Effect of PEI surface modification with PEG on cytotoxicity and transfection efficiency

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Polyethylenimine (PEI) is a cationic polymer with high transfection efficiency as non-viral gene delivery agent that exhibits promising features for gene therapy applications due to its cationic charge and thus favourable DNA-condensing abilities; however, its high cytotoxicity restricts its application. The cellular toxicity of PEI molecules depends on their structure, molecular weight and surface charge density. To improve the properties of branched 25 kDa PEI as a non-viral gene delivery agent, this polymer was conjugated to polyethylene glycol (PEG) molecules at three different molar ratios of 10, 20 and 30. The degree of PEG grafting was determined by 2, 4, 6-trinitrobenzene sulphonic acid assay. The effects of various PEGylation degrees on cellular toxicity and transfection ability of PEI polymer were assessed on BT-474 and MCF-10A cell lines. Compared to unmodified PEI, PEG-grafted PEI copolymers demonstrated reduced cytotoxicity, particularly at higher PEG grafting ratios. Among different PEG-grafted PEIs, the PEG–PEI copolymer which grafted to PEG at a molar ratio of 10:1 had the highest transfection efficiency in both cell lines. The findings of this Letter showed that these PEG-grafted PEI copolymers have desirable gene transfection efficiency and favourable biocompatibility for gene delivery.

1. Introduction: Gene therapy is a promising approach for the treatment of disorders that have been difficult to address by conventional therapeutic methods such as cancer. There are two major systems in gene delivery, viral and non-viral gene delivery system [1, 2]. Viral gene delivery vectors such as herpes simplex virus or adeno-associated virus have been studied for several years to transfer therapeutic genes into target cells due to their high transduction ability, but several restrictions have been led to limited use of viral gene delivery agents including relatively small size of transduced genetic material, safety concerns and non-specific interaction to undesirable cells [3, 4]. Non-viral gene delivery systems such as cationic polymers [5] and cationic lipids [6] have several advantages compared with viral gene carriers including low immunogenicity, ease of production and relatively fewer safety concerns [7].

Polyethylenimine (PEI) is one of the most efficient non-viral gene delivery vectors due to its high DNA-condensing ability. The high cationic surface charge of PEI can promote effective electrostatic interactions with the anionic charges of DNA molecules [8, 9]. 'Proton sponge effect' hypothesis describing that PEI/DNA complexes, when internalised into the cytoplasm via endocytosis, could promote proton and chloride influx into endosomes and leading to disruption of endosomes and release of DNA into the cytosol [10–13]. In spite of high transfection ability, application of PEI for gene delivery into target cells is restricted because of some drawbacks such as high cytotoxicity and unspecific interactions with normal cells or blood components *in vivo*, which decrease PEI/plasmid DNA (pDNA) polyplex half-life in blood circulation by fast opsonisation and entrapment in fine capillaries. Many factors such as molecular weight and composition, the degree of branching, size and zeta potential of polyplexes have been found to affect the transfection ability and cytotoxicity of PEI/DNA polyplexes [14, 15]. For example, the widely used commercial 25 kDa branched PEI, as one of the gold standards of non-viral gene delivery agents, exhibits high transfection efficiency, but it demonstrates high levels of cytotoxicity, too. In addition, PEI/pDNA polyplexes tend to aggregate under physiological conditions. Therefore, PEI has to be modified for reducing cytotoxicity and tendency toward aggregation and non-specific interaction for *in vivo* applications. Recently, investigators have tried to overcome these restrictions by physically or chemically adding biocompatible polymers such as poly (ethylene glycol) (PEG) on the surface of PEI [16–19]. Modification of PEI polymer with hydrophilic polymers such as PEG decreases undesirable aggregation due to secondary interactions between nanoparticles. Furthermore, this surface modification may reduce the recognition of the nanocarriers by blood components and other cells, and thus may lead to longer blood half-life, so increase the possibility of reaching the target site. In addition, targeting specificity of the PEI polyplexes may be further enhanced by conjugation of targeting ligands onto chemically reactive functional groups on the PEG chain. So the resulting PEG-grafted PEI is superior over the existing and unmodified cationic lipids or polymers, which are traditionally used for gene delivery such as lipofectamines.

To improve the biocompatibility of PEI polyplexes as a cancer gene delivery vector, different molar ratios of 3500 kDa *N*-hydroxysuccinimide ester–PEG–maleimide (NHS–PEG–MAL) chains to 25 kDa branched PEI and evaluate physicochemical characteristics, cytotoxicity and gene transfection efficiency of the resultant copolymers in BT-474 and MCF-10A, human breast cancer and non-cancerous cell lines, respectively.

2. Materials and methods

2.1. Materials: Branched PEI (molecular weight 25 kDa, the average degree of polymerisation 580), cysteine hydrochloride and 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). NHS–PEG_{3500}–MAL was obtained from Jenkem Technology (Beijing, China).

2.2. Cell culture: BT-474 (human breast cancer cell line) and MCF-10A (human non-tumourigenic mammary epithelial cell line) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). BT-474 cells were maintained in RPMI-1640 (Gibco/Invitrogen) supplemented with 20% foetal bovine serum (FBS) and 100 U/100 µg ml^{-1} penicillin/streptomycin. MCF-10A cell line was cultured in Dulbecco's Modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12) (1:1) (Gibco/Invitrogen) media supplemented with 5% donor horse serum, 365 µg/ml L-glutamine, 20 ng/ml epidermal growth factor, 10 µg/ml insulin,

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0.5 µg/ml hydrocortisone and 100 U/100 µg ml^{-1} penicillin/streptomycin at 37°C in a 5% carbon dioxide-humidified incubator.

2.3. Quantification of PEI surface primary amine group content: The branched PEI averagely has 25% primary amine groups, 50% secondary amine groups and 25% tertiary amine groups based on its structure. The number of primary amine groups was determined using TNBS using glycine as a reference according to standard protocol [20]. The degrees of PEG grafting were also calculated through TNBS assay by the differences in the contents of free primary amine groups remaining on PEGylated PEIs compared with unmodified PEI. Briefly, 25 µl of aqueous 0.03 M TNBS was added to 1 ml of each sample, completely mixed and then allowed to remain for 30 min at room temperature. The blank sample consisted of 25 µl of aqueous 0.03 M TNBS in 1 ml of 0.1 M borate buffer. TNBS interacts rapidly with the primary amino groups found in amino acids, peptides, proteins or other molecules. It results in the formation of yellow chromogenic derivatives. No colour products are produced with the secondary or tertiary amine groups. The amount of colour derivatives is measured at 420 nm.

2.4. Synthesis of PEG–PEI copolymers with different PEG–PEI molar ratios: PEG–PEI conjugates were synthesised by the grafting reaction between surface primary amino groups of PEI and NHS ester group of bi-functional PEG. Briefly, PEI (100 nmole) was dissolved in 2 ml of degassed reaction buffer (phosphate buffer: 0.15 M sodium chloride, 50 mM sodium phosphate and pH 7.5). Three different quantities of bi-functional NHS–PEG_{3500}–MAL were added to the solution of PEI with a molar ratio between PEG and PEI of 10:1, 20:1 and 30:1. The reaction mixtures were incubated at room temperature with stirring under nitrogen for 2 h. Remaining free PEG molecules were removed from PEG-grafted PEIs by ultrafiltration through a membrane (Amicon Ultra-15, MWCO 10,000, Millipore, Schwalbach, Germany) and centrifuged at 7000 rpm for 20 min for three times. The degrees of PEG grafting were calculated through TNBS assay by the differences in the contents of free primary amine groups remaining on PEGylated PEIs compared with unmodified PEI. Fourier transform infrared (FTIR) spectroscopy of PEG–PEI conjugates were taken using a Perkin-Elmer instrument at a resolution of 4.0 cm^{-1} on powder potassium bromide samples to further confirm the PEGylation reaction.

2.5. Preparation of polyplexes: pDNA and an appropriate amount of PEI or PEG–PEI copolymers were prepared separately in equal volumes of 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-buffered glucose (HEPES 20 mM, glucose 5% w/w, pH 7.4). The polyplexes were formed by mixing the two solutions followed by vortexing for 30 s, and then the mixtures were kept at room temperature for 30 min before use to allow polyplex formation with desired molar number of primary amines of PEI polymer/molar number of phosphate groups in the plasmid DNA (N/P) ratios.

2.6. *In vitro* cytotoxicity: Cytotoxicities of PEI/pDNA, PEG (10)–PEI/pDNA, PEG(20)–PEI/pDNA and PEG(30)–PEI/pDNA polyplexes were evaluated on BT-474 and MCF-10A cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In this colorimetric assay, yellow–green MTT is reduced by mitochondrial succinate dehydrogenase in live cells into a dark purple formazan product. The produced formazan crystals are solubilised in organic solvents such as dimethyl sulphoxide (DMSO) or acidic isopropanol, and the resultant colour intensity is determined by spectrophotometry at 570 nm. The cells were seeded in 96-well plates at an initial density of 5000 cells/well 24 h prior to cytotoxicity assay and then incubated at 37°C with different concentrations of PEI polymer (0, 5, 10, 15, 35, 75 and 150 µg/ml) or different N/P ratios (2, 4, 6, 10 and 15) of conjugates and the pDNA in the presence of fresh media. pDNA mass in each well was set to 0.2 µg. All experiments were conducted in triplicate. After cell incubation for 4 h in the presence of polyplexes, the medium replaced with fresh medium supplemented by FBS and further incubated for 48 h. MTT solution was added to the cells and the plates were kept in the incubator for 3 h at 37°C. Then, the supernatants were aspirated gently, the formazan crystals were solubilised in 100 µl DMSO and the colour intensity was evaluated. Control wells for naked DNA, cells without treatment (100% viability) and cells without the addition of MTT (as blank) were also prepared.

2.7. Gel retardation assays: Gel retardation assay was used to determine the ability of different PEI conjugates to complex and condense DNA. Plasmid encoding enhanced green fluorescent protein (pEGFP)–N1 pDNA (3 µg) was mixed with PEI and PEG–PEI at an N/P ratio of 6, by adding the conjugates into DNA as described above. The polyplexes then were subjected to electrophoresis on 1% (w/w) agarose gel containing 0.5 µg/ml ethidium bromide in 1 M tris–acetate–ethylenediaminetetraacetic acid buffer solution at 100 V for 45 min at room temperature. Free pDNA (pEGFP–N1) was loaded onto gel as a control.

2.8. Measurement of particle size and zeta potential: Polyplexes of PEI/pDNA, PEG(10)–PEI, PEG(20)–PEI and PEG(30)–PEI were prepared at an N/P ratio of 6. The particle size and zeta potential of the resulting polyplexes were determined by laser-light scattering using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

2.9. *In vitro* evaluation of PEGylation degree on transfection efficiency: Cells were seeded in 24-well plates at a density of 2×10^5 cells/well and allowed to incubate for 24 h prior to transfection. The cell culture media was removed, and replaced with 500 µl serum-free medium 2 h before transfection. PEI and PEI conjugate polyplexes with pEGFP–N1 pDNA were generated as described above (at an N/P ratio of 6) and then transfection mixtures were gently added to the cells. About 4 h later, the transfection mixtures were discarded and replaced with 1 ml fresh culture medium that supplemented with FBS, and further incubated for 48 h at 37°C. Then, cells were examined for the reporter gene (EGFP) expression. Qualitatively cells were visualised under fluorescence microscope and quantitatively the percentage of transfected cells was evaluated in terms of EGFP expression by flow cytometry analysis using fluorescence-activated cell sorting (FACS) Calibur flow cytometer equipped with CellQuest software (Becton Dickinson). Non-transfected cells were used as the control and statistics of cells fluorescing above the control level represent the transfection rate.

2.10. Storage stability of PEG-grafted PEI copolymers: PEG-grafted PEI copolymer at molar ratio of 10:1 (PEG:PEI) was prepared and evaluated as described above. Copolymer samples were stored in phosphate buffer pH 7 at 4°C and room temperature for a period of 6 weeks in the absence of any additional or supplementary reagent. Once a week, aliquots (100 µl) of the sample suspensions were examined for physicochemical properties in terms of particle size and zeta potential measurement.

2.11. Statistical analysis: Statistical analysis wherever needed was performed by GraphPad Prism 6.0. Data were presented as mean ± standard deviation of three experiments. Unpaired, Student's *t*-test and 2-way analysis of variance with Tukey correction were used to assess significance among groups.

3. Results and discussion

3.1. Synthesis and characterisation of PEG–PEI: After conjugation, the primary amine of PEI was coupled to the NHS ester of

MAL–PEG–NHS to generate the amide bond between the PEI and PEG molecules. PEGylated PEI was synthesised by reaction of the primary amine groups of PEI with MAL group on bi-functional MAL–PEG_{3500}–NHS. FTIR spectroscopy was carried out for characterisation of resultant copolymers. Result of this assay demonstrated a broad peak at about 3500 cm^{-1}, which represented NH– groups of PEI and the peak at 1107 cm^{-1}, which was the indicative of $–CH_2–O–CH_2–$ etheric bonds of PEG molecules. These findings proved the presence of both PEG and PEI in PEG–PEI copolymer. After PEGylation procedure, the reaction between primary amine of PEI and the NHS ester of PEG molecule generate the amide bond between the PEI polymer and PEG chains. The peak at 1666 cm^{-1} is due to the C=O stretching of carbonyl groups of PEG–PEI amide bonds and verified the successful grafting of PEG chains to PEI polymer (Fig. 1). TNBS and Ellman assays were used to determine the coupling amount of PEG chains on PEI molecules. On the basis of the findings of these assays, about 21, 48 and 82 bi-functional PEG chains were found to be successfully bound to each PEI molecule at PEG to PEI molar ratios of 10, 20 and 30, respectively. As our results showed, the average grafting ratios of PEG to PEI polymer at various molar ratios of 10, 20 and 30 were 3.72, 8.39 and 14.15%, respectively. In other words, the resulting copolymers, consisting 21.6, 48.74 and 82.21 PEG chains grafted to one PEI molecule for PEG(10)–PEI, PEG(20)–PEI and PEG(30)–PEI, respectively.

Merdan *et al.* [21] demonstrated that ten PEG monomethyl ether (2 kDa) chains grafted to one PEI 25 kDa molecule after a long reaction time. The more grafting degree attained in our work could be due to the different functional groups of NHS–PEG_{3500}–MAL used in our Letter compared with PEG monomethyl ether (2 kDa) and also different PEGylation procedure which we applied.

3.2. Toxicity assessment of PEI and PEI derivatives: Although PEI, has been extensively used as an efficient non-viral gene transfection vector, clarification of PEI-induced cytotoxicity has received much attention in recent years and restricts its application especially in gene therapy. A study conducted by Moghimi *et al.* has demonstrated that the mechanisms of PEI-induced cytotoxicity involve two major stages; polyplex-induced membrane damage with early necrotic changes to form phase I while initiation of apoptosis account for phase II [22]. Linear and branched PEI polyplexes can both affect cell membranes and change their permeability. There have been several attempts to reduce PEI-induced cytotoxicity via specific structural modifications of PEI. It has been demonstrated that such structural modifications could minimise the toxicity of PEI polymer while maintaining its high transfection ability [23]. Hashemi *et al.* [24] grafted 10 KDa PEI to several short lysine–histidine peptides. It has been shown that the amount and the distribution of lysine molecules among histidine residues could have a significant effect on decreasing PEI 10 KDa cytotoxicity.

The majority of these studies has been particularly focused on the structure of branched 25 KDa PEI. Some of PEI conjugates in such

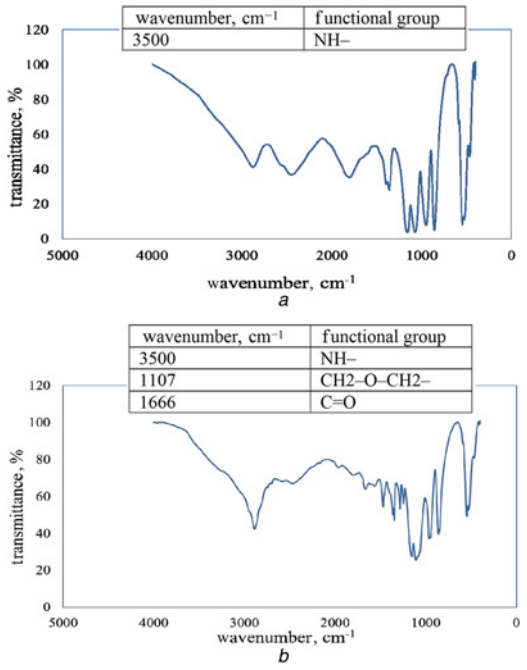

Fig. 1 *FTIR spectra of*
a PEI
b PEG(10)–PEI

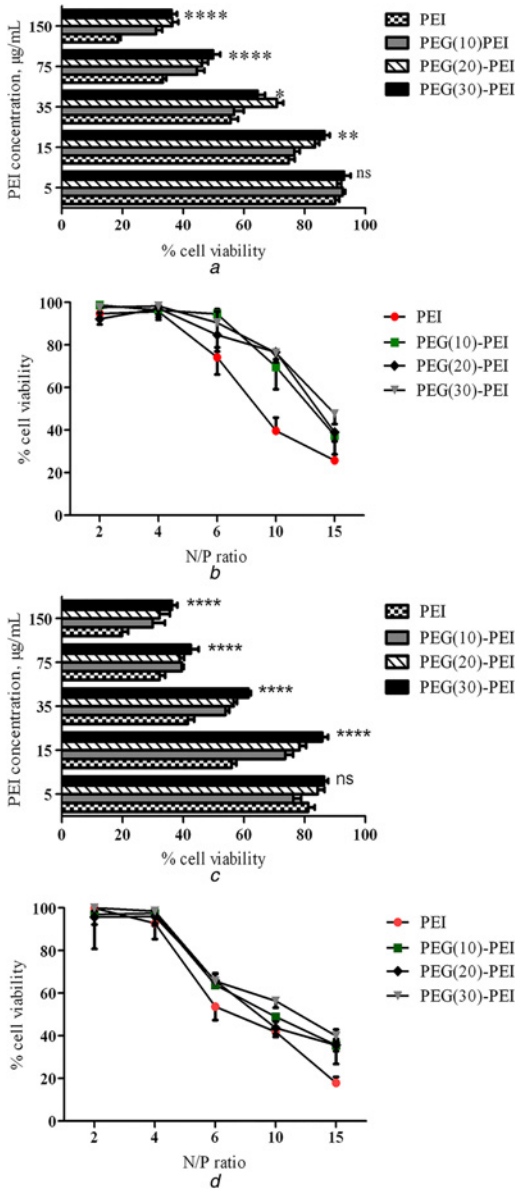

Fig. 2 *In vitro cytotoxicity evaluation of unmodified and PEGylated PEIs on BT-474 and MCF-10A cell lines*
a, *c* Cytotoxicity of the polymers at a concentration from to 150 µg/ml in BT-474 and MCF-10A cells, respectively
b, *d* Plots indicate cytotoxicity of the polyplexes at various N/P ratios of 2, 4, 6, 10 and 15 in BT-474 and MCF-10A cell lines, respectively. Untreated cells and 100% lysed cells were used as negative and positive controls, respectively

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studies involved permethylated PEI, perethylated PEI [25], PEI-polycylodextrin [26] and PEG PEI [27]. In this Letter, we applied PEGylation strategy to reduce branched 25 KDa PEI. The PEG grafting procedure was carried out as described before. To investigate the cytotoxicity of PEI and modified PEIs, viability of BT-474 and MCF-10A cell lines was examined by MTT assay after exposing the cells to different concentrations of PEI, PEG (10)–PEI, PEG(20)–PEI and PEG(30)–PEI including 5, 15, 35, 75 and 150 μg/ml calculated relative to the MTT value for untreated cells (100% viability).

The cell viability decreased with increase in polymer concentration. As demonstrated in Fig. 2, at lower concentrations, all PEGylated PEIs were almost non-toxic even at the lowest PEGylation degree. It is also demonstrated that higher degrees of PEGylation led to less cytotoxicity. Results showed that at the highest PEI concentration (150 μg/ml), the highest PEGylation degree (30:1 PEG–PEI molar ratio) could approximately decrease cytotoxicity of PEI in all cell lines by approximately two folds. In general, PEGylated polyplexes showed lower toxicity toward cell lines than non-PEGylated polyplexes regardless of cell lines. These observations are in agreement with the findings of other scientists [28, 29] and possibly can be attributed to the shielding effect of PEG chains on the positive charges of PEI. In this N/P ratio, cell viability of PEG–PEI-treated BT474 cell line was >80% for all different PEGylated PEIs, whereas PEG(10)–PEI showed slightly greater viability rate than the other PEGylated PEIs. In MCF-10A cells, at an N/P ratio of 6, viability percentage was about 70% for all PEG–PEI copolymers and was higher than that of PEI-treated cells. In this cell line, no significant difference was observed between cytotoxic effects of different PEGylated PEI copolymers. The cytotoxicity effect was increased at N/P ratios of 10 and 15, for the majority of different treatments in all cell lines, though PEG–PEI nanocarrier exhibited lower cytotoxicity than the unmodified carrier, but as it is demonstrated in Fig. 2, at these N/P ratios PEG(30)–PEI exhibited the lowest cytotoxic effect in both cell lines. These findings suggest that higher degree of PEG grafting improved the biocompatibility of PEI polymer.

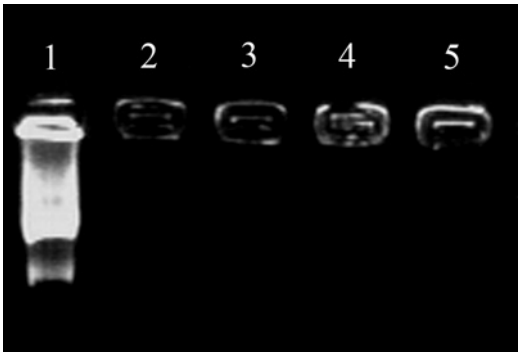

Fig. 3 *Agarose gel electrophoresis of PEI and PEGylated PEI polyplexes at an N/P ratio of 6. Lanes 1–5 represent pDNA, PEI/pDNA, PEG (10)–PEI/pDNA, PEG(20)–PEI/pDNA, PEG(30)–PEI/pDNA, respectively*

3.3. Agarose gel electrophoresis retardation assay: The gel retardation assay demonstrated that PEI, PEG(10)–PEI, PEG(20)–PEI and PEG(30)–PEI could effectively and completely condense the pDNA at N/P ratios of 6 (Fig. 3). On the basis of the results, this ratio was an appropriate ratio for gene delivery experiments.

3.4. Evaluation of particle size and zeta potential: Hydrodynamic diameters of PEG–PEI polyplexes were determined at an N/P ratio of 6 using laser-light scattering (Table 1). PEI–PEG exhibited reduced size compared with the unmodified PEI, particle size was decreased from 221 ± 4.7 nm for unmodified PEI to 189.2 ± 6.9, 141.1 ± 3.1 and 106.3 ± 7.8 for PEG(10)–PEI, PEG (20)–PEI and PEG(30)–PEI, respectively. These findings are in agreement with other studies, where PEG–PEI was used to form polyplexes [30].

The PEGylation process decreased the aggregation of PEI/pDNA complexes due to the increased hydrophilicity of the polyplexes. These observations are in coordinance with the results of Germershaus and co-workers research on PEG–PEI–Trastuzumab complexes [30]. Complex sizes of <200 nm are appropriate for endocytosis [31]; therefore, all PEG-grafted polyplexes at an N/P ratio of 6 are favourable for cellular uptake via endocytosis. Interactions between polymer/pDNA polyplexes and cell membrane are dependent on the surface charge of the nanoparticle. As demonstrated in Table 1, zeta potential measurements revealed that PEI/pDNA polyplexes exhibited relatively high positive surface charge of +29.3 mV. PEGylation also significantly decreased zeta potential of PEG–PEI copolymers in comparison with PEI polymer. Zeta potential was reduced from 29.9 ± 1.4 to 18.8 ± 2.1, 12.2 ± 1.1 and 9.7 ± 1.5 mV for PEG(10)–PEI, PEG (20)–PEI and PEG(30)–PEI, respectively. This reduced zeta potential is possibly due to the masking effect of PEG chains over primary amine groups of PEI.

3.5. Gene transfection: The ability of PEG-grafted PEI to efficiently transfer exogenous genes to the cells was evaluated using a EGFP, pEGFP–N1 (4733 bp) and few cytometry analysis. Two cell lines (BT474 and MCF-10A) were incubated with the polyplexes at an N/P ratio of 6 for 4 h. Cells were then assayed for expression of the GFP reporter gene 48 h post-transfection and then GFP expression was analysed by fluorescence microscopy (Fig. 4) and flow cytometry analysis (Fig. 5). In both cell lines, PEI/pEGFP–N1 polyplexes resulted in the highest GFP expression than other pDNA vectors, whereas GFP fluorescence was rarely observed in PEG(30)–PEI/pEGFP–N1 polyplex-treated cells. Among different PEGylated PEI copolymers, PEG(10)–PEI/pEGFP–N1 polyplexes led to nearly the highest GFP expression (Fig. 4). Flow cytometry analysis confirmed the microscopy imaging findings. As exhibited in Fig. 5, gene transfection ability of PEI polyplexes was higher than all PEGylated polyplexes. In BT-474 cell line, the GFP expression of PEI/pEGFP–N1 polyplexes was about 21.8% while the GFP expression rate was about 15.8% for PEG(10)–PEI/pEGFP–N1 polyplexes, 12.5% for PEG(20)–PEI/pEGFP–N1 polyplexes and 12.1% PEG(30)–PEI/pEGFP–N1 polyplexes. As exhibited by these results, there was no very significant difference between the various PEG grafting ratios, but PEG(10)–PEI

Table 1 Mean particle hydrodynamic diameter and zeta potential of the polyplexes formed between pEGFP–N1 and unmodified PEI and PEGylated PEI at an N/P ratio of 6

Polymer/copolymer	PEI	PEG(10)–PEI	PEG(20)–PEI	PEG(30)–PEI
size, nm	198 ± 3.5	176.2 ± 7.2	156.1 ± 4.9	132.1 ± 6.9
zeta potential, mV	+29.3 ± 1.7	+15.4 ± 0.2	+13.84 ± 0.7	+11.1 ± 1.1
feed ratio PEG:PEI (by mole)	—	10:1	20:1	30:1
number of conjugated PEGs	—	21	48	82

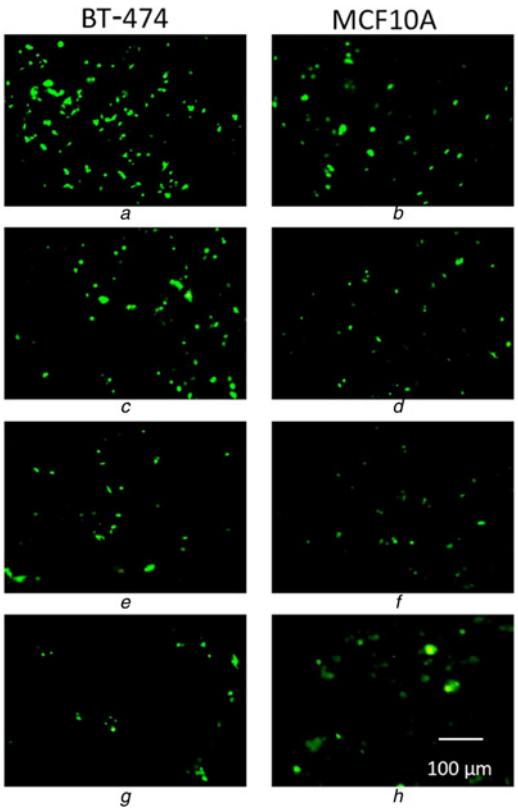

Fig. 4 *Fluorescent microscopy images of EGFP-N1 gene transfected cells*
a, *b* PEI/pEGFP–N1 transfected BT474 and MCF-10A cell lines respectively
c, *d* Micrographs show PEG(10)–PEI/pEGFP–N1 transfected BT474 and MCF-10A cell lines, respectively
e, *f* PEG(20)–PEI/pEGFP–N1 transfected BT474 and MCF-10A cell lines, respectively
g, *h* Micrographs demonstrate PEG(30)–PEI/pEGFP–N1 transfected BT474 and MCF-10A cell lines, respectively

demonstrated slightly higher transfection ability. Almost similar results were obtained in MCF-10A cell line. In these cells, PEI/pEGFP–N1 polyplexes led to the highest GFP expression rate among all the delivery agents (22.8%). PEG(10)-PEI/pEGFP–N1 polyplexes led to the expression of GFP in 16.9% of cells which was slightly higher than that of PEG(20)–PEI/pEGFP–N1 polyplexes (14.0%) and PEG(30)–PEI/pEGFP–N1 polyplexes (11.8%). PEG(30)–PEI/pEGFP–N1 complexes were the less effective gene vector compared with other nanocomplexes in both cell lines, possibly due to the less accessible surface primary amine groups compared with unmodified PEI and hence less pDNA-condensing ability and less interaction with negatively charged cell membranes. As it is calculated by TNBS assay at the PEG–PEI polar ratio of 10, about 21 PEG chains were conjugated to one PEI molecule, the PEGylation rate is 48 PEG chains per one PEI molecule for PEG(20)–PEI and 82 PEG substitutions per one PEI molecule for PEG(30)–PEI. So it seems that the best PEG substitution rate for branched 25 kDa PEI is 21 PEG chains per one PEI molecule which were obtained at the molar ratio of 10 (PEG–PEI), which led to the acceptable cytotoxic effect and gene transfection efficiency, indicating that in this PEGylation degree, PEI molecules still retain their DNA-condensing ability through their unbound primary amines, but by increasing the number of grafted PEG chains, this ability is decreased.

3.6. Stability storage determination: Stability of PEG-grafted PEI copolymer was determined over a storage period of 6 weeks. Over the total storage time, no significant alteration in particle

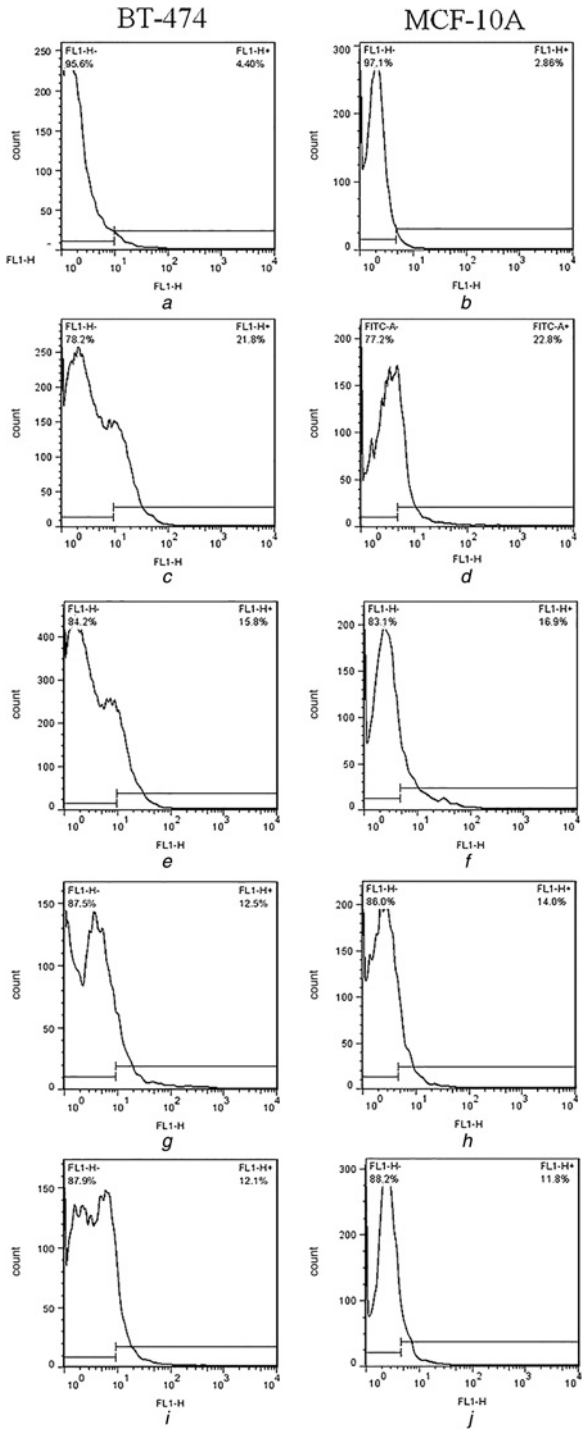

Fig. 5 *Transfection efficiency of PEI/pEGFP–N1, PEG(10)–PEI/pEGFP–N1, PEG(20)–PEI/pEGFP–N1 and PEG(30)–PEI/pEGFP–N1 polyplexes by flow cytometry analysis in BT-474 cells and cells at an N/P ratio of 6*
a, *b* Untreated BT-474 and MCF-10A cells, respectively
c, *d* PEI-treated BT-474 and MCF-10A, respectively
e, *f* PEG(10)–PEI-treated BT-474 and MCF-10A, respectively
g, *h* PEG(20)–PEI-treated BT-474 and MCF-10A, respectively
i, *j* PEG(30)–PEI-treated BT-474 and MCF-10A, respectively. The FL1 (*x*-axis) channel represents the EGFP intensity

size was detectable at 4°C and even after 6 weeks, PEG(10)–PEI copolymer size was appropriate for cell internalisation. In this storage condition, zeta potential was slightly decreased, but steel is relatively in convenient positive charge for cell binding. In the same way, the physicochemical properties of copolymer did not change particularly at room temperature (Fig. 6). Although particle size has relatively demonstrated further enhancement

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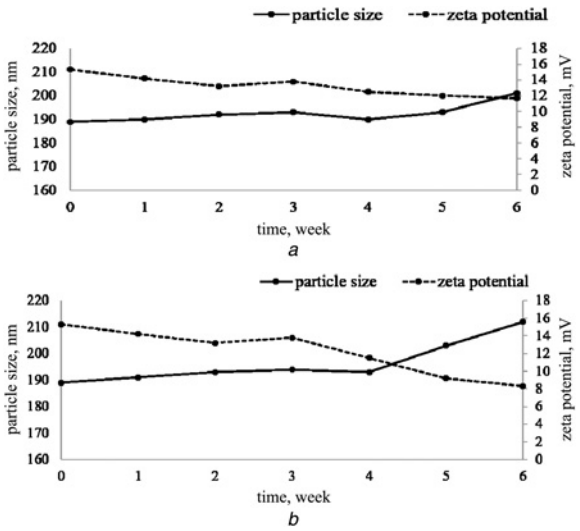

Fig. 6 *Storage stability of PEG(10)–PEI copolymer at*
a 4°C
b Room temperature

after 4 weeks of storage at room temperature. The zeta potential also showed a further reduction at this temperature compared with 4°C. These observations are probably due to the further aggregation of the particles at room temperature in comparison with 4°C. Storage stability of the synthesised PEG–PEI copolymer over 6 and 4 weeks was demonstrated at 4°C and room temperature, respectively. This stability is appropriate for applications in the cell culture studies and *in vivo* assays as well.

4. Conclusion: We successfully grafted NHS–PEG_{3500}–MAL chains to 25 kDa branched PEI polymer at various degrees of PEGylation and confirmed the ability of resultant copolymers to efficiently package and condense pDNA. Among different PEGylated copolymers, PEG–PEI copolymer which was prepared at molar ratio of 10 (PEG–PEI molecules) exhibited the best transfection ability together with favourable biocompatibility. We concluded that PEG(10)–PEI copolymer is a promising gene delivery vector with desirable characteristics for *in vitro* applications.

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