed, and maintained at room temperature under dark with constant stirring for 20 h. After the occurrence of gel, the dichloromethane was removed in vacuum at room temperature. The residue was thoroughly mixed with 40 ml of ice cold water by vortexing, the little water-insoluble gel was removed by filtration, and the filtrate was dialyzed using Spectra/Pro membrane (MWCO = 3500) against deionized water at 4°C for 24 h. The dialyzed solution was lyophilized, and the solid was stored at −20°C.

2.4 Characterization of PEG-*cr*-PEI

The compositions of PEG-*cr*-PEI were estimated by ^{1}H-nuclear magnetic resonance (^{1}H NMR) (Avance™ 500, Bruker, Germany). For NMR measurement, the sample concentration in D_2O was 10 mg/ml. The molecular weights of samples were measured by gel permeation chromatography (GPC) (Agilent 1200, Agilent Technologies Inc.). Agilent 1200 refractive index detector and aqueous GPC start-up kit were used. Chromatography column (PL aquagel–OH 30, 8 μm, Polymer Laboratories Ltd., Amherst, MA, USA) was calibrated with poly(ethylene glycol) standard samples

(MW: 2–20 kDa). The column temperature was maintained at 25°C. Mobile phase was 0.2 M $NaNO_3$ and 0.01 M NaH_2PO_4 (pH 7.0), and the flow rate was 1 ml/min.

2.5 Degradation of copolymer

Degradation of copolymer in different pH buffer was characterized by measurement of molecular weight of the degraded copolymer. Briefly, copolymers were dissolved in different buffer solution (0.5 g/ml) at pH 5.6 (0.2 M HAc/NaAc) and 7.4 (0.2 M PBS), respectively, and incubated at 37°C with constant shaking at 100 rpm. The molecular weight of the degraded copolymer was measured by GPC at an appropriate time intervals.

2.6 DNA condensation ability assay

To confirm DNA condensation ability of the copolymer, electrophoresis was performed. Polymer/DNA complexes with various N/P ratios from 0.5 to 20 were prepared freshly before use by mixing the pGL3-control (0.1 μg/well) and polymer solution. The complexes were incubated at room temperature for 30 min and then followed by addition of 2 μl of 6 × agarose loading dye mixture (Biosesang, Korea). After further incubation for 10 min, the mixture solution was loaded onto 0.8% agarose gel with EtBr (0.1 μg/ml) and run with Tris–acetate–EDTA (TAE) buffer at 100 V for 30 min. The gel was analyzed on UV illuminator to show the location of the DNA.

2.7 Protection and release assay of DNA

Protection and release of DNA in complexes were evaluated by agarose gel electrophoresis according to the similar method previously described by Gebhart et al. [35]. Briefly, 2 μl of polymer/DNA complexes with N/P ratio of 10 (pGL3-control, 0.1 μg) were separately incubated with or without (as a control) 1 μl of DNase-I (1 unit) in DNase/Mg^{2+} digestion buffer (50 mM Tris–Cl, pH 7.6 and 10 mM $MgCl_2$) at 37°C with shaking at 100 rpm for 120 min. For DNase inactivation and DNA release, all samples were treated with 4 μl of 250 mM EDTA for 10 min and mixed with 8 μl of sodium dodecyl sulfate (SDS) dissolved in 0.1 M NaOH (pH 7.2). Finally, samples were incubated at room temperature for 2 h and then followed by addition of 3 μl of 6× agarose loading dye mixture (Biosesang, Korea). After further incubation for 10 min, the mixture solutions were loaded onto 0.8% agarose gel with EtBr (0.1 μg/ml) and run with TAE buffer at 100 V for 30 min. The gel was analyzed on UV illuminator to show the location of the DNA.

2.8 Particle size and zeta potential measurement

The zeta potential, particle sizes and size distribution of polymer/DNA complexes were measured by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd., UK). Polymer/DNA complexes were prepared in water at N/P ratios of 5, 10, 15, 20 and 30, respectively, and allowed to be incubated at room temperature for at least 25 min. The volume of each sample was 2 ml containing 80 μg of calf thymus DNA. Each sample was measured three times and data were fit using Gaussian intensity distribution.

2.9 Morphology observation with energy-filtering transmission electron microscopy

For energy-filtering transmission electron microscopy (EF-TEM) measurement, the final concentration of DNA in complex solution was 20 μg/ml. A 10 μl of polymer/DNA complexes with N/P ratio 10 was carefully dropped onto clean copper grids and negatively stained with 1.5 wt% phosphotungstic acid (pH 7.4) for 5 s. The copper grids surface was dried at room temperature for 5 min before observation on EF-TEM (LIBRA 120, Carl Zeiss, Germany).

2.10 Cell viability assay

In vitro the cell viability study was evaluated by MTT proliferation kit as described by the supplier. In brief, cells were seeded in 96 well plate at an initial density of 1×10^4 (HeLa and 293T) or 2×10^4 (HepG2) cells/well in 200 μl growth medium and incubated for 18–20 h to reach 80% confluency at the time of treatment. Cells were transfected with various N/P ratios of complexes and further incubated for 48 h, then 5 mg/ml MTT solution were added, and incubation for 2–4 h, the absorbance was measured at 570 nm, using an ELISA plate reader (GLR 1000, Genelabs Diagnostics, Singapore). Each dosage was tested in triplicate.

2.11 In vitro cell transfection

2.11.1 Luciferase activity assay

Cells were seeded in 24 well plate at an initial density of 1×10^5 (HeLa and 293T) or 2×10^5 (HepG2) cells per well in 1 ml growth medium and incubated for 18–20 h to reach 70–80% confluency at the time of transfection. The medium was replaced with 500 μl serum-free media with polymer/pGL3-control (1 μg) complexes at various N/P ratios (5, 10, 20 and 30) and additionally incubated for 6 h. Then, the media were changed with fresh media containing

serum and allowed to incubate for 24 h. The luciferase assay was performed according to manufacture's protocols. Relative light units (RLUs) were measured with a chemiluminometer (Autolumat LB953, EG and G, Berthold, Germany). Protein quantification was determined by the BCA method, and RLUs were normalized to protein concentration in the cell extracts [36]. Each transfection experiment was carried out in triplicate, and transfection activity was expressed as relative light units.

2.11.2 Serum resistant ability study

The stability of the C1/DNA complexes in the serum was investigated by measuring the turbidity of the complexes using a UV–vis spectrophotometer (Uvikon923, Kontron Instruments, Italy) at 500 nm. The method used was reported by Park et al. [37]. Briefly, C1/DNA complexes at N/P 10 were prepared and diluted with water or 10% serum, then incubated at 37°C. The turbidity was evaluated for 4 days.

The transfection efficiency of C1/DNA complexes to the HeLa cells in the presence or absence of serum was also studied. C1/pGL3-control (1 μg) complexes at N/P 10, 20 and 30 were diluted in 10% serum-containing media or serum free medium, respectively, then transfected to the HeLa cells. The luciferase assay was carried out as mentioned above.

2.11.3 Transfection mechanism assay

To deduce the gene transfection mechanism of PEG-*cr*-PEI, HeLa cells were incubated with 500 μl of 200 nM bafilomycin A1 (2 μg of bafilomycin A1 was dissolved in 1 ml DMSO, then 15 ml serum-free media was added and sterilized by filtration) for 10 min before transfection with polymer/pGL3-control complexes at N/P ratios of 10 and 20.

2.12 Statistical data analysis

Statistical data analysis was performed using the Student's *t*-test with $P < 0.05$ as the level of significance.

3 Results and discussion

3.1 Preparation and characterization of PEG-*cr*-PEI

PEG-*cr*-PEI was successfully synthesized through Michael addition reaction between PEGDMA (MW: 550) and various LMW branched PEIs (MW: 0.6, 1.2 and 1.8 K) (Fig. 1 and Table 1). Nucleophilic addition easily occurred between amino groups of PEI and acrylate ones of PEGDMA due to the autocatalytic nature of mild basic amino groups. To control the crosslink density of PEG-*cr*-PEI, the reaction should be finished within 20 h. At this time point, the PEG-*cr*-PEI can be swollen in dichloromethane. After removal of dichloromethane, almost all the gel can be dissolved in ice-cold water due to its low crosslink density. The reproducibility of synthesis is good. The lyophilized copolymer based on PEI 0.6K was viscous gel, while copolymers based on PEI 1.2K or PEI 1.8K were waxy solids at room temperature. Representative ^{1}H NMR of purified PEG-*cr*-PEI was shown in Fig. 2a. The appearance of the peak at 1.95 ppm assigned to –OC(O)*CH*(CH_3)CH_2NH clearly indicated the Michael addition. Molar ratios of PEI to PEGDMA in copolymers were calculated from the integral area ratios of methylene peak at 2.68–3.02 ppm (–NHCH_2) to 3.49–3.86 ppm (–OCH_2) in ^{1}H NMR, and variable depending on the feed molar ratios. As shown in Table 1, A3, B2 and C1 had almost equal molar percents of –CH_2CH_2N– repeating units, they were selected for further studies as gene carriers.

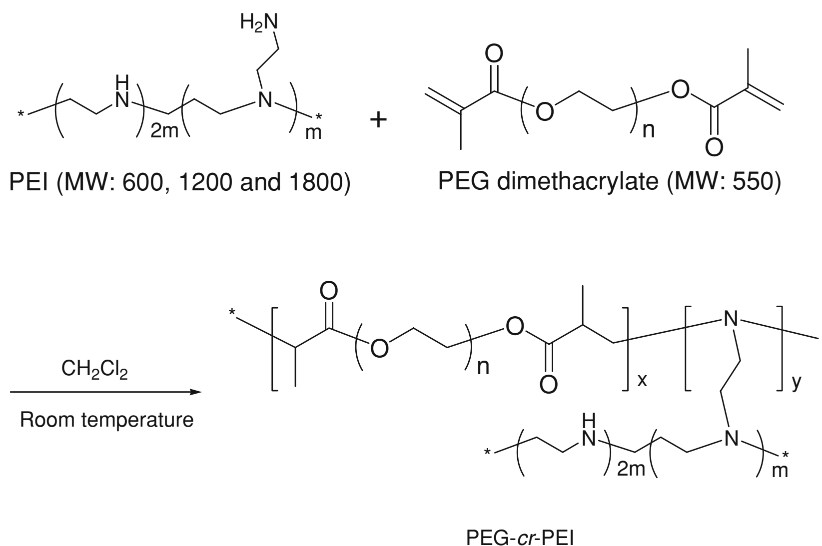

Fig. 1 The synthesis scheme of PEG-*cr*-PEI

Table 1 Characteristics of prepared PEG-*cr*-PEI

Sample No.	MW of PEI (kDa)	Feed ratio of PEI to PEGDMA	Molar ratio of PEI to PEGDMA[a]	Yield of copolymer (%)	MW of copolymers ($MW \times 10^{-4}$)[b]
A1	0.6	1:1	1.0:1	56	0.9
A2	0.6	2:1	1.5:1	49	0.8
A3	0.6	4:1	3.6:1	35	1.1
B1	1.2	1:1	0.9:1	55	1.4
B2	1.2	2:1	1.6:1	31	1.5
B3	1.2	4:1	3.1:1	42	1.3
C1	1.8	1:1	1.0:1	47	1.6
C2	1.8	2:1	1.6:1	39	1.3
C3	1.8	4:1	3.9:1	33	1.4

[a] Determined by ^{1}H NMR (Avance™ 500, Bruker, Germany, D_2O)

[b] Determined by Agilent 1200 GPC (column temperature: 25°C; mobile phase: 0.2 M $NaNO_3$ and 0.01 M NaH_2PO_4 (pH 7.0); flow rate: 1 ml/min, detector: refractive index detector). PEG was used as a standard sample

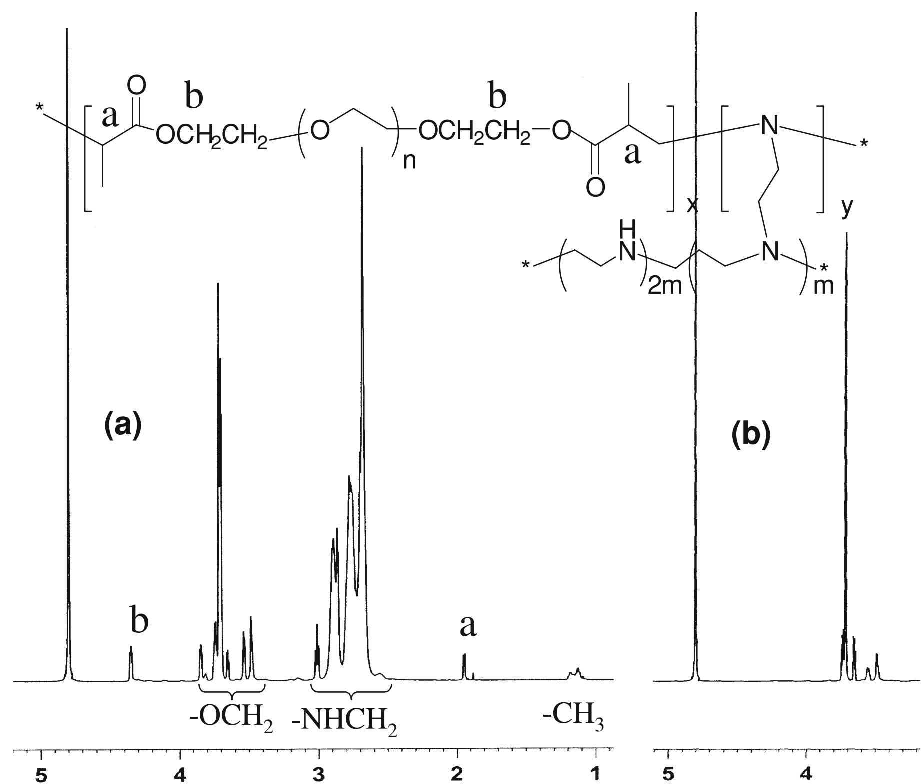

Fig. 2 (a) ^{1}H NMR spectrum of A3 in D_2O: 1.13–1.19 (–CH_3); 1.95 (OCO*CH*(CH_3)CH_2NH); 2.68–3.02 (–NH*CH_2*); 3.49–3.86 (–O*CH_2*) and 4.35–4.36 (COO*CH_2*); (b) ^{1}H NMR spectrum of A3 after degradation in PBS at 37°C for 18 days. The signal at 4.35–4.36 ppm assigned to ester bonds (COO*CH_2*) disappeared

It was noteworthy that the molecular weight of PEG-*cr*-PEI measured by GPC tended to decrease with increasing the feed molar ratio of PEI to PEGDMA from 1:1 to 4:1, just similar to the results reported by Langer group [33, 34]. The molecular weights of all the PEG-*cr*-PEI reached around 1×10^4, which were high enough for polycations as gene carriers.

As an ideal gene carrier, it is expected that the cationic polymer is stable in serum, whereas acid-catalytic degradation occurs in lysosome, which facilitates strong DNA condensation capacity outside cells and good DNA unpackage ability inside cells, leading to improvement of gene transfection efficiency as well as reduction of cytotoxicity. PEG-*cr*-PEI was designed to be degraded by acid catalyzation due to the ester bonds. Degradation curves of copolymer A3, B2 and C1 were shown in Fig. 3. It was found that the copolymers degraded rapidly at pH 7.4, especially at early stages. After 18 days, the disappearance of the signal at 4.35–4.36 ppm assigned to ester bonds (–COO*CH_2*CH_2O) clearly indicated the

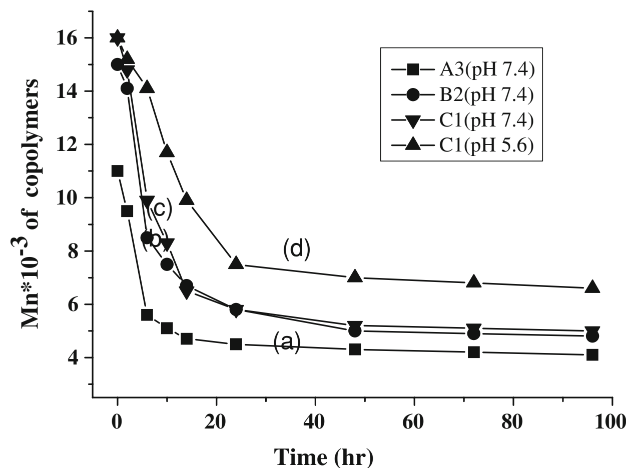

Fig. 3 Degradation curves of copolymers: (a) A3 [PEG-*cr*-PEI600] at pH 7.4 PBS; (b) B2 [PEG-*cr*-PEI1200] at pH 7.4 PBS; (c) C1 [PEG-*cr*-PEI1800] at pH 7.4 PBS and (d) C1 at pH 5.6 HAc/NaAc buffer solution

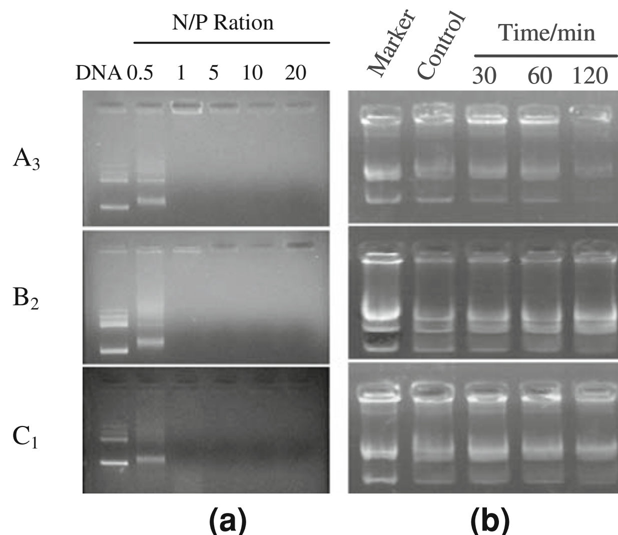

Fig. 4 (**a**) Agarose gel electrophoresis of polymer/DNA (pGL3-control) complexes at various N/P ratios; (**b**) protection and release assay of DNA. Polymer/DNA (pGL3-control) complexes at N/P ratio of 10 were treated with DNase-I enzyme for different times, and DNA were released by adding 1% SDS to the polymer/DNA complexes

complete degradation of A3 at pH 7.4 (Fig. 2b). The degradation half-life of A3 was 6.8 h at pH 7.4, B2 and C1 were 10 h, whereas the degradation half-life of C1 at pH 5.6 reached 22 h. That suggested the polymer degradation is non-acid-catalysis. This phenomena may be the reason that more free (unprotonated) amino groups are present at pH 7.4 than at pH 5.6 therefore proton from water and/or tetrahedral intermediately transfer to the free amine and act as general base catalysts in the hydrolysis reaction [38].

3.2 Characterization of copolymer/DNA complexes

As aforementioned, the DNA condensation ability is one of prerequisite for polymeric gene carriers. Polycations are able to interact with the negatively charged phosphate groups of DNA, which results in the formation of neutral polyelectrolyte complexes unable to migrate under the influence of electric field in agarose gel. As shown in Fig. 4a, when the N/P ratio of polymer/DNA complexes was around 1, the migration of DNA was completely retarded, suggesting the good DNA condensation property of PEG-*cr*-PEI.

Effective condensation is a key issue for DNA stability against degradation by nucleases [38]. Agarose gel electrophoresis assay also demonstrated that A3, B2 and C1 were able to protect DNA from enzymatic hydrolysis at N/P ratio 10, even treated with DNase-I for 2 h (Fig. 4b), indicating the introduction of PEGDMA to PEI did not affect its bind capability. Zhang et al. [14] got the same result when introducing methoxy poly(ethylene glycol) and galactose to chitosan.

Effective condensation is also expected to result in the formation of polymer/DNA nanoparticles. Their sizes are known to dramatically affect gene transfection efficiency. As shown in Fig. 5a, all the polymers condensed DNA into nano-sized complexes. The sizes of the complexes decreased sharply with increasing N/P ratios. When N/P ratio was above 10, all the particle sizes were less than 250 nm, suggestion of their effective endocytosis potential [39]. And it is thought that the sizes of complexes became smaller at N/P ratio of 10 owing to net electrostatic repulsive forces between complexes although the polymer retarded the DNA migration at N/P ratio of 1. Among A3, B2 and C1, it was ranked in the order of A3/DNA < B2/DNA < C1/DNA complexes according to their sizes at the same N/P ratios, respectively, suggesting that the DNA condensation ability of PEG-*cr*-PEI was positively correlative to the molecular weights of parent PEI. It should be noticed that the sizes of B2/DNA and C1/DNA complexes showed a tendency to increase with increasing N/P ratio above 10. It is thought that the excessive polymer aggregated on the surface of polymer/DNA nanoparticles, leading to the increased particle sizes.

The formation of polymer/DNA nanoparticles was also confirmed by the observation of their morphology. Representative EF-TEM images of C1/DNA complexes at N/P ratio 10 demonstrated the relatively homogenous complex particles with spherical shape, compact structure and good dispersity (Fig. 5b). Sizes observed from EF-TEM images

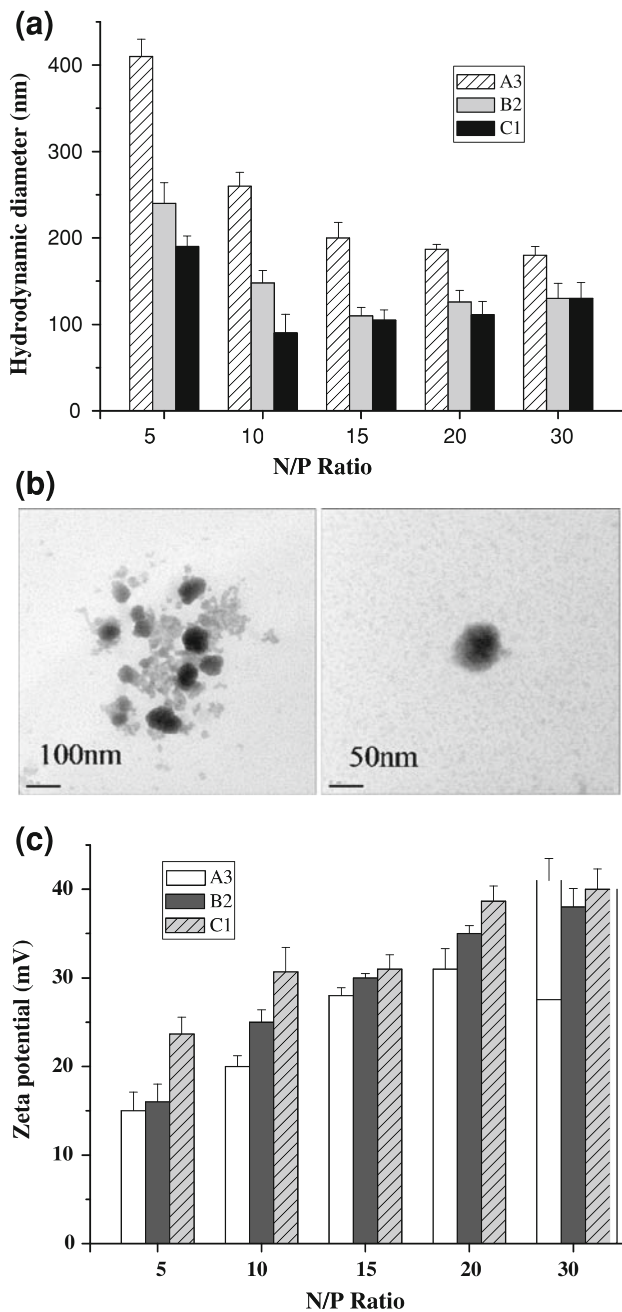

Fig. 5 (**a**) Particle sizes of polymer/DNA complexes in distilled water at various N/P ratios (mean ± SD $n = 3$), (**b**) EF-TEM images of C1/DNA complexes at N/P ratio 10 (about 88 nm), phosphotungstic acid was used as negative staining agent and (**c**) Zeta potential of polymer/DNA complexes in distilled water at various N/P ratios (mean ± SD $n = 3$)

were very similar to those measured by dynamic light scattering.

To know better the zeta potential of polycation/DNA complexes depending on N/P ratios, the zeta potentials of complexes at various N/P ratios were measured. As shown in Fig. 5c, with the increase of N/P ratios from 5 to 30, the zeta potentials of complexes rapidly increased from +15 to +40 mV. It is deemed that the positive zeta potentials of untargeted polyplexes are necessary for the attachment to anionic cell surfaces, which consequently facilitates cell uptake.

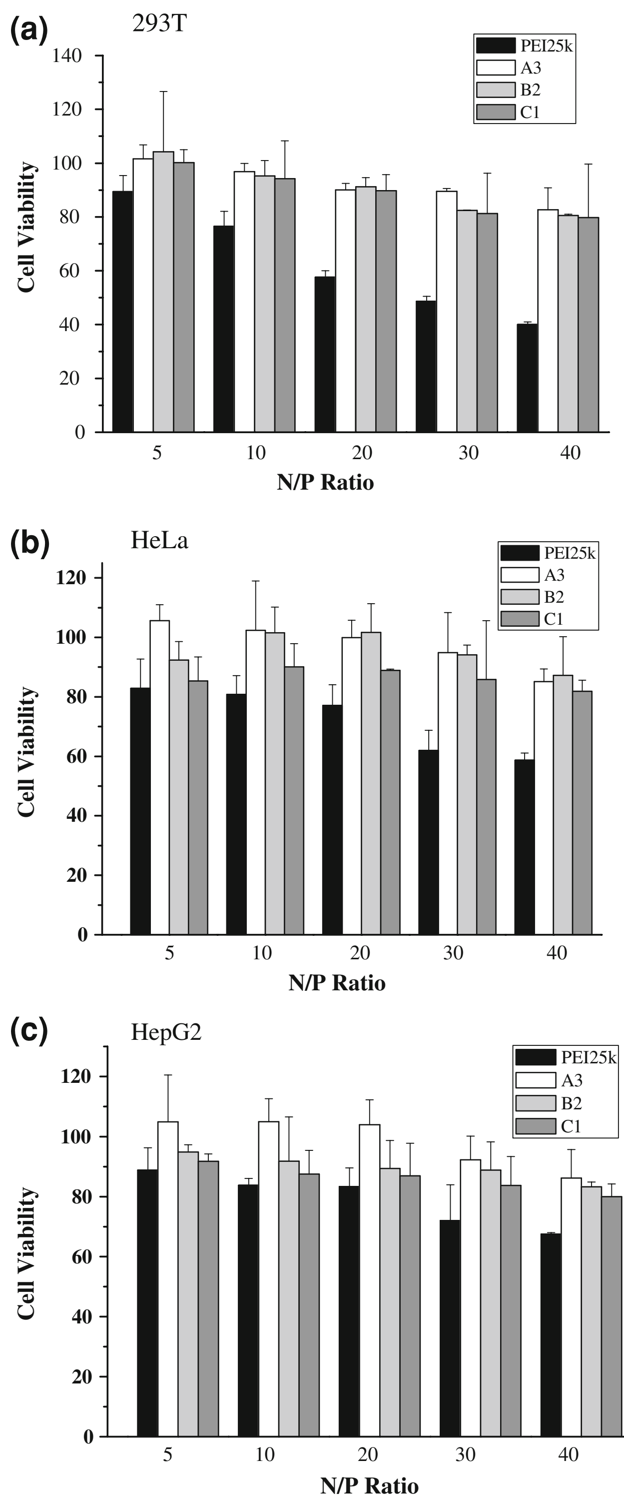

Fig. 6 Cell viability of polymer/DNA complexes at various N/P ratios in different cell lines (mean ± SD $n = 3$): (**a**) 293T, (**b**) HeLa and (**c**) HepG2

3.3 Cell viability assay

As illustrated in Fig. 6, with increasing the N/P ratio of the complexes, the cytotoxicity of complexes were increased, owing to the increased polymer composition, whereas those of polymer A3, B2 and C1 showed higher cell viability than PEI 25K at the same N/P ratio. The low cytotoxicity of A3, B2 and C1 is due to their good degradability due to the hydrolyzable ester bonds and the positive charges of the polymer/DNA nanoparticles were

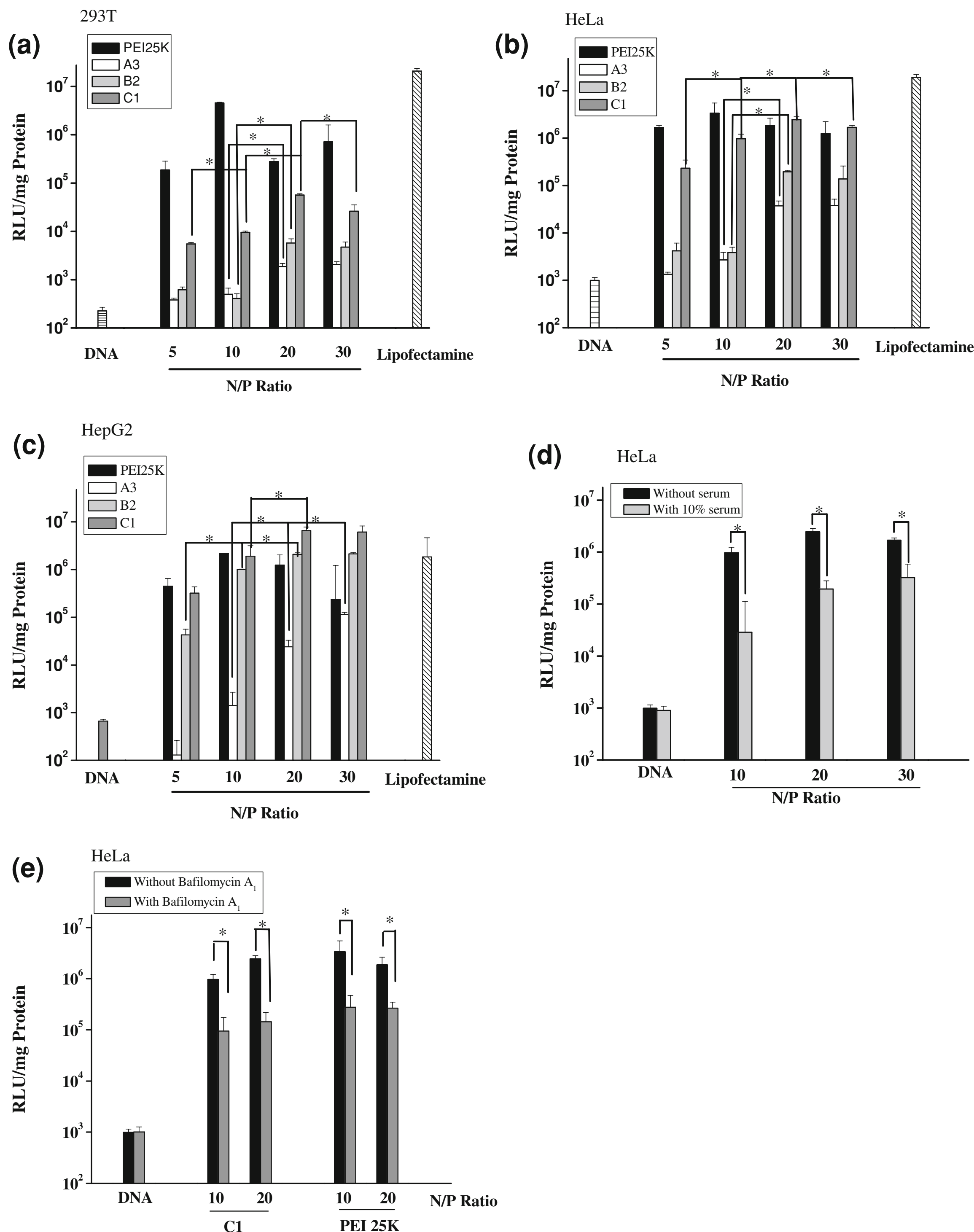

Fig. 7 Transfection efficiency of (**a**) 293T, (**b**) HeLa and (**c**) HepG2 by copolymer/pGL3-control complexes at various N/P ratios in serum-free media (mean ± SD $n = 3$); Effect of (**d**) serum and (**e**) bafilomycin A1 on gene transfection efficiency in HeLa cell line (mean ± SD, $n = 3$; $P < 0.05$)

partially shielded by nonionic PEG moiety in their molecule chains.

3.4 In vitro transfection efficiency and mechanism assay of PEG-*cr*-PEI

In vitro transfection efficiency of PEG-*cr*-PEI was shown in Fig. 7a–c. Their cell line-dependent nature was remarkable. The most efficient transfection of PEG-*cr*-PEI was higher than that of PEI 25K in HeLa or HepG2, whereas lower in 293T cells. The transfection efficiency of B2 and C1 increased with increasing the N/P ratios from 5 to 20, due to the increased surface charge of polymer/DNA complexes. Accordingly, the interaction between the complexes and the cell membrane will be increased, which might trigger more endocytosis and ultimately affect the gene expression [26]. Then a decline was observed due to the increased cytotoxicity resulted from the superfluous polycations [40]. However, the transfection efficiency of A3 showed a tendency to increase monotonously with the increase of N/P ratios from 5 to 30. It is thought that the different optimum N/P ratios in the transfection of polymer/DNA complexes were associated with the difference of cytotoxicity or charge density.

At the same N/P ratio, the transfection efficiency of PEG-*cr*-PEI increased with increasing the molecular weight of parent PEI in PEG-*cr*-PEI, and followed the order of A3/DNA < B2/DNA < C1/DNA complexes. As aforementioned, among A3, B2 and C1, although the cytotoxicity of A3 was the lowest one, the interaction of cell and polymer/DNA complexes is a crucial factor to gene expression of polymer/DNA complexes. It is thought that with increasing the molecular weight of parent PEI in PEG-*cr*-PEI, the charge density increased, resulting in the increase of positive surface charge of polymer/DNA nanoparticles, facilitating the attachment of complexes to the negatively charged cell surfaces along with their good gene transfection efficiency.

As we known, it is a key issue to develop gene delivery systems that are stable even in serum for in vivo use of non-viral delivery systems. Therefore transfection efficiencies in the presence of serum or without serum at different N/P ratios were evaluated. As shown in Fig. 7d, the transfection efficiency of C1/DNA complexes showed a tendency to remarkable decrease in the presence of serum. The turbidity of the C1 in the presence or absence of the serum were also measured. The values were 0.036 ± 0.009 and 0.227 ± 0.025, respectively, after 4 days incubation with or without serum.

Bafilomycin A1, as a specific inhibitor of vacuolar type proton ATPase, inhibits the endo-/lysosomal proton pump, which results in the decrease of PEI-mediated gene transfection [41]. As shown in Fig. 7e, transfection of C1/DNA complexes at N/P ratios of 10 and 20 were drastically decreased when the HeLa cells were treated with 200 nM of bafilomycin A1, just similar to that of PEI, suggesting that the mechanism of PEG-*cr*-PEI-mediated gene transfection is based on the "proton sponge effect" due to the presence of low molecular weight branched PEI.

4 Conclusions

PEG-*cr*-PEI was successfully synthesized by Michael addition and characterized as a gene carrier. The polymer shows good DNA condensation and protection ability as well as low toxicity. The high positive zeta potential and appropriate particle size and enhanced gene transfection efficiency of polymer/DNA nanoparticles demonstrated the potential use of PEG-*cr*-PEI as an effective gene carrier. In particular, the transfection efficiency of PEG-*cr*-PEI increased with increasing the molecular weight of parent PEI in PEG-*cr*-PEI.

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