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Revisit complexation between DNA and polyethylenimine — Effect of length of free polycationic chains on gene transfection

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ABSTRACT

Our revisit of the complexation between DNA and polyethylenimine (PEI) by using a combination of laser light scattering and gel electrophoresis confirms that nearly all the DNA chains are complexed with PEI to form polyplexes when the molar ratio of nitrogen from PEI to phosphate from DNA (N:P) reaches ~3, irrespective of the PEI chain length and solvent. Each solution mixture with N:P>3 contains two kinds of PEI chains: bound to DNA and free in the solution. It has been shown that it is those free PEI chains that play a vital role in promoting the gene transfection. The effects of the length of the bound and free chains on the gene transfection were respectively studied. Both short and long PEI chains are capable of condensing DNA completely at N:P~3 but long ones are ~10^2-fold more effective in the gene transfection, apparently due to their fast endocytosis and intracellular trafficking. The cellular uptake kinetics studied by flow cytometry reveals that long free chains increase the uptake rate constant of the DNA/PEI complexes. In the intracellular pathway, they are able to prevent the development of the later endolysosomes, and facilitate the subsequent release of the polyplexes from the endosomes. Our result shows that the "proton sponge" effect is not dominant because the shut-down of the proton pump only partially attenuates the transfection efficiency. A possible mechanism is speculated and presented.

1. Introduction

The gene therapy, considered as the treatment of genetically-caused diseases by transferring exogenous nucleic acids into specific cells of patients, has attracted great interests over the past few decades [1]. It has been gradually realized that the development of safe, efficient and controllable gene-delivery vectors has become a bottleneck in clinical applications. The gene transfection vectors can be generally divided as viral and non-viral ones. The non-viral vectors, including cationic polymers and lipids, offer several advantages in comparison with the viral ones, such as low immune toxicity, construction flexibility and facile fabrication [1–3]. However, their up-to-now low efficacy greatly limits their potential clinical applications. The introduction of polyethylenimine (PEI) as a non-viral vector represented a big leap because of its much higher efficiency in promoting the gene transfection [4–6]. After such a discovery, PEI has been modified in various ways to reduce its high cytotoxicity by shielding its cationic charges, increase its efficiency by coupling short PEI chains with intracellular biodegradable linkers [7,8], increase its serum stability by grafting poly(ethylene glycol) (PEG) on it [9,10], and enhance its cell targeting by attaching some functional molecules on its surface [9–12]. However, less attention has been paid to even ask why PEI remains one of the best non-viral vectors, how it facilitates the intracellular trafficking of the polyplexes, and when and where DNA is released from the polyplexes [13–24].

The first step in the preparation of non-viral vectors is to package long anionic DNA chains into a small particle, *i.e.*, the DNA complexation and condensation. Previous studies have accumulated many data about the effects of size, density and zeta-potential of the polyplexes on their final transfection efficiency [25–28], but a coherent picture remains lacking. Previous studies have attributed the high transfection efficiency of PEI to the so-called "proton sponge" effect; namely, further protonation of the PEI chains inside the endolysosomes due to their primary and secondary amines, which should lead to an influx of counter (chloride) ions so that the raising of the osmotic pressure inside could burst the endocytic vesicle and release the polyplexes [4,29]. Many people, especially those who jumped into this research field later, have taken this mechanism as granted. However, a number of researchers have always questioned

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whether such a "proton sponge" effect plays a dominant role in the high efficiency of PEI because of some contradictive results [23,30]. For example, long PEI chains are much more effective than short ones; and on the other hand, if only considering the colligativity, we know from thermodynamics that for a given weight concentration (g/mL), both short and long PEI chains should lead to a very similar number of chloride influx but short chains themselves should generate a slightly higher osmotic pressure inside the endocytic vesicles. Therefore, one could not put these two well-known facts together.

Recently, using a combination of different methods to characterize the size, molar mass and surface charge of the DNA/PEI polyplexes formed under different conditions, we previously found that nearly all the DNA chains are condensed by PEI to form the DNA/PEI polyplexes when the molar ratio of nitrogen from PEI to phosphate from DNA (N:P) reaches ~3, but the polyplexes have a high *in-vitro* gene transfection efficiency only when N:P ≥ 10 [31]. Putting these two facts together, we concluded and confirmed that (1) the extra 7 portions of the PEI chains are free in the solution mixture; and (2) it is these 7-portion free PEI chains that greatly promote the gene transfection no matter whether they are applied hours before or after the administration of the polyplexes (N:P = 3). Therefore, there are two kinds of PEI chains in the solution mixture: bound to DNA and free in the solution. Note that such an effect of free PEI chains was reported before but most of the results were only qualitative [32–36].

In the current study, we have further evaluated the effect of the length of both the bound and free PEI chains on the gene transfection by using different quantitative experimental methods. Particularly, we have explored how the chain length affects the cellular uptake and the subsequent intracellular trafficking. More importantly, we have investigated how the free chains with different lengths possibly prevent the development of the later endolysosomes and help the release of the polyplexes from the endosomes. In addition, we have also studied the effects of the chain topology and chemical structure.

2. Materials and methods

2.1. Materials and cell lines

Three branched PEIs (M_w = 800, 2000, and 25,000 g/mol, denoted as bPEI-0.8 K, bPEI-2 K, and bPEI-25 K) and two linear PEIs (M_w = 2500 and 25,000 g/mol, denoted as lPEI-2.5 K and lPEI-25 K) were respectively purchased from Sigma-Aldrich and Polysciences, and used without further purification. FITC-labeled bPEI-25 K was prepared as described [31]. Initial plasmid DNA pGL3-control vector (5256 bp) encoding modified firefly luciferase was purchased from Promega (USA). A large amount of this plasmid was prepared by ourselves using a Qiagen Plasmid Maxi Kit (Qiagen, Germany). Bafilomycin A1 (Baf-A1) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Deutschland). POPO-3 iodide was purchased from Invitrogen (USA). The Bright-Glo assay and CytoTox 96 non-radioactive cytotoxicity assay kits were purchased from Promega (Madison, USA). The fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and penicillin–streptomycin were products of GIBCO (USA). 293 T cells were grown at 37 °C, 5% CO_2 in DMEM supplemented with 10% FBS, penicillin at 100 units/mL and streptomycin at 100 μg/mL.

2.2. Formation of DNA/PEI polyplexes

The plasmid DNA was complexed with different PEIs in either distilled water or phosphate buffered saline (PBS) to form the DNA/PEI polyplexes as follows. Different amounts of the PEI solution ($C = 10^2$–10^3 μg/mL) were added dropwise into a dilute DNA solution (C = 14.5 μg/mL), resulting in different molar ratios of nitrogen from PEI to phosphate from DNA (N:P). Each resultant dispersion was incubated for 5 min at room temperature before being added to the cell culture medium. The DNA/PEI polyplexes were analyzed by the gel-shift assay (110 V for 20 min), in which the polyplexes were mixed with 6× loading buffer and then loaded on a 0.8% (w/v) agarose gel containing ethidium bromide in TBE buffer and the result was photographed under UV. The DNA binding was also characterized using laser light scattering.

2.3. Formation of phospholipid vesicles

Chloroform solutions of soybean phospholipids (0.09 g, the injectable grade, Shanghai Taiwei Incorporation) and cholesterol (0.03 g, Chengdu Kelong Incorporation) were mixed in a 25 mL round-bottom flask and the chloroform was removed at room temperature by rotary evaporator to form a uniform film. The flask was placed under vacuum for an additional 6 h. The dried film was hydrated by vortex with 20 mL of warm DI water and then subjected forty ultrasonic rounds (a total of 10 min) using an ultrasonic cell crusher machine. The solution mixture was further subjected to extrusion through a 450-nm PTFE hydrophilic filter to remove the large vesicles. Vesicle solutions were stored at 4 °C and diluted as needed for the laser light scattering characterization and zeta-potential measurement.

2.4. POPO-3 exclusion assay

The plasmid DNA was labeled with POPO-3 dye at a ratio of 1 dye per 100 bp. The DNA/PEI dispersion with different chosen N:P ratios were prepared as described above. The final DNA concentration was kept a constant (1.6 μg DNA per 100 μL PBS). After the 5-min incubation at room temperature, the relative fluorescence intensity (F) of each solution mixture was recorded using a Hitachi F7000 fluorescence spectrophotometer (excitation 534 nm, emission 568 nm). The fraction of the uncomplexed DNA chains (x) free in the solution mixture was determined by $x = (F_{polyplex} - F_{blank})/(F_{DNA} - F_{blank})$.

2.5. Laser light scattering (LLS)

A commercial LLS instrument (ALV5000) with a vertically polarized 22-mV He–Ne laser (632.8 nm, Uniphase) was used to characterize the polyplexes mediated by different PEIs. In static LLS [37], we can obtain the weight-averaged molar mass (M_w) and the z-averaged root-mean square radius of gyration ($<R_g>_z$) of scattering objects in a sufficiently dilute solution/dispersion from the angular and concentration dependence of the excess absolute scattering intensity (Rayleigh ratio $R_{vv}(q)$) as

$$\frac{KC}{R_{vv}(q)} \cong \frac{1}{M_w}\left(1 + \frac{1}{3}q^2R_g^2\right) \quad (1)$$

where $K \equiv 4\pi^2(dn/dC)^2/(N_A\lambda_0^4)$ and $q \equiv (4\pi n/\lambda_0)\sin(\theta/2)$ with dn/dC, N_A, λ_0, n and θ the specific refractive index increment, the Avogadro number, the incident wavelength in vacuum, the refractive index of solvent, and the scattering angle, respectively. Note that we have neglected the concentration correction here. The values of $(dn/dC)_{632.8\ nm}$ of DNA and different PEIs in water were determined using a novel laser differential refractometer [38]. $(dn/dC)_{632.8\ nm}$ of the polyplexes was calculated by using $(dn/dC)_{polyplex} = w_{DNA}(dn/dC)_{DNA} + w_{PEI}(dn/dC)_{PEI}$, where w_{DNA} and w_{PEI} are two normalized weight fractions of DNA and PEI inside the polyplexes, *i.e.*, $w_{DNA} + w_{PEI} = 1$. The measurable angular range was 20–50° for the polyplexes to ensure that $qR_g < 1$. All of the measurements were carried out at 25.0 ± 0.1 °C.

In dynamic LLS [39], the Laplace inversion of each measured intensity–intensity time correlation function ($G^{(2)}(q,t)$) in the self-beating mode can be related to a line-width distribution $G(\Gamma)$. For a diffusive relaxation, Γ is further related to the translational diffusion coefficient D by $(\Gamma/q^2)_{C\to 0,\ q\to 0} = D$. Therefore, $G(\Gamma)$ can be converted

into a translational diffusion coefficient distribution $G(D)$ or a hydrodynamic radius distribution $f(R_h)$ using the Stokes–Einstein equation, $R_h = k_B T/(6\pi\eta D)$, where k_B, T, and η are the Boltzmann constant, the absolute temperature, and the solvent viscosity, respectively.

2.6. Zeta-potential measurement

The average mobility (μ_E) of the polyplexes under an electric field in an aqueous solution was determined from the frequency shift in a laser Doppler spectrum using a commercial zeta-potential spectrometer (ZetaPlus, Brookhaven) with two platinum-coated electrodes and one He–Ne laser as the light source. Each data point presented in the mobility measurement was averaged over 10 times at 25 °C. The zeta-potential ($\zeta_{potential}$) can be calculated from μ_E using $\mu_E = 2\varepsilon\zeta_{potential} f(\kappa R_b)/(3\eta)$, where ε is the permittivity of water and $1/\kappa$ is the Debye screening length [40]. When $\kappa R_b \ll 1$ (the Hückel limit), $f(\kappa R_b) \approx 1$; while $\kappa R_b \gg 1$ (the Smoluchowski limit), $f(\kappa R_b) \approx 1.5$. In the current study, the Hückel and Smoluchowski limits are respectively used to calculate $\zeta_{potential}$ in water and PBS.

2.7. In vitro gene transfection

The *in vitro* gene transfection efficiency was quantified by using the luciferase transfection assays, in which plasmid pGL3 was used as an exogenous reporter gene. 293T cells were seeded in a 48-well plate at an initial density of 120,000 cells per well, 24 h prior to the gene transfection. Each DNA/PEI dispersion with a desired N:P ratio was further diluted in serum-free medium and then administered to the cells at a final concentration of 0.4 μg DNA per well. The complete DMEM medium (600 μL/well) was added 6 h after the administration of the DNA/PEI polyplexes. Using a GloMax 96 microplate luminometer (Promega, USA) and the Bio-Rad protein assay reagent, we respectively determined the transgene expression level and the corresponding protein concentration in each well at 48 h post-administration of the polyplexes. The gene transfection efficiency is expressed as a relative luminescence unit (RLU) per cellular protein (mean ± SD of triplicates).

2.8. Cytotoxicity assay

The cytotoxicity of free PEI chains and the corresponding DNA/PEI dispersions was evaluated on 293T cells by using the MTT assay. 293T cells were seeded in a 96-well plate at an initial density of 60,000 cells per well. After 24 h, free PEI chains alone or with DNA to form the DNA/PEI dispersions were respectively added to cells at different chosen concentrations and N:P ratios. For DNA/PEI dispersion, the final DNA concentration is 0.2 μg/well in a total volume of 100 μL. The treated cells were incubated in a humidified environment with 5% CO_2 at 37 °C for 48 h. The MTT reagent (in 20 μL PBS, 5 mg/mL) was then added to each well. The cells were further incubated for 4 h at 37 °C. The medium in each well was then removed and replaced by 200 μL of DMSO. The plate was gently agitated for 15 min. In each measurement, 100 μL of the DMSO mixture from each well of the plate was carefully transferred into a new 96-well plate before the absorbance (A) at 490 nm was recorded by a microplate reader (Bio-rad, USA). The cell viability (y) was calculated by $y = (A_{treated}/A_{control}) \times 100\%$, where $A_{treated}$ and $A_{control}$ are the absorbance of the cells cultured with polymer/polyplex and fresh culture medium, respectively. Each experiment condition was done in quadruplicate. The data was shown as the mean value plus a standard deviation (± SD).

2.9. Cellular uptake of polyplexes by flow cytometry

Plasmid pGL3 was covalently labeled with the fluorophore Cy5 using a Label IT nucleic acid labeling kit (Mirus, Madison, WI) as the manufacturer's instructions. The gel electrophoresis results confirmed that the labeling has no visible interference on the formation of DNA/PEI polyplexes (data not shown). 293T cells were seeded in a 12-well plate at an initial density of 500,000 cells per well. After 24 h, Cy5-pGL3/bPEI-25 K polyplexes (N:P = 3) without and with 7 portions of free bPEI-2 K or bPEI-25 K chains (N:P = 10) were added to the 293T cells in serum-free DMEM, respectively. The cells were incubated at 37 °C and harvested after different desired incubation times. The harvested cells were briefly rinsed twice with PBS containing 0.001% SDS and then with only PBS to remove the polyplexes remained outside of the cells [41]. The cells were further detached by 0.05% trypsin/EDTA supplemented with 20 mM sodium azide to prevent further endocytosis [42]. Finally, the cells were washed twice by pelleting and then resuspending in ice-cold PBS containing 2% FBS. The confocal microscopic images of the cells were taken to ensure that the removal of the polyplexes from the extracellular space and the cell surface was complete (data not shown). The cellular uptake of polyplexes was assayed by using a FC 500 flow cytometry system (Beckman Coulter, USA). The fluorophore Cy5 was excited at 635 nm and detected at 675/15 nm. To discriminate the viable cells from dead ones and to exclude possible doublets, the cells were gated by the forward/side scattering and pulse width. 10,000 gated events per sample were collected at least in duplicate.

2.10. Measurement of intracellular pH around polyplexes

Plasmid pGL3 was covalently double-labeled with pH-sensitive FITC and pH-insensitive Cy5 using a Label IT nucleic acid labeling kit (Mirus, Madison, WI). 293T cells were seeded in a 6-well plate at an initial density of 1,000,000 cells per well. After 24 h, double-labeled pGL3/bPEI-25 K polyplexes (N:P = 3) without and with 7 portions of free bPEI-2 K or bPEI-25 K chains (N:P = 10) were added to the 293T cells in serum-free DMEM, respectively. After 6-h incubation at 37 °C, the cells were harvested as described before and pelleted into six separate Eppendorf vials. The cells in two vials were resuspended with PBS containing 2% FBS, whereas the cells in the rest four vials were respectively resuspended with four intracellular pH clamping buffers (pH = 5.33, 6.03, 6.73 and 7.43) [43]. The cells in each vial were further washed by pelleting and resuspending in an appropriate and corresponding buffer. The FITC/Cy5 fluorescence ratio of each sample was measured using the flow cytometer. The fluorophores FITC and Cy5 were excited at 488 nm and 635 nm, respectively. The corresponding emissions were detected at 525/10 and 675/15 nm, respectively. The FITC/Cy5 fluorescence ratio was calculated using the median values of the FITC and Cy5 fluorescence intensities of the total cell population (10,000 cells). The four intracellular pH clamped samples generate a linear pH calibration, from which the FITC/Cy5 ratio of each of the other two duplicate samples corresponds to an average pH around the DNA/PEI polyplexes.

2.11. Lactate dehydrogenase (LDH) membrane integrity assay

293T cells were seeded in a 96-well plate at an initial density of 60,000 cells per well. After 24 h, cells were treated with bPEI-2 K or bPEI-25 K chains at different desired concentrations. The treated cells were incubated in a humidified environment with 5% CO_2 at 37 °C for 6 h. To determine the maximum LDH release, the 10× lysis solution was added to the control group 2 h prior to the usage of a CytoTox 96 Non-radioactive cytotoxicity assay kit (Promega, USA). In each measurement, 50 μL of the cell culture solution from each well of the plate was aspirated and mixed with 50 μL of the reconstituted substrate mix in a new 96-well plate. After 30-min incubation at the room temperature, 50 μL of the stop solution was added to each well before the absorbance (A) at 490 nm was recorded by a microplate reader (Bio-Rad, USA). The degree of LDH Release, defined as $(A_{treated} - A_{control})/(A_{maximum} - A_{control}) \times 100\%$, represents the membrane disruption induced by different PEIs,

where $A_{treated}$ and $A_{control}$ are the absorbance values of the cells cultured with and without PEI, and $A_{maximum}$ is the absorbance value of the cells in the maximum LDH release group. Each experiment condition was done in quadruplicate. The data were shown as the mean value plus a standard deviation (±SD).

3. Results and discussion

Fig. 1 shows the complexation profiles of DNA and branched PEI with different chain lengths, respectively evaluated by the gel-shift and POPO-3 exclusion assays. Progressive condensation can be revealed from the retarded mobility of the DNA bands and their reduced fluorescence intensity (Fig. 1A). Clearly, the two DNA bands (supercoiled and relaxed forms) disappear when N:P~2–3, independent of the PEI chain length. Further, we quantitatively estimated the extent of free and uncomplexed DNA chains at each given N:P ratio by using a double-strand DNA intercalating dye (POPO-3) whose fluorescence emission is dramatically enhanced upon its binding to DNA. The complexation of DNA with PEI excludes POPO-3 so that the fluorescence intensity decreases. Fig. 1B shows that over 90% of the DNA chains have been condensed into the polyplexes at N:P~3, irrespective of the PEI chain length.

On the other hand, we explored the N:P ratio dependence of the weight-averaged molar mass (M_w), the average hydrodynamic radius ($<R_h>$), the average chain density ($<\rho> \equiv M_w/[N_A(4/3)\pi<R_h>^3]$), and the zeta-potential ($\zeta_{potential}$) of the DNA/PEI complexes with different PEI chains using a combination of laser light scattering (LLS) and zeta-potential measurement (Fig. 2). There is no significant difference among the complexation profiles of DNA and PEI with different chain lengths. Namely, the polyplexes have a reversed positive $\zeta_{potential}$ at N:P~2–2.5 and further addition of more PEI chains in the solution mixture (*i.e.*, N:P>3) has nearly no effect on M_w, $<R_h>$, $<\rho>$ and $\zeta_{potential}$, revealing that those PEI chains added after N:P>3 are free in the solution mixture, which are invisible in LLS because the scattered light intensity is proportional to the square of mass of a scattering object. It is worth noting that short chains (bPEI-0.8 K) lead to larger DNA/PEI polyplexes (M_w~2.5×10^8 g/mol and $<R_h>$~110 nm) but a lower $\zeta_{potential}$ (~25 mV) than long chains (bPEI-25 K, M_w~1.8×10^7 g/mol, $<R_h>$~45 nm, and $\zeta_{potential}$~35 mV), presumably due to the previously proposed viscoelastic effect [44,45]. For comparison, we also used linear PEI chains with different lengths to complex with DNA in both salt-free water and salt-rich PBS and obtained similar results (not shown). Namely, (1) when N:P~3 most of DNA chains are complexed with PEIs; and (2) it is those uncomplexed PEI chains free in the solution mixture that greatly promote the gene transfection. On the basis of these results, we can use a combination of two different PEI chains: one to complex and condense DNA and the other with a different length as free chains. Before studying the gene transfection, we have known whether there is an exchange between bound chains inside the polyplexes and free chains in the solution mixture. Theoretically, short PEI chains free in the solution mixture are not able to replace long PEI chains inside the polyplexes because the immobile of short chains leads to relatively more loss of the translational entropy so that the total entropy gain is less in comparison with the immobile of long chains. Table 1 shows that the addition of free PEI with different chain lengths has nearly no effect on physical parameters of the polyplexes made of long bPEI-25 K chains. This result is further supported by the fluorescence assay, where long FITC-labeled bPEI-25 K chains were used to complex DNA to form the polyplexes. The fluorescence intensity decreases due to the quench of fluorophores in proximity. Fig. 3 shows that the addition of free PEI chains with different lengths has no effect on the

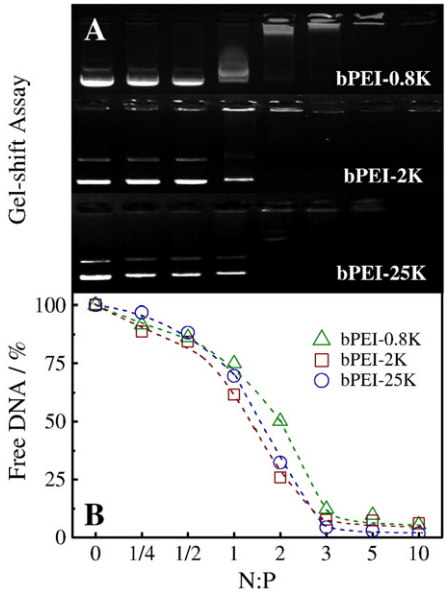

Fig. 1. Complexation of DNA by PEI with different chain lengths, evaluated by (A) gel-shift assay and (B) POPO-3 exclusion assay.

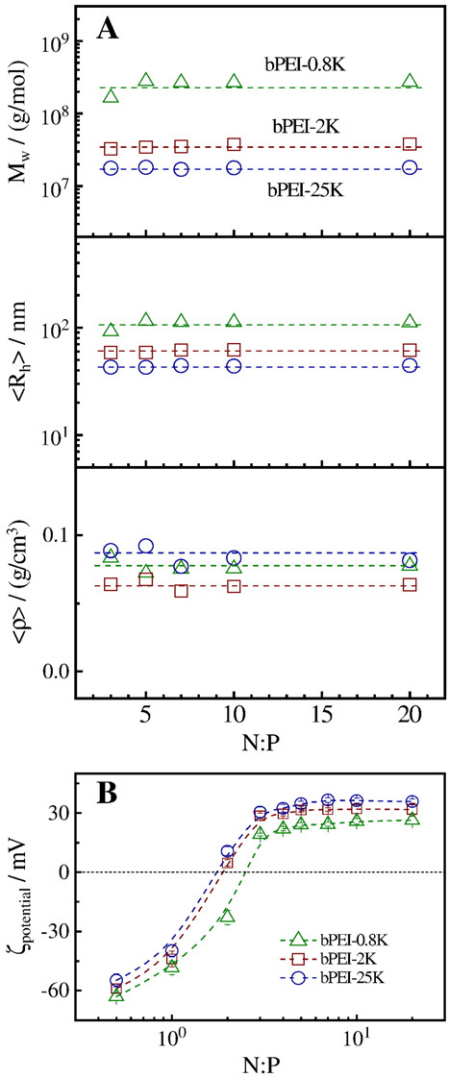

Fig. 2. (A) N:P ratio dependence of weight-averaged molar mass (M_w), average hydrodynamic radius ($<R_h>$) and average chain density ($<\rho> \equiv M_w/[N_A(4/3)\pi<R_h>^3]$) of different PEI-mediated polyplexes formed in water. (B) N:P ratio dependence of zeta-potential ($\zeta_{potential}$) of different PEI-mediated polyplexes in water.

Table 1
Molecular characterization of DNA/bPEI-25 K polyplexes (P) before and after addition of 7 portions of different free PEI chains.

Sample [a]	M_w/(g/mol) [b]	$<R_h>$/nm [b]	$<\rho>$/(g/cm^3) [b]	$\zeta_{potential}$/mV
P	2.2×10^7	44	0.11	32.9 ± 2.4
P + free b0.8 K	2.2×10^7	43	0.11	32.0 ± 2.9
P + free b2 K	2.4×10^7	44	0.11	33.2 ± 2.2
P + free lin2.5 K	2.3×10^7	42	0.13	31.4 ± 2.2
P + free lin25 K	2.5×10^7	46	0.10	32.1 ± 3.0

[a] The total and final N:P ratio was kept at N:P = 10, identical for all the samples.
[b] Determined by laser light scattering.

fluorescence intensity of the DNA/FITC-bPEI-25 K polyplexes (N:P = 3), clearly indicating that there is no interchange between the bPEI-25 K chains inside the polyplexes and short added branched or linear PEI chains free in the solution mixture. Otherwise, we would see an increase in the fluorescence intensity if there was some exchange between them.

Fig. 4 shows the effect of the length of free PEI chains on the gene transfection when long bPEI-25 K chains are used to complex and condense DNA, where t = 0 means a simultaneous addition of the DNA/bPEI-25 K polyplexes (N:P = 3) and 7 portions of free PEI chains with different lengths; and t > 0 represents that free PEI chains were added after administrating the polyplexes. Fig. 4 shows that the addition of free PEI chains essentially increases the gene transfection efficiency, irrespective of the chain length and topology (linear or branched). However, long chains, such as bPEI-25 K and lPEI-25 K, free in the solution mixture are much more effective (~10^2 fold) than their short counterparts. A combination of Figs. 1–4 shows that both short and long PEI chains are capable of condensing DNA completely at N:P ~ 3 but free short PEI chains are less effective in enhancing the gene transfection, indicating that the chain length plays an important role in the PEI-mediated gene transfection. On the other hand, it is interesting to note that for long chains (25 K), the chain topology makes no big difference in the transfection efficiency, but for short chains (~2 K), linear PEI chains are ~10 times more effective than branched ones. We will come back to this point later. Fig. 4 also shows that the efficacy of long free chains only slightly decreases if they are added after the administration of the polyplexes, implying that the DNA/bPEI-25 K polyplexes are fairly stable in the extra- and intracellular space up to 4 h.

Fig. 5A shows the effect of free PEI concentration on the gene transfection, where a pair of short and long branched chains (bPEI-2 K and bPEI-25 K) is used. Taking N:P = 3 (no free chains) as a reference, we can see that for short chains, the transfection efficiency increases with the PEI concentration, from ~10-fold at N:P = 7 to ~80-fold at N:P = 27; but for long chains, the transfection efficiency slightly decreases as N:P increases, which can be attributed to the fact that long chains are much more cytotoxic than short ones, especially at higher concentrations, as shown in Fig. 5B. Quantitatively, it is worth

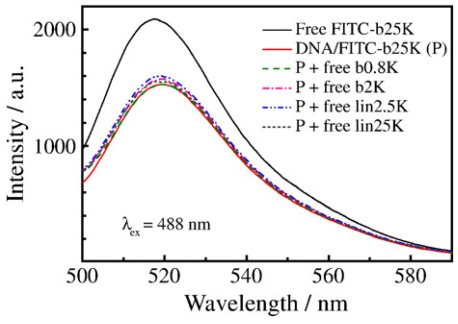

Fig. 3. Effect of addition of 7 portions of different free PEI chains on the fluorescence intensity of DNA/FITC-bPEI-25 K polyplexes (N:P = 3), where $C_{FITC\text{-}bPEI\text{-}25K}$ = 9.0 μg/mL, identical for all the tests.

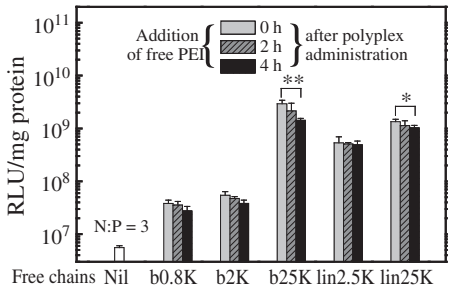

Fig. 4. Effect of length and topology of free PEI chains on the gene transfection efficiency in 293T cells, where 7 portions of different PEIs were added at 0, 2 or 4 h after administrating the DNA/bPEI-25 K polyplexes (N:P = 3). The total and final N:P ratio is 10, identical for all the tests. "Nil" means that no free PEI chains were added. **indicates $p<0.01$, and *indicates $p<0.05$, $n=3$, Student's *t*-test.

noting that when C_{bPEI} > 0.8 μg/mL, corresponding to N:P > 3, both the PEI solution and the DNA/PEI solution mixture exhibit a similar toxicity profile (Fig. 5B, $p>0.05$, $n=4$, Student's *t*-test). Therefore, it is those free PEI chains, especially long ones, that lead to the cytotoxicity at high N:P ratios. On the other hand, we should note that N:P ~ 10 is typically used for the gene transfection, corresponding to C_{bPEI} ~ 3 μg/mL at which both bPEI-2 K and bPEI-25 K chains exhibit little cytotoxicity and the cell viability is well above 85%. The pronounced cytotoxicity of long PEI chains is also reflected in their ability to alternate the cell morphology, which is attributed to the PEI-induced

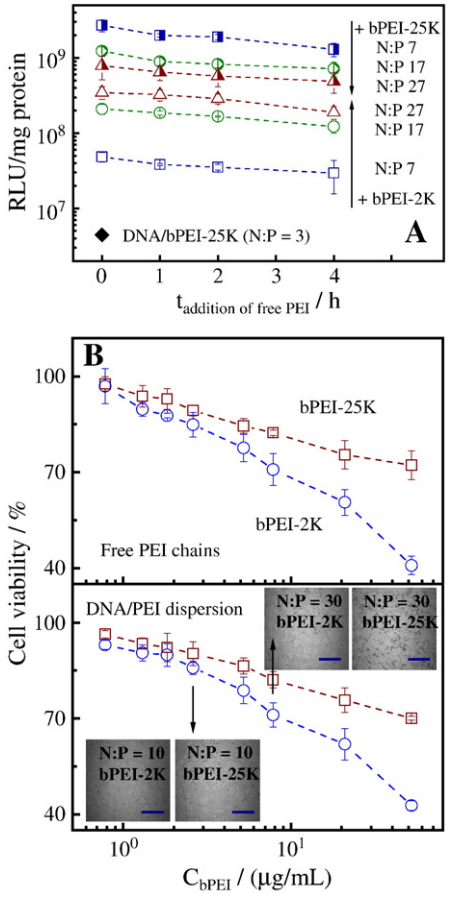

Fig. 5. (A) Effect of free PEI concentration on gene transfection efficiency, where free short (bPEI-2 K) and long (bPEI-25 K) chains were respectively added at different times after administrating DNA/bPEI-25 K polyplexes (N:P = 3). (B) Concentration dependence of 293T cell viability when PEI solution and DNA/PEI dispersion were respectively used, where MTT assay was used and C_{bPEI} = 2.7 μg/mL corresponds to N:P = 10. Insets: Microscopic images of 293T cells 6 h after the administration of DNA/bPEI-2 K and DNA/bPEI-25 K dispersions, respectively. Scale bar: 200 μm.

membrane disruption (also shown in Fig. 10) and apoptosis via mitochondrial pathway [46]. The inset of Fig. 5B reveals that after 6-h incubation with bPEI-25 K at N:P = 30, some of the cell contours turn round and many cell debris appear, leading to a reduced cellular metabolic activity and a decrease of the transgene expression inside the cells.

Fig. 6 shows the effect of the length and topology of the bound PEI chains on the gene transfection when long free PEI chains (bPEI-25 K) are used. Very surprisingly, in spite that the polyplexes (N:P = 10, the filled symbols) made of different PEI chains have very different transfection efficiencies, the replacement of 7 portions of free chains with long ones (bPEI-25 K) greatly enhances their efficiencies and reduces their differences when the polyplexes and long free chains are added at the same time (*i.e.*, t = 0). To generalize such an issue, we repeated this experiment with the HeLa cells by using lPEI-25 K as free chains. The results confirm that upon a simultaneous addition of free lPEI-25 K chains, the polyplexes made of different PEIs display a similar high gene transfection efficiency, regardless of the length and topology of the bound PEI chains used (Fig. S1).

Fig. 6 also shows that the gene transfection efficiency generally decreases as the delay time of adding free bPEI-25 K chains increases. For the polyplexes made of long bound chains, the delay time has much less effect on the transfection efficiency, but for those made of short chains, especially bPEI-0.8 K, the delayed addition of free chains significantly reduces their transfection efficiency. Likely, the addition of free bPEI-25 K chains to the dispersion of the polyplexes made of bPEI-0.8 K leads to a chain interchange so that long chains replace short ones, resulting in the formation of more stable DNA/bPEI-25 K polyplexes. To confirm it, we respectively added naked DNA and different PEI-mediated polyplexes (N:P = 3) to a solution containing 7 portions of free FITC-bPEI-25 K chains and monitored its fluorescence emission.

Fig. 7 shows that ~10 min after the polyplexes administration, the fluorescence intensity of FITC-PEI in each case decreases and reaches equilibrium after 1.5–2.0 h, indicating the interchange between long bPEI-25 K chains free in the solution and short branched or linear PEI chains inside the polyplexes. Namely, long branched PEI chains can replace most of short chains inside the polyplexes. In the gene transfection, when long bPEI-25 K chains were added hours after the administration of the polyplexes made of short chains, the chain replacement should not be a problem because the polyplexes have already moved inside the cell. Previous studies showed that short PEI chains have a lower binding affinity to DNA than long ones. Therefore, the polyplexes made of short PEI chains would be more likely to release DNA in the presence of other long anionic chains, such as proteins or RNAs, in the intracellular space [47,48], resulting in a lower transfection efficiency. Consequently, these less stable polyplexes made of short PEI chains were entrapped and digested inside

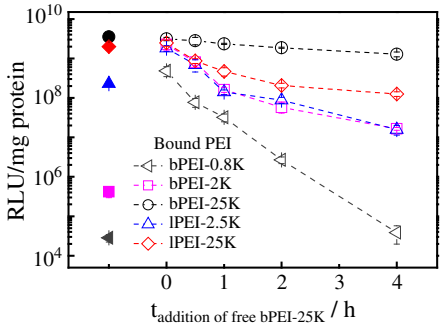

Fig. 6.

the later endolysosomes before the addition of long free PEI chains. To exclude the influence of such a chain interchange, we mainly used long PEI chains (bPEI-25 K) to condense DNA in the current study so that we can concentrate on the effect of length of free chains, mainly bPEI-2 K and bPEI-25 K, on the extra- and intra-cellular gene delivery. The real challenge is to elucidate how the long free cationic PEI chains prevent the development of the later endolysosomes, help the escape of the polyplexes from the endosomes, and promote the gene transfection in the intracellular space, including the nuclear localization/entry, and finally the de-assembly and transcription.

Since long free PEI chains significantly enhance the gene transfection, it is natural to ask whether they promote the cellular association and endocytosis in the extracellular space. To answer this question, we used the flow cytometry to monitor the cellular uptake kinetics of the DNA/PEI polyplexes in the presence of free PEI chains with different lengths. Generally, three characteristics can be extracted from one flow cytometry measurement, namely, (1) the fraction of cells containing Cy5-labeled DNA (Fig. 8A); (2) the median of the fluorescence intensity of Cy5-positive cell population ($F_{Cy5\text{-}DNA,\ Cy5+}$, Fig. 8B); and (3) the median of the fluorescence intensity of total cell population ($F_{Cy5\text{-}DNA,\ tot}$, Fig. 8C).

Fig. 8A shows that in the presence of 7 portions of free short bPEI-2 K or long bPEI-25 K chains much more cells can internalize the polyplexes within 1 h. In each comparison group, the cellular uptake nearly ceases and the fluorescence intensity approaches a plateau 6 h after the incubation. The corresponding percentages of the cell internalization respectively reach 75%, 95% and 65%. On the other hand, Fig. 8B shows that $F_{Cy5\text{-}DNA,\ Cy5+}$ (an indication of the average amount of polyplexes inside each Cy5-positive cell) in the first 6-h incubation remains ~2- or 10-fold higher when free bPEI-2 K or bPEI-25 K chains are added. A combination of Fig. 8A–C reveals that the addition of free PEI chains enhances the rate constant of the cellular uptake and long free PEI chains can make the uptake much faster than short ones. However, previous results demonstrated that the major play of free cationic PEI chains is mainly in the intracellular space [31]. The confocal live cell imaging revealed that most of the free PEI chains were co-localized together with the polyplexes in the endolysosomes [34], indicating that free chains might involve in the endolysosomal release. To have a more quantitative elucidation of such an involvement, we used a specific inhibitor for vacuolar ATPase proton pump (bafilomycin A1) to shut off the acidification of the early endosomes and prevent the

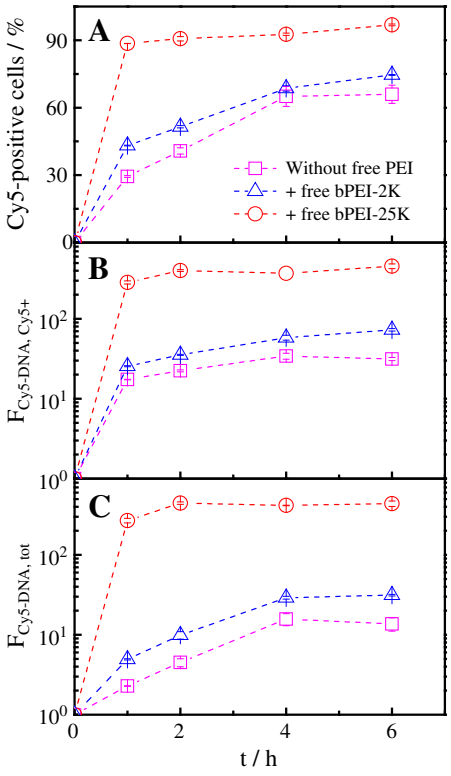

Fig. 8. Flow cytometry study of effect of length of free PEI chains on cellular uptake of Cy5-DNA/bPEI-25 K polyplexes, where 293 T cells were used, the transfection was respectively carried without (N:P = 3) and with 7 portions of free bPEI-2 K or bPEI-25 K chains (Final and total N:P = 10), and cellular uptake extent is expressed as (A) percentage of Cy5-positive cells; (B) median of Cy5-DNA fluorescence intensity of Cy5-positive cell population ($F_{Cy5\text{-}DNA,\ Cy5+}$); and (C) median of Cy5-DNA fluorescence intensity of total cell population ($F_{Cy5\text{-}DNA,\ tot}$).

development of the later endolysosomes [49,50]. Naturally, the shut-off of the proton pumps on the endolysosomes should also eliminate the so-called "proton-sponge" effect if any.

Fig. 9A shows that the addition of bafilomycin A1 reduces the gene transfection efficiency by a factor of ~4, ~6 and ~16, respectively, for N:P = 3 without free chains, N:P = 10 with short bPEI-2 K and long bPEI-25 K free chains. Relatively, the reduced transfection efficiency with long bPEI-25 K free chains (N:P = 10) is still ~20 times higher than that without free chains (N:P = 3). It clearly indicates that even without the help of the so-called "proton pump" effect, long free PEI chains can still help the polyplexes to avoid the entrapment of the endolysosomes, presumably via the prevention of development of the later endolysosomes or the promotion of the escape from the endosomes or both. Further, we tried to estimate when the polyplexes escape from the endolysosomes, respectively, without and with the addition of short bPEI-2 K and long bPEI-25 K free chains by adding bafilomycin A1 at different times after the administration of the DNA/PEI polyplexes.

Fig. 9B shows that ~6 h after the polyplexes administration, the addition of bafilomycin A1 has nearly no effect on the gene transfection efficiency when N:P = 10 (with long bPEI-25 K free chains), implying that most of the polyplexes are able to escape from the endolysosomes after ~6 h with the help of long free chains. Such an escaping time increases to ~10 h when short bPEI-2 K free chains are used. Without free chains, the escaping time is longer than 12 h. To further elucidate the escape from the endolysosomes, we monitored the environmental pH near the polyplexes in the intracellular space, as shown in Fig. 9C. Our study reveals that 6 h after the polyplexes administration, without free PEI chains (N:P = 3), the intracellular pH near the polyplexes dramatically decreases from ~7.4 to ~4.3, clearly showing that the polyplexes alone are unable to escape from the acidic lysosomes. In contrast, with short bPEI-2 K and long bPEI-25 K free chains (N:P = 10), the intracellular pH near the polyplexes slightly decreases to ~6.6 and ~7.1, respectively, implying that the polyplexes have escaped into the cytoplasm or still reside inside the endosomes that are not matured into the later endolysosomes. Relatively speaking, short cationic PEI chains are not as effective as long ones. Our current results should convince readers that the "proton sponge" effect might not be related to the osmotic pressure and is only partially responsible for the safe trafficking of the polyplexes inside the cell. We have to consider that free PEI chains might play positive roles via other mechanisms in the development of the later endolysosomes, the escape of the polyplexes from the endosomes into the cytosol, and the nuclear localization, release and transcription.

As discussed before, the role of PEI in facilitating the endolysosomal release was previously attributed to its ability to increase the osmotic pressure inside the endolysosomes via the proton sponge effect [4,29] or to destabilize the endolysosomal membrane via electrostatic interaction [15,51]. Our previous results showed that a simple chemical coupling of few bPEI-2 K chains via a linker can enhance the gene transfection by a factor of ~10^4 times [8], which could not be explained by the increase of

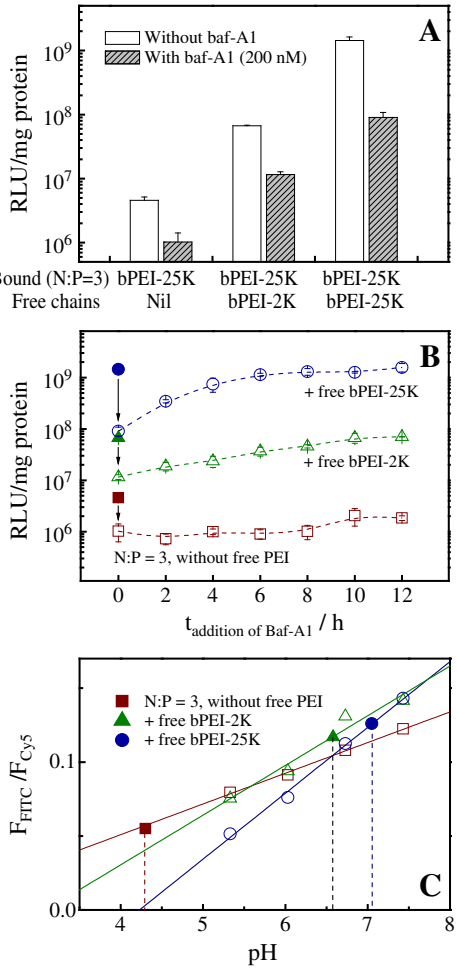

Fig. 9. (A) Effect of simultaneous addition of bafilomycin A1 on the gene transfection efficiency of the DNA/bPEI-25 K polyplexes (N:P = 3) without and with 7 portions of free bPEI-2 K or bPEI-25 K chains (N:P = 10). (B) Time-dependent effect of bafilomycin A1 treatment on the gene transfection efficiency of the DNA/bPEI-25 K polyplexes (N:P = 3) without and with 7 portions of free bPEI-2 K or bPEI-25 K chains (N:P = 10), where filled symbols denote that no bafilomycin A1 was added. (C) pH calibration curves (hollow symbols), where each symbol represents one study of DNA/bPEI-25 K polyplexes (N:P = 3) without and with 7 portions of free PEI chains, and filled symbols show the intracellular pH near the polyplexes in each case, which were respectively obtained from the normalized relative fluorescence intensity via each calibration curve.

the internal osmotic pressure (the "proton sponge" effect) because we did not alter the chemical structure. Therefore, we have to further explore how different PEI chains interact with the phospholipid membrane by using narrowly distributed phospholipid vesicles ($<R_g>=48.3$ nm, $<R_h>=42.2$ nm, and $\zeta_{potential}=-38.6\pm0.9$ mV) and 293T cells.

Thermodynamically, the attraction of cationic PEI chains to anionic vesicles is also entropy-driven due to the release of small counter ions (the gain of translational entropy). Therefore, long PEI chains lead to relatively less translational entropy lose than short ones so that the total gain in entropy is higher. Fig. 10 shows that long bPEI-25 K chains are much more disruptive to the cellular membrane than short bPEI-2 K chains for a given polymer concentration, especially when $C_{bPEI}\geq2.7$ μg/mL, corresponding to N:P≥10 in our gene transfection experiments. Our study of the synthetic phospholipid vesicles also reveal that long cationic bPEI-25 K chains can reverse the charge of the vesicles at a much lower concentration (~2 μg/mL) than short bPEI-0.8 K or bPEI-2 K chains. It seems that the destabilization and disruption of the cellular membrane by long free PEI chains is correlated to the release of the polyplexes from the endosomes in the intracellular space, and to some extent, the uptake of the polyplexes into the cells in the extracellular space.

Our previous and current results lead us to propose a hypothesis about why long free PEI chains are more effective in enhancing the gene transfection as follows. When long PEI chains are added into the cell culture medium, they are able to quickly penetrate different membranes of the cell and across the cytosol all the way into nucleus within less than one hour (data not shown). Some of them are embedded inside the membranes. Typical phospholipid bi-layer membranes with two anionic surfaces have a thickness of 5–6 nm. It is those embedded cationic chains that weaken/destabilize the anionic membrane via electrostatic interaction to facilitate the escape of the polyplexes from the endosomes. More importantly, in order to differentiate the endosomes entrapping foreign subjects from those vesicles generated by different organelles inside the cell, lysosomes normally use the membrane proteins attached to the inner surface of the cell membrane as a signal. The embedment of long cationic PEI chains on the cell membrane can shield those signal proteins so that the fusion among the endosomes and lysosomes and the development of the later endolysosomes are prevented or delayed. Using this hypothesis, we can explain all of those observed differences in the gene transfection efficiency, such as (1) why short free PEI chains are less effective because they are too short to shield the membrane proteins (*e.g.* bPEI-0.8 K~4 nm and bPEI-2 K~6 nm); (2) why lPEI-2.5 K is more effective than bPEI-2 K because linear PEI-2.5 K chains (~18 nm if stretched) are much longer than their branched counterparts even they have an identical molar mass; (3) why the coupling of 3–4 short bPEI-2 K chains into a long one (~18 nm) can enhance the transfection efficiency by a factor of ~10^4 times; and (4) why linear PEI-25 K and branched PEI-25 K chains have a similar transfection efficiency even though linear ones have a much longer contour length because linear chains are coiled in solution so that they have a similar size as the branched ones. It should be noted that the longer the cationic chains, the more cytotoxic they become. Therefore, there is a dedicate balance between the gene transfection efficiency and the cytotoxicity. Our hypothesis essentially suggests that one should choose cationic chains with a proper length (~15–20 nm if stretched) so that they are able to (1) insert themselves into the cell membrane via electrostatic interaction, and at the same time, (2) stick out their end(s) to shield the membrane proteins attached on the inner surface of the cell membrane.

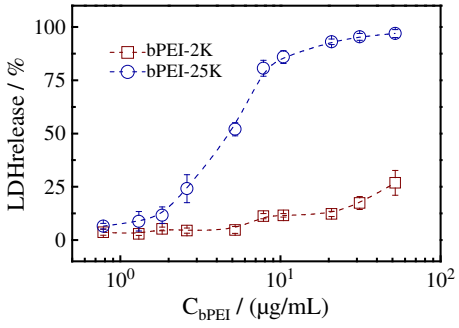

Fig. 10. Concentration dependence of LDH release from 293T cells, 6 h after they are treated with bPEI-2 K and bPEI-25 K chains, where $C_{bPEI}=2.7$ μg/mL corresponds to N:P = 10 used in an effective gene transfection.

4. Conclusion

A combination of laser light scattering (LLS) and gel electrophoresis results confirm that most of anionic DNA chains are complexed and condensed by cationic PEI chains when the molar ratio of nitrogen from PEI to phosphate from DNA (N:P) reaches ~3, irrespective of the chain length and solvent (pure water or PBS). In the solution mixture (N:P>3), there exists two kinds of PEI chains: bound to DNA and free in the solution mixture. The bound chains mainly provide the charge neutrality, resulting in the condensation and collapse of long plasmid DNA chains into 10^2-nm particles. It is those free PEI chains, rather than other physical properties of the DNA/PEI polyplexes, that play a vital role in promoting the gene transfection by a factor of $10–10^3$ times, depending on the chain length. Our results reveal that long bPEI-25 K free chains are ~10^2 more effective than their short counterparts (bPEI-0.8 K and bPEI-2 K). Long free cationic chains can significantly increase the uptake rate constant of the polyplexes, presumably due to their ability to disrupt the anionic cell membrane via electrostatic interaction. However, the main contribution of these long free PEI chains is in the intracellular space; namely, they prevent the entrapment of the DNA/PEI polyplexes into the endolysosomes. We can conclude that the increase of the osmotic pressure inside the later endolysosomes due to the "proton sponge" effect plays a certain role, but not dominant, in promoting the gene transfection. The current study leads to a hypothesis as follows. It is those free cationic PEI chains with a proper length (~15–20 nm) embedded inside the anionic cell membrane via electrostatic interaction that are able to (1) destabilize/weaken the endosome membrane (the "proton sponge" effect might help here) and promote the escape of the polyplexes entrapped inside; and (2) prevent the fusion between the endosomes and lysosomes so that further development of the later endolysosomes is hindered. This is because the stick-out cationic chain end(s) effectively shield those "signaling" anionic proteins embedded on the inner surface of the cell membrane (*i.e.*, the outer surface of the endosomes). Note that the development of non-viral vectors is an extremely complicated and multi-dimensional problem. Therefore, some fundamental understanding is not only needed but also a must. We should do a hypothesis-driven research instead of a "lottery-like" trial-and-error approach. The huge amount of literature (>10^5 publications) generated in the past 3–4 decades have already taught us a great lesson. On the basis of our hypothesis, we will modify short PEI chains (~5 nm) with a proper hydrophobic molecule (chain segment) (~2–5 nm in size) so that they can effectively stick inside the cell membrane and expose short PEI chains on its surface; and use them as the 7 portions of free cationic chains to promote the gene transfection. In this way, we hope to be able to solve the catch-22 "transfection efficiency"-*versus*-"cytotoxicity" problem because it has been known that short PEI chains are nearly not cytotoxic in a normal concentration range used for the gene transfection. Further, we will develop some methods to pack them together with the polyplexes so that they can be safely delivered to the targeted cells or organs in *in vivo* experiments.

Acknowledgements

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