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Structure–transfection activity relationships with glucocorticoid–polyethylenimine conjugate nuclear gene delivery systems

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ABSTRACT

Efficient nuclear gene delivery is essential for successful gene therapy. It was previously reported that the transport of DNA into nucleus may be facilitated by glucocorticoid (GC). In this study, five glucocorticoids with different structures and potencies were conjugated with low molecular weight PEI 1800, and the degree of substitution of glucocorticoids was controlled to be close to each other. The glucocorticoid–polyethylenimine (GC–PEI)/pDNA complexes were prepared and their physico-chemical properties and transfection efficiency were investigated. The results showed that the complexes had similar physico-chemical properties, but their transfection activities were different statistically. In order to explore the reason of this difference, the affinity of GC–PEI polymer with GC receptor was analyzed by the application of molecular docking, and the correlation between transfection activity and the potency of five GC was investigated. The result showed that receptor binding of five GC was different and transgene expression enhanced linearly with the increasing GC potency, but log *P*. In addition, confocal microscopy examination confirmed that GC–PEI/DNA complexes were more effectively translocated in the nucleus than PEI 25 K or PEI 1800 complexes and the cytotoxicities of the GC–PEI polymers were lower than that of PEI 25 K. These results demonstrated that transfection activity of GC–PEI polymer correlated with its GC potency, and this regularity might be useful for the development of more efficient GC substituted polymer as promising nuclear-targeting carrier.

1. Introduction

The success of gene therapy is waiting for efficient delivery vectors. Admittedly, transfection efficiency of viral gene carriers is higher than that of non-viral gene transfer agents. But viral gene carriers have severe side effects which resulted from insertion of the recombinant virus in the initial coding region of a gene [1,2]. Non-viral carriers have advantages such as non-immunogenicity, low cytotoxicity and low cost, but poor transfection efficiency compared to viral carriers limits their application to clinical gene therapy. There were three main barriers in the intracellular delivery of genetic materials using non-viral gene carriers, namely, cellular membrane, endosomal membrane and nuclear membrane [3]. Poor access of plasmids DNA to the nucleus represents the major barrier to the success of non-viral gene therapy [4].

In the last two decades, several strategies were developed to overcome this barrier [5]. Using glucocorticoid ligand as nuclear localization signal (NLS) to enhance transgenic expression was an alternative strategy [6]. Glucocorticoid receptor (GR) is a nuclear receptor, which mainly locates in cytoplasm in its inactive form associated with heat shock proteins (HSPs) in the absence of its ligand. When GR binds to the ligand, it dissociates from the HSPs and translocates from the cytoplasm into the nucleus by means of the endogenous nuclear transport machinery [7]. In addition, the nuclear pore is dilated up to 60 nm during this process to facilitate the uptake of transfected DNA into the nucleus [8]. Moreover, glucocorticoids have a wide range of pharmacological effect, leading to pronounced anti-inflammatory and immunosuppressive effects and extensive clinical application. And recent studies indicate that glucocorticoid encapsulated in long-circulating liposomes exerts strong inhibitory effects on tumor growth in a low dose and low frequency schedule after intravenous administration [9–11]. Some studies have conjugated glucocorticoids (GCs) to polycation in order to improve transfection efficiency [6,12–15]. The results of those studies had confirmed the enhancement of gene delivery by using steroid polymers.

PEI is a well-characterized, commercially available organic macromolecule with the highest cationic-charge-density potential

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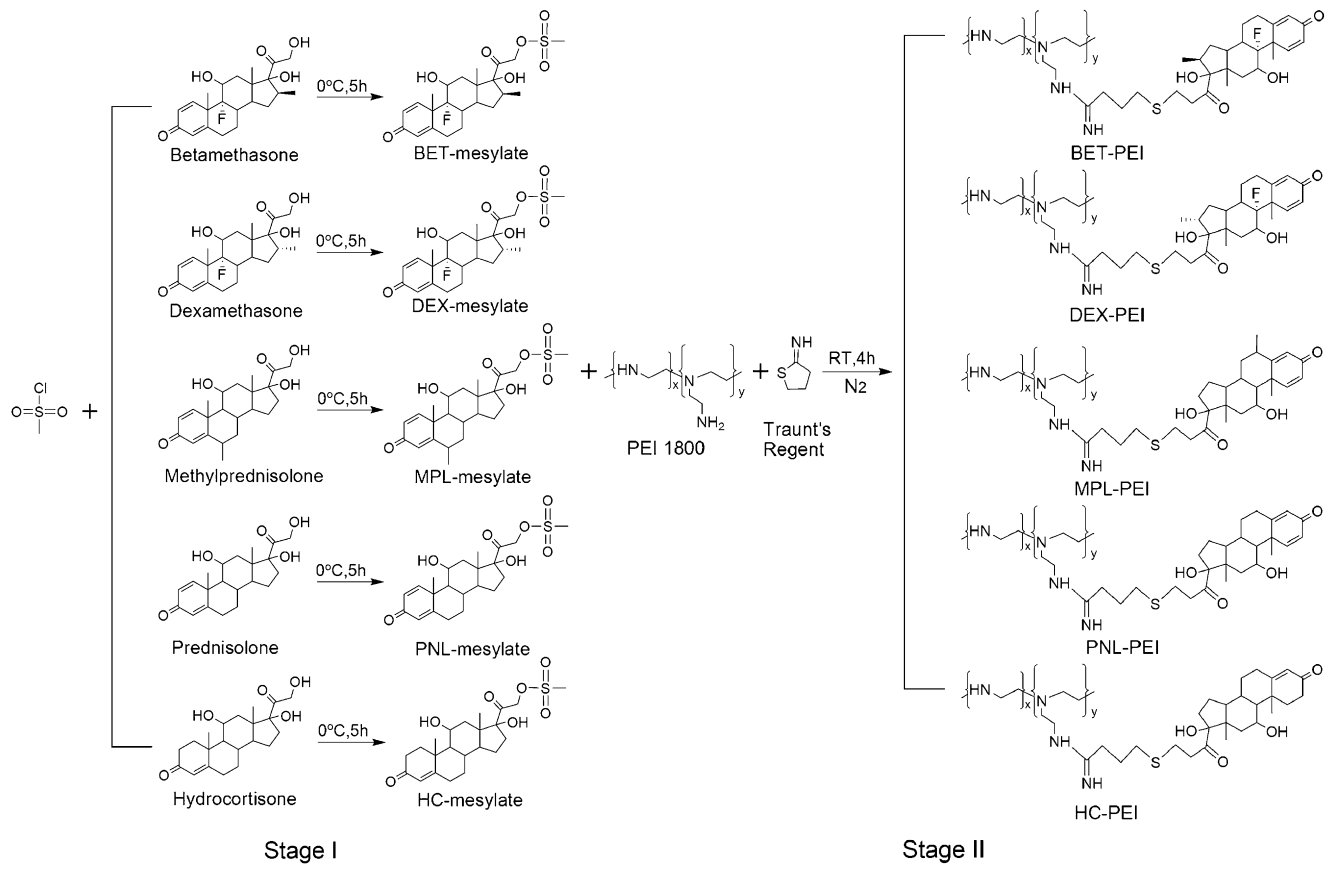

Fig. 1. Synthesis of betamethasone–PEI (BET–PEI), dexamethasone–PEI (DEX–PEI), hydrocortisone–PEI (HC–PEI), methylprednisolone–PEI (MPL–PEI) and prednisolone–PEI (PNL–PEI).

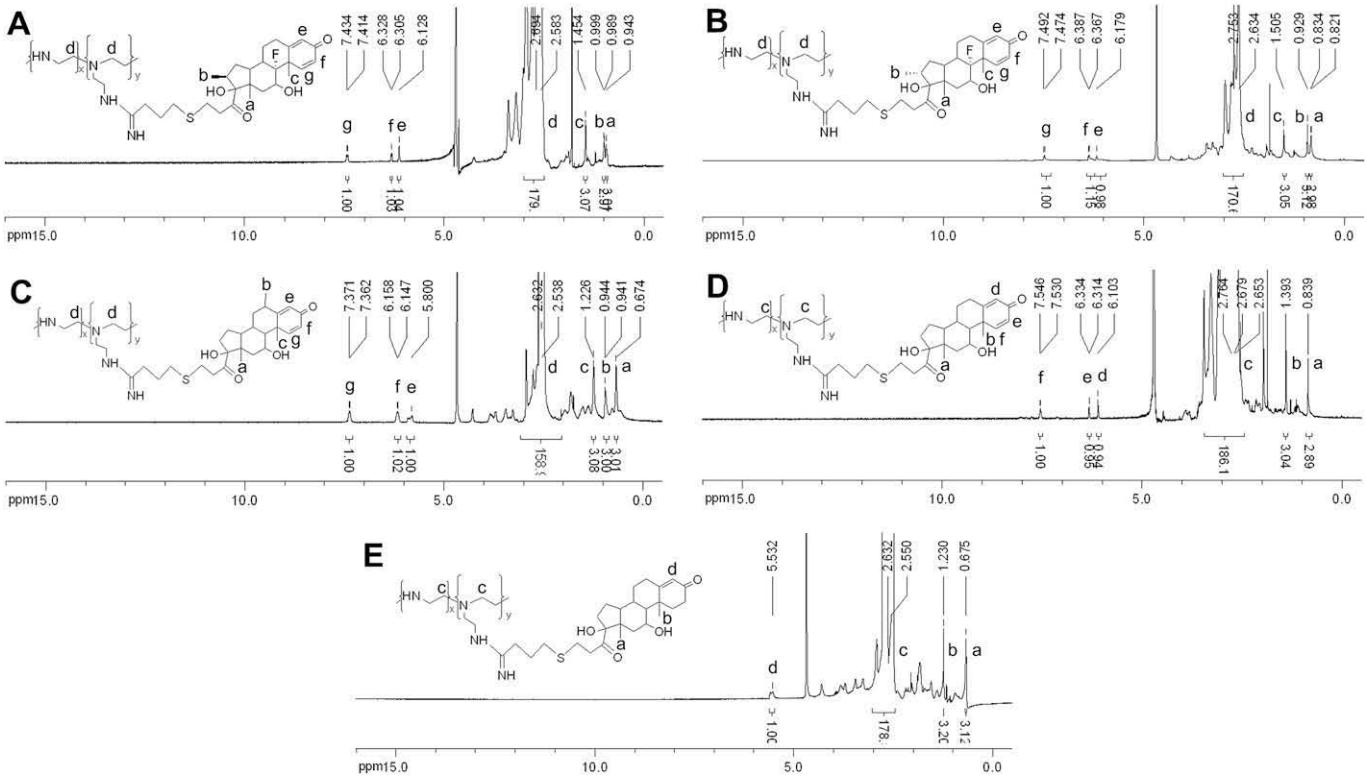

Fig. 2. The ^{1}H NMR spectrum of five GC–PEI (A, BET–PEI; B, DEX–PEI; C, MPL–PEI; D, PNL–PEI; E, HC–PEI).

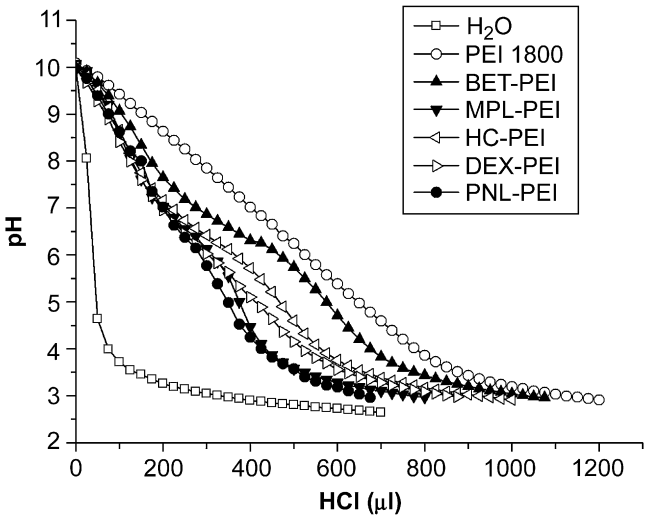

Fig. 3. Determination of the buffer capacity of PEI 1800 and five GC–PEI by acid–base titration.

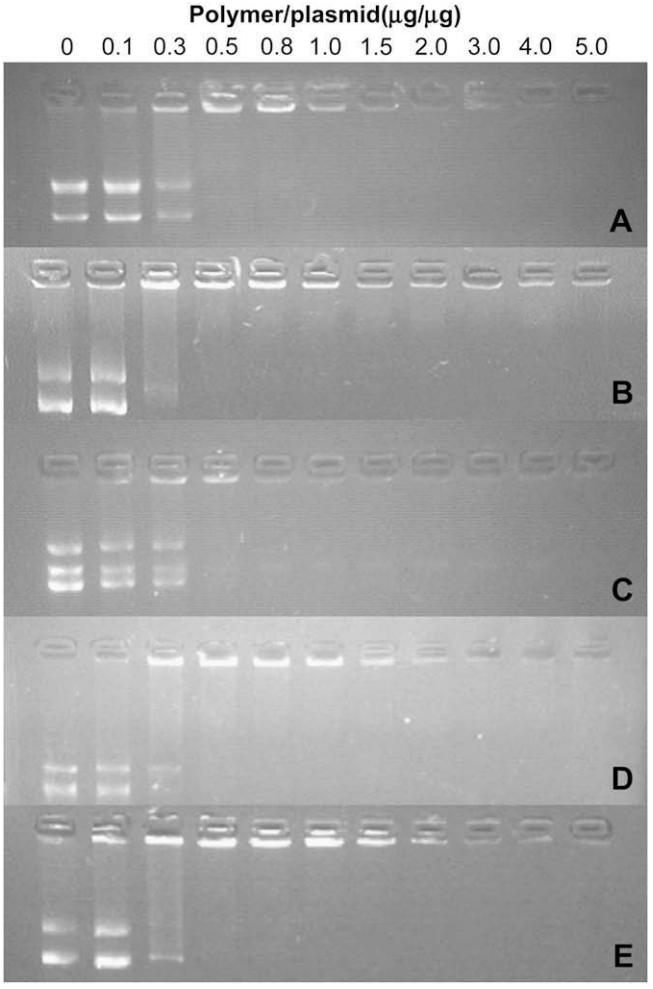

Fig. 4. Gel retardation assay: polymer/pDNA complexes were prepared at various weight ratios (A, BET–PEI; B, DEX–PEI; C, MPL–PEI; D, PNL–PEI; E, HC–PEI). The mixtures were incubated at room temperature for 30 min and electrophoresis on 1% (w/v) agarose gel and stained with ethidium bromide.

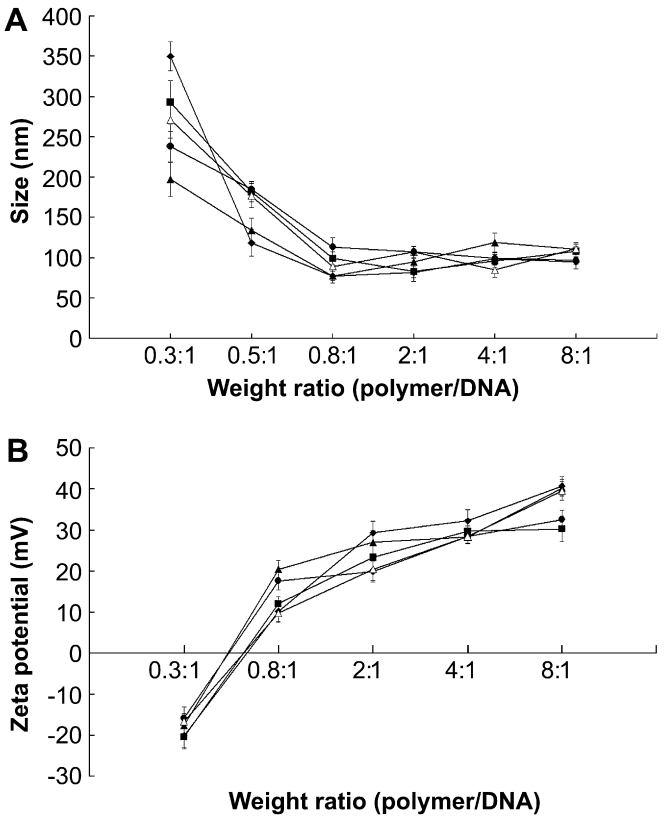

Fig. 5. Mean particle size (A) and zeta potential (B) of various polymers (BET–PEI (◆), DEX–PEI (△), MPL–PEI (▲), PNL–PEI (●), and HC–PEI (■)) complexed with pDNA at various weight ratios (polymer/pDNA ratios). The pDNA concentration was 10 μg/mL. Each point is the mean ± SD ($n = 3$).

and can ensnare DNA efficiently to form stable polyplexes via electrostatic interaction. PEI retains a substantial buffering capacity at virtually any pH and it protects DNA from endosomal degradation. PEI 25 kDa showed the higher gene transfection efficiency than other polymer vectors that have been reported so far. However, the cytotoxicity of the high molecular weight PEI is a major obstacle for their clinical application. So, because of its relative nontoxicity, low molecular weight PEI has been investigated as a gene carrier. Many studies have been conducted to decorate low molecular weight PEI for enhanced functionality and targeting activity using the polymer as a starting material [16,17]. In our study, several commonly used GCs with different potencies were conjugated to low molecular polyethyleneimine (PEI) (1800D) to create a series of polymeric gene carriers. Their physico-chemical properties and transfection activities were investigated and compared systematically. To gain optimal transfer material and to explore the reason of their transfection activity difference were the goals of this paper.

2. Materials and methods

2.1. Materials

Polyethylenimine (PEI, branched, 1800 and 25 kDa), 2-iminothiolane (Traut's reagent), fluorescein isothiocyanate (FITC), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), ethidium bromide were purchased from Sigma–Aldrich Chemical Co., Ltd. (Milwaukee, WI, USA). RPMI 1640, penicillin–streptomycin (PS, 10,000 U/ml), trypsin–EDTA (TE, 0.5% trypsin, 5.3 mM EDTA tetra-sodium) were obtained from Gibco BRL (Gaithersberg, MD, USA). Fetal bovine serum (FBS) was purchased from sijiqing Biologic Co., Ltd. (Hangzhou, China). Methanesulfonyl chloride was purchased from Shanghai Jiachen Chemical Co., Ltd. (Shanghai, China).

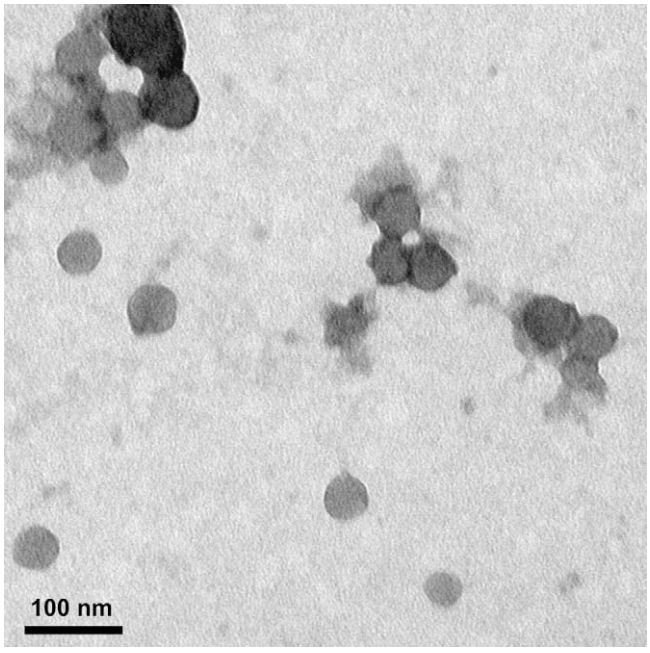

Fig. 6. Morphology of BET–PEI/DNA complex at weight ratio 4 as observed by transmission electron microscope. Complexes were negatively stained by a 2% aqueous solution phosphotungstic acid for 30 s. Scale bar: 100 nm.

Betamethasone (BET), dexamethasone (DEX), methylprednisolone (MPL), prednisolone (PNL) and hydrocortisone (HC) are kindly granted by Zhejiang Xianju Pharmaceutical Co., Ltd. (Hangzhou, China), and purity of those chemical compounds was over 99%. Promega Luciferase Assay Kit containing luciferase cell culture lyses reagent and luciferase substrates was obtained from Promega (Madison, WI, USA). The BCA Protein Assay Kit and Hoechst 33342 were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Plasmid DNA (pEGFP-N1 and pGL-3) was kindly provided by Institute of Infectious Diseases, Zhejiang University (Hangzhou, China). The plasmids were propagated in *Escherichia coli* DH5α, isolated, and purified using Axyprep Plasmid DNA Maxiprep Kit (Axygene Biotechnology Limited, Hangzhou, China). The purity and concentration of DNA were determined by measuring a UV absorbance 260 and 280 nm. All other chemicals were of analytical grade.

2.2. Synthesis of glucocorticoid–PEI (GC–PEI)

Five kinds of GC–PEI were synthesized via two-step reaction as shown in Fig. 1.

2.2.1. Activation of glucocorticoid

To activate five kinds of glucocorticoid, we substituted their 21-hydroxyl groups with mesylates. Using the activation of BET for an example, to a solution of BET (105.2 mg, 0.268 mmol) in anhydrous pyridine (2.5 ml) at 0 °C under N_2 was added dropwise methanesulfonyl chloride (41.5 μL, 0.536 mmol) with stirring. After reacting for 5 h at 0 °C, ice water (50 mL) was added. The precipitate was filtered, washed with ice water, crude betamethasone mesylate (BET-mesylate) as a white solid powder was obtained and dried. Using recrystallization from ethanol–acetic ether to purify the crude mesylate. Other four glucocorticoid mesylates, which were also white powder, were synthesized by the same method. TLC (ethyl acetate/ligroin/methanol = 10/10/1, v/v/v) was performed at the end of the activation. The product was solubilized in DMSO-d_6 for ^{1}H NMR analysis (500 MHz, Bruker, Germany). HPLC (Agilent 1100 series, water/methanol = 25/75, v/v) was used to determine the purity of five glucocorticoid-mesylates. The wavelength of the UV detector was chosen at 234, 235, 238, 241, 242 nm for DEX-mesylates, BET-mesylates, HC-mesylates, MPL-mesylates and PNL-mesylates, respectively.

2.2.2. Conjugation of PEI and GC-mesylate

The conjugation reaction was followed by the procedures as earlier reported with some modification [14]. Traut's reagent and betamethasone mesylate (0.29 mmol, both 2 equiv. to PEI) in 3.0 mL anhydrous DMSO were added slowly 1 equiv. of PEI 1800 in 2.0 mL anhydrous DMSO. The reaction was allowed to proceed under N_2 at room temperature under continuous stirring for 4 h. The product was quenched by the addition of an excess amount (20-fold volume, 100 mL) of cold ethyl acetate. The precipitated product was solubilized in pure water and filtered by 0.45 μm micropore film. Then dialyzed against pure water using dialysis membrane (MWCO 1000) for 48 h, the dialysis medium was refreshed every 12 h. A pale yellow product (BET–PEI) was obtained after further freeze-dried. Other four GC–PEIs were synthesized via the same procedure. The structure of product was analyzed by using ^{1}H NMR (500 MHz, Bruker, Germany, D_2O/DMSO-d_6).

2.3. Polymer buffer capability

Each polymer solution was prepared in a 50 mL flask (0.2 mg/mL, 30 mL) and pure water was used as a control. After adjusting an initial pH to 10 with 0.1 N NaOH or 0.1 N HCl if necessary, 25 μL increments of 0.1 N HCL were titrated into the solution and the pH response was measured with a micro-pH electrode at the same time. The whole pH variation was recorded from 10 to 3.

2.4. Preparation and physico-chemical properties of GC–PEI/DNA complex

A GC–PEI/DNA complex was induced to self-assemble in distilled water by mixing plasmid DNA with polymer solution at various weight ratios, and incubated for 30 min at room temperature before use. The complex formation between GC–PEI and pDNA was analyzed by agarose gel electrophoresis. The mean particle size and zeta potential of polyplex were determined by laser diffraction spectrometry (Malvern Zetasizer 3000HS, Malvern, UK). The morphology of BET–PEI/pDNA (pEGFP-N1) polyplex with a weight ratio of 4 was observed using TEM (JEM 1230, JEOL, Tokyo, Japan).

2.5. Cytotoxicity assay

Human embryonic kidney 293 cells and human liver carcinoma HepG2 cells were incubated in RMPI 1640 supplemented with 10% FBS, streptomycin at 100 μg/mL, and penicillin at 100 U/mL at 37 °C in an incubator with a humidified atmosphere containing 5% CO_2. Evaluation of cytotoxicity was performed by MTT assay. Briefly, the HEK 293 and HepG2 cells were seeded respectively at a density of 5000 cells/well in 96 well plates (Corning incorporated, NY, USA) overnight. After the polymers were added, the cells were further incubated for 4 h at 37 °C. Then the medium was replaced with 20 μL MTT (5 mg/mL) solutions and 180 μL of fresh medium without serum and further incubated for 4 h. After that, the medium was removed and 100 μL DMSO was added. The absorbance at 570 nm was measured by using an ELISA plate reader (Thermo Multiskan Spectrum, USA).

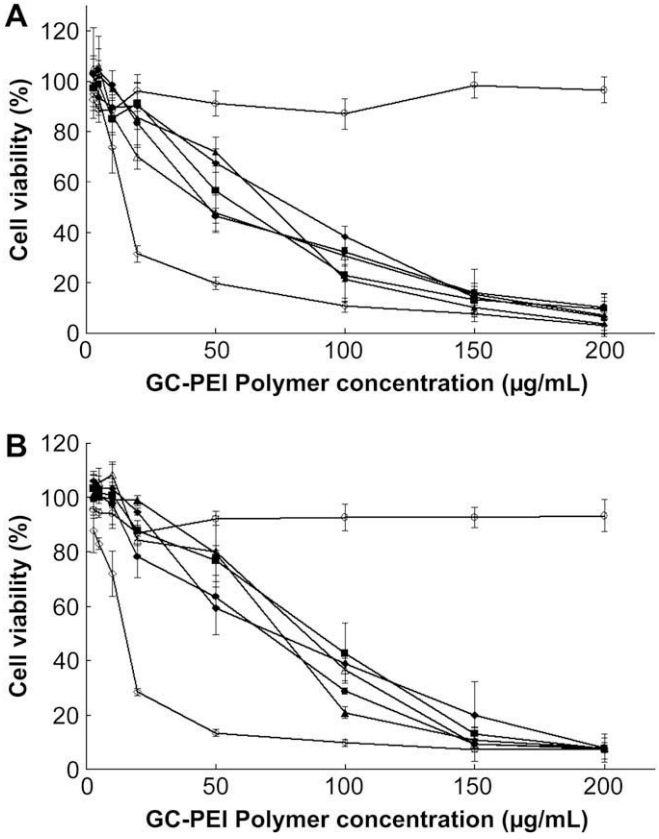

Fig. 7. Viability of 293 cells (A) and HepG2 cells (B) treated with various amounts of polymers ((BET–PEI (◆), DEX–PEI (△), MPL–PEI (▲), PNL–PEI (●), and HC–PEI (■), PEI 25 K (◇), PEI 1800 (○)) and as determined by MTT assay. Each point is the mean ± SD ($n = 3$).

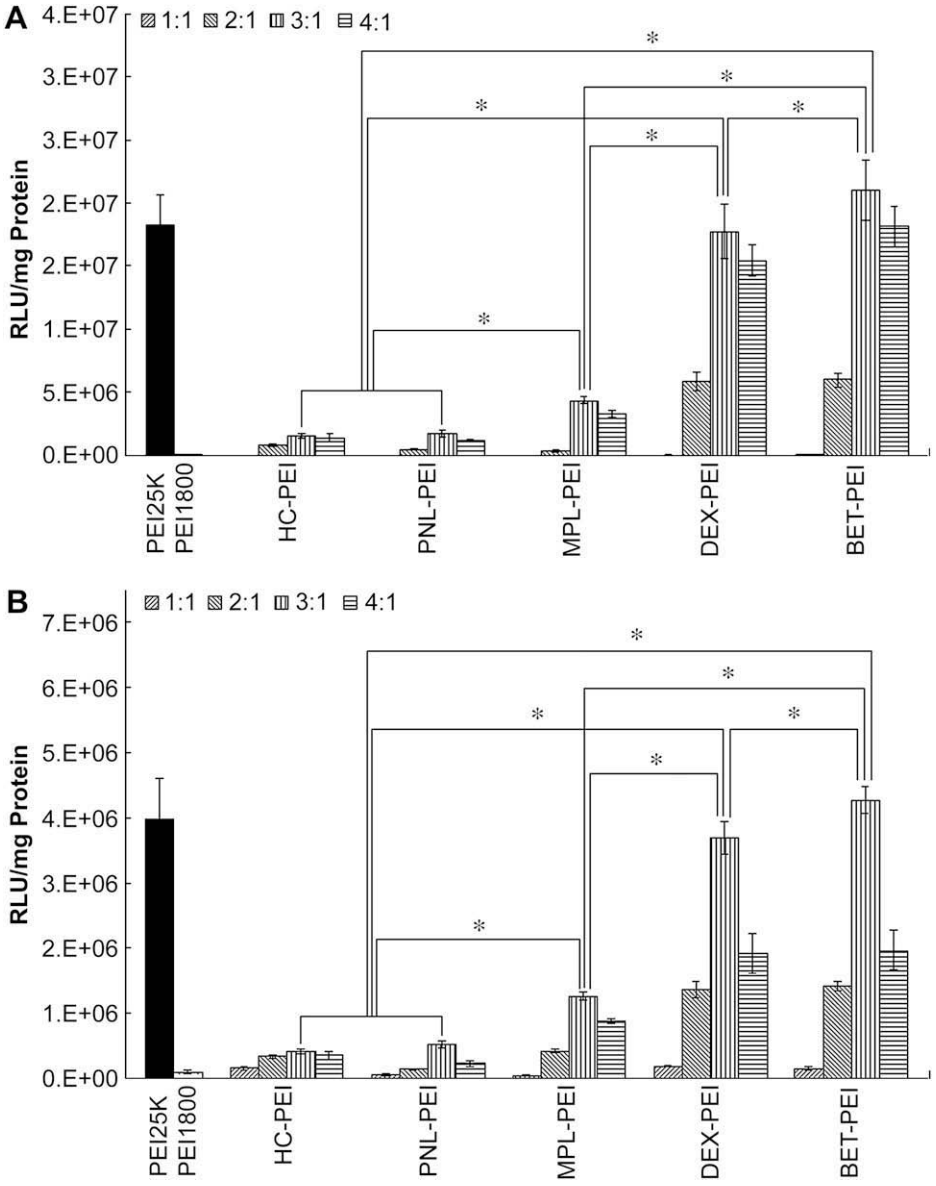

Fig. 8. Transgene activity obtained with various GC–PEI polymers in HEK 293 (A) and HepG2 (B). The polymer was complexed with pDNA at various weight ratios and incubated for 30 min. The complexes containing 2 μg of plasmid DNA in 0.5 mL were added to 1×10^5 cells per 24-well plate for 6 h, cells were harvested 48 h post-transfection. Luciferase activity is shown as means ± SD of triplicates (RLU, relative light units). The DNA's concentration was 2 μg/well. * Indicates statistically significant difference ($P < 0.05$).

2.6. Transfection efficiency

For the transfection experiments, the pGL-3 and pEGFP-N1 plasmid DNA were both used to evaluate the transfection efficiency of various GC–PEI polymers. The cells were seeded in the 24-well plate at a density of 1×10^5 cells/well and incubated overnight. The GC–PEI/DNA complexes at various w/w ratios ranging from 1 to 4 were added. The amount of pDNA was fixed at 2 μg/well. The cells were then incubated for 6 h at 37 °C. Then, the serum-free RMPI 1640 was replaced with fresh RMPI 1640 containing 10% FBS and the cells were further incubated for 48 h. For luciferase assay, the medium was removed and the cells were washed with PBS twice, and 200 μL reporter lysis buffer was added to each well. After 30 min of incubation at room temperature, the cells were harvested and transferred to tubes, and centrifuged at 13,000 rpm for 5 min. The relative light units (RLUs) were measured with a luminometer (Turner Designs Luminometer Model TD-20/20, Promega). The total protein was measured according to a BCA protein assay kit and luciferase activity was expressed as RLU/mg protein. Inverted fluorescent microscope (Leica DMI 4000 B, Leica, Germany) was also used to observe the transfection effect of the polyplexes.

2.7. FITC-labeling of polymers

For fluorescent labeling, PEI 1800, PEI 25 k, BET–PEI (10 mg) were reacted with FITC (2 mg) in DMSO at room temperature and incubated for 24 h in the dark with stirring. Separation of unreacted FITC was performed by dialysis (MWCO 1000) against PBS (pH 7.4) for 48 h and then distilled water for 48 h until free FITC could not be detected by TLC (chloroform/methanol = 1/1, v/v) [18].

2.8. Confocal microscopy experiments

For confocal microscopy, HepG2 cells were grown on glass cover slips in 6-well plates and incubated at 37 °C for a day. FITC-labeled PEI 1800, PEI 25 kDa, and BET–PEI were complexed with pGL-3 at weight ratio of 5.3/1, 1.3/1 and 3/1 respectively. After 24 h, the cells were fixed with 4% paraformaldehyde for 30 min. To stain the cell nuclei, the cells were incubated with Hoechest 33342 for 15 min at room temperature after washed three times with 1 ml of PBS, and then the cover slips were mounted on glass slides with a drop of 0.1 M glycerine in PBS placed in between to keep the cells from drying out. The fluorescence was examined with an LSM 510 META confocal laser scan microscope (Carl Zeiss, Co., Ltd.). A UV laser (405 nm excitation) was used to induce the blue fluorescence of Hoechest 33342 and an argon (488 nm) to excite the green fluorescence of FITC.

2.9. Molecular docking

The crystal structure of the glucocorticoid receptor was supplied by the RCSB PDB: protein data bank, and its resolution is 2.70 Å [19]. We predicted the

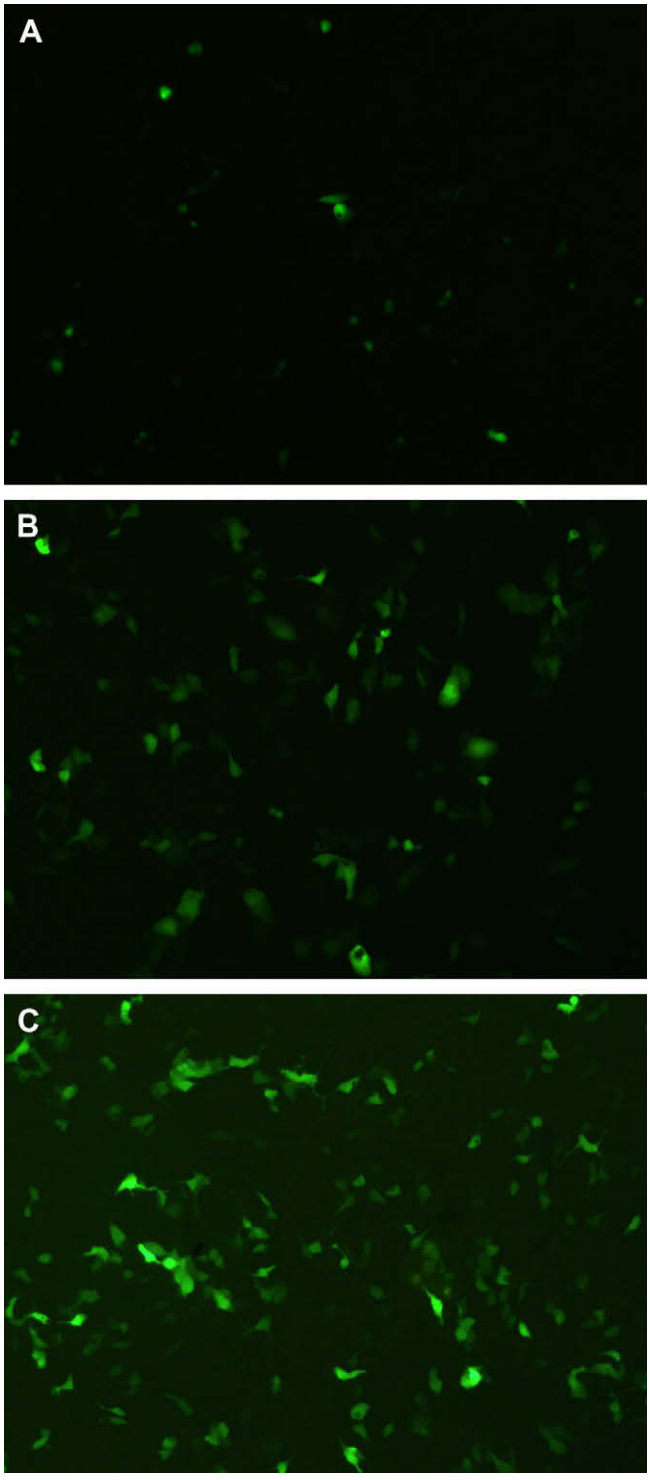

Fig. 9. Comparison of transfection of HEK 293 cells mediated by the parent PEI 1800 (A), PEI 25 K (B) and BET–PEI polymers (C) at optimum weight ratio using EGFP gene. HEK 293 cells (1×10^5) were treated with polyplex containing 2 μg of plasmid DNA containing EGFP gene.

interactions between five different GCs substitutes which conjugated to the backbone of PEI and GC receptor by the application of Autodock 3.0.5. Since hydrogen bonds are frequently important in ligand binding, we compared not only the docking energy but also the hydrogen bonds in the binding domain. The optimal conformation was elaborately chosen from fifty best modeling results according to the lowest docking energy and binding energy. All parameters were applied with default ones.

2.10. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). Differences between groups were considered statistically significant at $P < 0.05$ (*).

3. Results

3.1. Synthesis of GC–PEI

TLC was performed on the solution of the first stage reaction, using corresponding glucocorticoid as control. The spots of the products (glucocorticoids-mesylates, $R_f = 0.5$–0.6) and the reactants (glucocorticoids, $R_f = 0.2$–0.3) could be distinguished by direct observation. After approximately 5 h, the spot of the solution became a single clear one which indicates the termination of the stage I reaction. The peaks of methyl protons on mesylate were at δ 3.2 ppm. The purity of the glucocorticoids-21-mesylates was determined by HPLC method. The crude products showed purity around 93%, and increased to above 99% after the process of recrystallization. Thus the quality of stage II reaction can be ensured.

The ^{1}H NMR spectrum of the five GC–PEIs was shown in Fig. 2. The degree of glucocorticoids grafting on each PEI polymer chain was obtained by calculating the peak intensity ratio between the ethyl protons of PEI backbone. It was observed that each mole of PEI conjugated 0.97 mol of BET, 0.98 mol of DEX, 1.05 mol of MPL, 0.90 mol of PNL or 0.94 mol of HC, respectively.

3.2. Polymer buffer capability

PEI 1800 has greater protonation capability than its grafted polymer (Fig. 3) since the amino groups increase and also appear to buffer better at neutral medium compared to GC–PEI due to the presence of more free prime amines. Molecules entering cells on endocytic pathways will experience a drop in pH from neutral to 5.0 [20]. Buffer capability of polymers which serve as therapeutic gene vectors is crucial to the molecules entering cells, the proton sponge nature of PEI ensures buffering inside the endosome, resulting in the escape of the vector from the degradative lysosomes so that the genes can be protected and functional.

The five kinds of polymers had relatively high buffer capability in the pH range of 4–6 compared to pure water. Although it is slightly lower than PEI 1800, we still considered it is sufficient for gene transfection.

3.3. Characterization of GC–PEI/pDNA complexes

Polymer/pDNA complexes at various weight ratios were prepared and analyzed by agarose gel electrophoresis as shown in Fig. 4. The result indicates that pDNA was retarded completely at the weight ratio 0.5:1 (GC–PEI/pDNA, W/W), and there was no significant difference among various GC–PEI polymers. It was not consistent with the former report where DEX–PEI retarded pDNA at a lower weight ratio (0.3:1) [14]. The discrepancy possibly resulted from the difference of substitute degree of DEX onto the backbone of PEI. The higher was the substitute degree of DEX–PEI, the less primary amino groups had PEI, and the more DEX–PEI was needed to bind with pDNA to form stable complexes.

The particle size and zeta potential of the polyplexes were examined at the various weight ratios (Fig. 5). The particle sizes of the complexes decrease with increasing weight ratios. After reaching the weight ratio of 0.8, all five GC–PEI polymers can compact pDNA into nanoparticles of 80–120 nm in diameters and no obvious change of sizes for those complexes was observed along with further increase of weight ratios. The size of the complex was

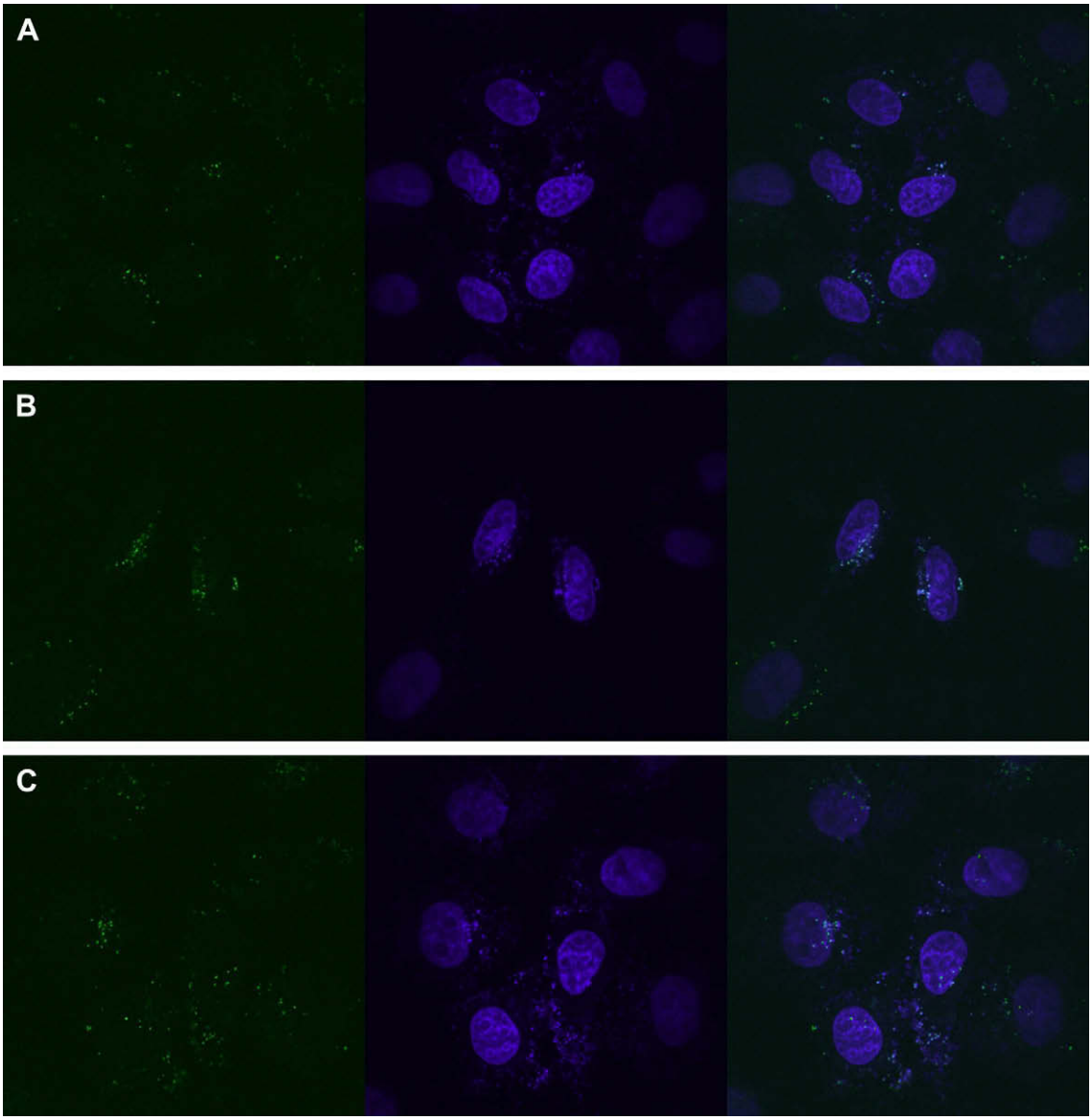

Fig. 10. Subcellular distribution of complexes was visualized by fluorescence confocal microscopy in HepG2 cells. FITC-labeled PEI 1800 (A), PEI 25 K (B) and BET–PEI (C) complexes were prepared as described in Materials and methods. Cells were fixed 24 h after transfection and observed by confocal microscopy (left row: FITC-labeled polymer (green), middle row: Hoechest 33342 labeled nuclei (blue), right row: merged images of left and middle row).

confirmed by TEM (Fig. 6). At the weight ratio 4:1, the compacted complexes of pDNA with BET–PEI exist in the form of spherical nanoparticles of about 100 nm which is consistent with the result of dynamic light scattering. The net surface charge of the polymer/pDNA complexes was negative at weight ratio 0.3:1, but as the polymer/pDNA weight ratio increases from 0.8 to 8, the zeta potential of the pDNA complexes increases gradually. After reaching the weight ratio of 2, the zeta potentials of the pDNA complexes of all five GC–PEIs are strongly positive above 20 mV and their difference was within a narrow range of 10 mV.

3.4. Cytotoxicity assay

The cytotoxicity of polymer was examined by means of MTT assay in HEK 293 and HepG2 cells. As shown in Fig. 7A,B, PEI 1800 showed no toxicity at the concentration ranges tested in this experiment. All GC–PEI polymers exhibited a dose-dependent cytotoxic effect. No significant cytotoxicity was found among various polymers at the low concentration. But with the increasing concentration, polymer showed cytotoxicity. Nevertheless, all GC–PEI polymers exhibit lower toxicity than PEI (25 kDa) (the controls) in the HEK 293 and HepG2 cells, which indicated more extensive dosage range of polymer for gene transfer could be selected. The reason, that the cytotoxicity of polymer was discrepancy with the previous report, was possibly the difference of the substitution degree of GC [14].

3.5. Transfection efficiency

Transfection activity of these polymers at different weight ratios was investigated in HEK 293 and HepG2 cells by using pGL-3 and pEGFP-N1 reporter gene, and PEI 25 K and PEI 1800 were used as control (Fig. 8). The results indicated all polymers showed greater increased level of gene expression compared to native PEI 1800 at all weight ratios tested. The transfection efficiency depended on the weight ratio, and the highest level of luciferase expression of various polymers occurred at the weight ratio 3:1, and it was disagree with former report about DEX–PEI polymer [14]. This discrepancy was

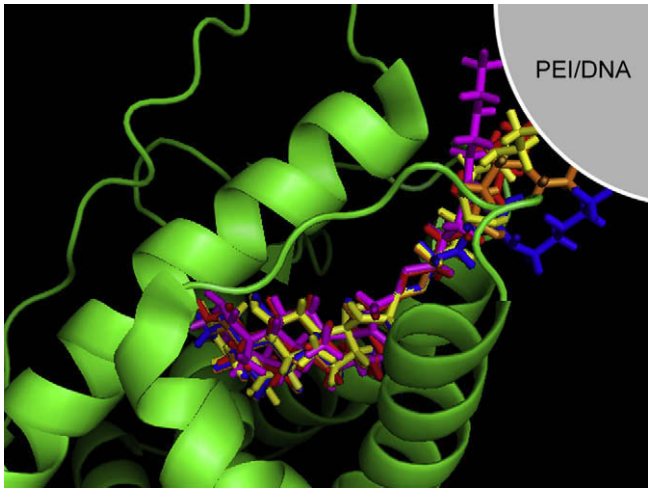

Fig. 11. The crystal structure of five glucocorticoid grafting substitutes binding glucocorticoid receptor (BET substitute-red, DEX substitute-magenta, MPL substitute-yellow, PNL substitute-blue, HC substitute-orange, by PyMol 0.99rc2).

possibly caused by the difference of substitute degree. In our report, the substitution degree of GC was higher than former research. The higher substitution degree of GC, the less weight of polymer was needed to obtain higher transfection. As Fig. 7 shows, the different polymers provided maximum transfection efficiency with remarkable distinction. The transfection results in both cells indicated that, among the all polymers examined, the increase in transfection capability generally follows the order, HC–PEI < PNL–PEI < MPL–PEI < DEX–PEI < BET–PEI, with more pronounced enhancements in BET–PEI and DEX–PEI. The maximum transfection efficiency mediated by BET–PEI and DEX–PEI polymers was higher than that mediated by PEI 25 K at the best weight ratio while their cytotoxicities were lower than PEI 25 K.

The transfection activity of BET–PEI polymer was further confirmed using enhanced green fluorescent protein (EGFP) gene. Fig. 9 depicts fluorescence microphotographs of HEK 293 cells treated with BET–PEI polymer, PEI 25 K and PEI 1800 at optimum weight ratio. Apparently, cellular fluorescence mediated by BET–PEI polymer derived from EGFP was stronger than PEI 25 K and PEI 1800.

3.6. Confocal microscopy experiments

Many studies showed that in the presence of GC, the reporter gene expression was improved drastically [21–23] because pDNA was translocated efficiently into the cellular nucleus. To directly monitor the cellular localization of the GC–PEI polymer, FITC-labeled BET–PEI complexes mixed with pDNA were prepared and transfected in HepG2 cells, and imaged with a confocal laser scanning microscope. As presented in Fig. 10, more BET–PEI/pDNA complexes were found inside the nucleus which stained in blue, compared to FITC-labled PEI 25 K or PEI 1800 complexes. These results clearly indicated that BET–PEI/pDNA complexes were more effectively translocated into the nucleus than native PEI 25 K or PEI 1800. This efficient translocation into the nucleus may result in the high transfection efficiency of the GC–PEI polymer.

3.7. Molecular docking

The results and data of docking program were presented in Fig. 11 and Table 1. In Fig. 11, the best conformation was demonstrated as protein (cartoon) and GC substitutes (sticks). We found out that the GC parts of the grafting substitute all embedded into the pocket of glucocorticoid receptor, and had several interactions with the binding domain, e.g. hydrogen binding. In the meantime, the other side of the grafting substitutes, which created by Traunt's reagent, stretched out of the glucocorticoid receptor so that guaranteed the PEI it conjugated could combine and compress the therapeutic or reporting DNA.

The docking energy and binding energy of five GC substitutes showed slight difference. We considered the very similar structure of five GCs and the insufficient discrimination of Autodock itself may be the cause of this discovery, so we could not confidently conclude that there is a direct relationship between docking results and transfection results. What we could confirm is that the ligand–receptor complex possesses low energy and this ensures their stability in the process of transfection in ex vivo. However, the docking energy and binding energy of five GC could be divided into two main groups—DEX–PEI, BET–PEI (relatively high) and HC–PEI, MPL–PEI, PNL–PEI (relatively low). Hence, we were inspired to investigate relationships between the potency of GC and the transfection efficiency.

4. Discussion

The 21-hydroxy group is not required for pharmacological activity and was therefore a reasonable choice for conjugation to a polycation. The GC with 21-OH was reacted with methanesulfonyl chloride under mild condition to generate C_{21}-substituted GCs [24]. Then a one-pot reaction between PEI, 2-iminothiolane (Traut's reagent) and glucocorticoid mesylate yielded GC–PEI polymer. The proportion of reagents was remained same in every reaction so that the substitute degree of glucocorticoids was close to each other. This polymer possesses merits of both GC and PEI. PEI/DNA complexes that are taken up by the cell into acidified endosomal compartments are efficiently released into the cytoplasm, via the so-called 'proton sponge mechanism' and exhibit high transfection efficiency. The buffer capability of the five GC–PEI polymers resembled and they could escape from endosome and enter into cytoplasm efficiently without degradation.

The result of gel electrophoresis of GC–PEI/pDNA complexes showed that different polymers retarded DNA migration at same weight ratio, and there were no differences among the polymers to form complex with DNA. All GC–PEI polymers can efficiently condense pDNA into small nanoparticles. The sizes of polymer/pDNA complexes were around 100 nm above weight ratio of 0.8 which can readily undergo endocytosis [25]. After reaching the weight ratio of 2, the zeta potential of all five GC–PEI/pDNA complexes was positively charged surface within 10 mV which will give rise to similar affinity for anionic cell surfaces and in turn facilitate uptake into the cell.

Generally, lower molecular weight PEI was shown to have a reduced toxicity as compared to high molecular weight PEI. In this essay, the cytotoxicity of PEI 1800 modified with GC was higher

Table 1
Results of molecular docking of glucocorticoid grafting substitutes and glucocorticoid receptor.

polymer	Binding energy	Docking energy	Number of hydrogen bond	Ligands involved in hydrogen binding
BET substitute	−24.36	−26.69	4	ASN 564, GLN 570, ARG 611, GLN 642
DEX substitute	−24.92	−27.41	4	ASN 564, GLN 570, ASP 638, GLN 642
HC substitute	−22.19	−26.26	3	GLN 642, MET 604, ARG 611
MPL substitute	−22.96	−26.74	4	SER 746, GLN 642, ARG 611, GLN 570
PNL substitute	−22.53	−26.53	4	GLN 642, ASP 638, ARG 611, MET 604

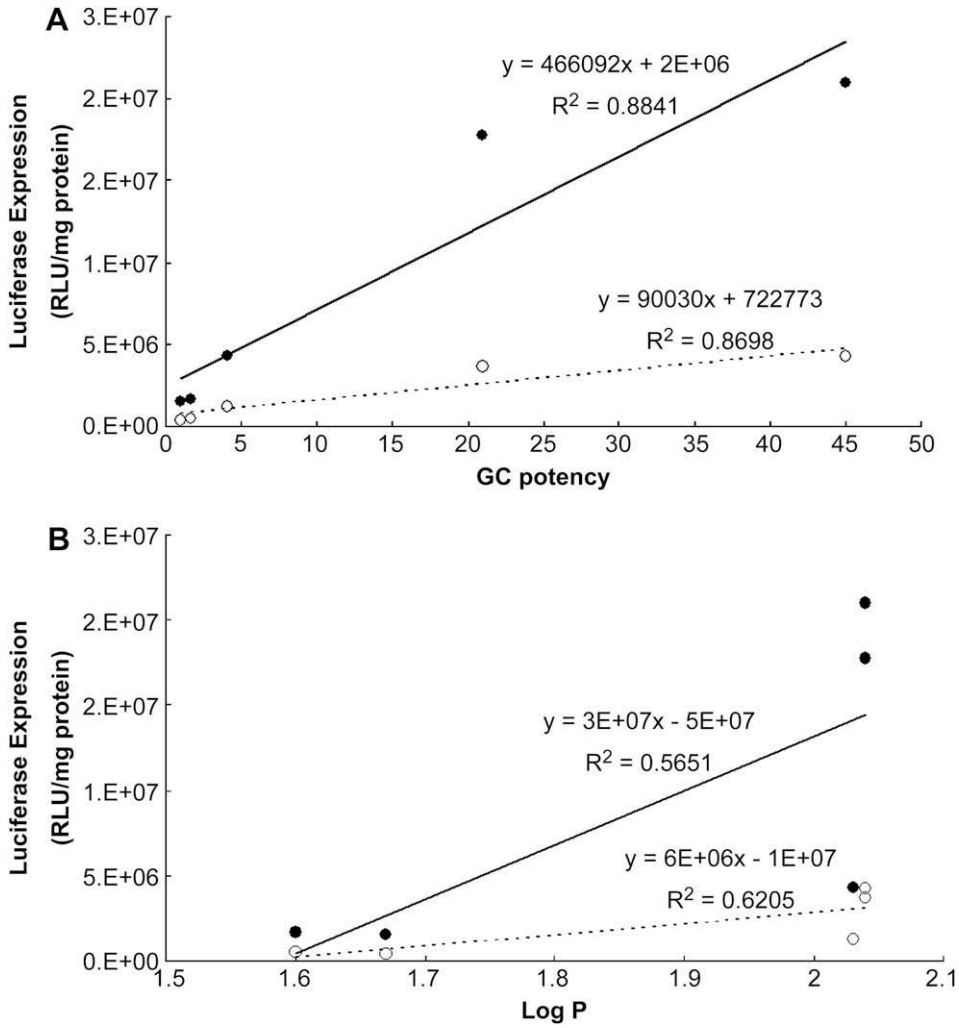

Fig. 12. The correlation between the five polymers' luciferase expression vs. GC potency (A) or log *P* (B) in HEK 293 cells (closed circles) and HepG2 cells (open circles). Linear regression was used to fit the lines to the data in HEK 293 cells (bold) and in HepG2 cells (dashed).

than that of native PEI 1800. We reasoned that more PEIs modified with GC were guided into cell nucleus by GC and in turn interfered with the cells viability. Furthermore, viability and proliferation of cells were also affected by GC. But, in general, the cytotoxicities of the GC–PEI polymers were lower than that of PEI 25 K.

It is well known that GC can be used as a nuclear translocation signal to facilitate the translocation of GC decorated complex into nucleus. From the confocal microscopy observations, it was confirmed that BET–PEI/DNA complexes were more effectively accumulated in the nucleus than PEI 25 K/DNA and PEI 1800/DNA complexes. This suggests that GC in the conjugate might have contributed to the efficient nuclear localization effect of the complexes causing greater increased transfection efficiency.

The conjugation of GC to polymeric gene carriers for increasing transfection efficiency has been studied [6,12–15,21–23], but deepgoing research was waiting.

In this essay, five commonly used GCs were conjugated to low molecular weight PEI. The transgenic expressions of the five GC–PEI polymers were evaluated. Comparison of transfection activities among these polymers showed that the transfection activity increases in the same order with their potency, irrespective of the cell type, so a correlation between binding affinities and transactivation properties can be assumed.

The MC was not selected. The physiological human ligands of the mineralocorticoid receptor (MR) and GR are aldosterone and cortisol respectively, and they have different chemical structures, manifest various binding affinity with the two receptors, and evoke respective distinct physiological effect. The important position for GC activity is the 11-hydroxy group, presents in the GCs but not in aldosterone (hemiacetal form), which consequently possesses a low GC potency. The potency of the five GCs is in an order followed by HC < PNL < MPL < DEX < BET [26]. A 6α-methyl (in MPL), 16α-methyl or 16β-methyl group (in BET and DEX) and a Δ1-dehydro-configuration (without in HC) of GCs resulted in an enhanced GC activity via the GR. However, opposite to the effect observed with the GR, the Δ1-dehydro-configuration and the 16α-methyl and 16β-methyl groups attenuate MC potency [27]. The 9α-fluorination (in BET and DEX) also leads to increased GC transactivation. For GC, the receptor binding is only a prerequisite of the much more complex process of gene regulation. Within the nucleus, the ligand-activated receptors regulate transcription via various pathways. A correlation between binding affinities and transactivation properties cannot be assumed a priori for all GCs. Thus, the binding affinity between different GCs and GRs was mocked by the molecular docking to demonstrate the magnitude of the bonding force.

The molecular docking certified that the binding affinity between GR and the five GCs was different. DEX–PEI and BET–PEI were relatively higher than that HC–PEI, MPL–PEI and PNL–PEI, which consisted with their potency and the transfection results.

In general, a hydrophobic residue may increase GC activity of a steroid so that it seems that log *P* of GC would influence its transfection efficiency. In fact, BET and DEX possess same value of log *P*, but their luciferase expressions were dissimilar. MPL has a similar log *P* with BET and DEX, but its luciferase expression was lower than that of the latter ones.

In order to reveal the relationship of GC–PEI polymers transgenic expression and GC potency or log *P*, the correlation between luciferase expression of the five polymers and the GC potency or log *P* in literature [27–29] was inhibited in Fig. 12. As can be seen from Fig. 12, the transgenic expression correlated linearly with GC potency. The higher was the potency of GC substituted on the polymer, the more effective was its transfection. The luciferase expression has no obvious relationship with the values of log *P*.

Furthermore, the molecular quantity of GC on every PEI backbone and the physico-chemical property of GC–PEI/pDNA nanoparticle were similar and could not impact on their transfection activity significantly. Therefore, one can conclude that receptor binding affinity is possibly a main factor to determine transactivation property for a GC–PEI polymer, and a GC-polymer with higher binding affinity with GR might lead to a better transfection activity for a series of GC-polymer with same properties in other sides.

5. Conclusion

In this paper, five GC–PEI polymers were synthetized. The physico-chemical properties and transfection activity of the GC–PEI/pDNA nanoparticles were investigated. Their physico-chemical properties resembled each other, but their transfection activity increased in the order of HC–PEI < PNL–PEI < MPL–PEI < DEX–PEI < BET–PEI. Their potency was surprisingly found to increase in the same order, suggesting that their binding affinity with GR closely correlated with their transfection activity. Therefore, it is likely that the GC–PEI polymers possessing higher binding affinity with GC might transfer more efficiently into cellular nucleus and produce significant gene expression. The molecular docking confirmed the presumption and the potency of binding affinity with GR is possibly determinative factor for their transfection activity. The complex with the highest binding affinity, BET–PEI/DNA complex, showed the most satisfactory transfection activity among all five transfection reagents. The materials, GC–PEI polymer, could not only promote gene expression in many cells, but also educe various pharmacological effects due to GC's versatile pharmacological actions. The study of GC–PEIs transfection efficiency in vivo is going to be done and their application as gene non-viral vector is highly promising.

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Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular Figs. 9–11, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.03.042.

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