Journal of Controlled Release 140 (2009) 40–46

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Revisit the complexation of PEI and DNA — How to make low cytotoxic and highly efficient PEI gene transfection non-viral vectors with a controllable chain length and structure?

Rui Deng [a,*], Yanan Yue [a], Fan Jin [a], Yangchao Chen [b], Hsiang-Fu Kung [b], Marie C.M. Lin [c], Chi Wu [a]

[a] Department of Chemistry, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong
[b] Stanley Ho Center for Emerging Infectious Diseases, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong
[c] Institute of Molecular Biology, Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong

ARTICLE INFO

Article history:
Received 20 January 2009
Accepted 9 July 2009
Available online 19 July 2009

Keywords:
Gene delivery
Polyethyleneimine
Disulfide
Laser light scattering
Chain structure

ABSTRACT

The commercially available branched polyethyleneimine (PEI) with a molar mass of 25 kD (PEI-25K) is an effective *in vitro* vector to transfer genes, but its cytotoxicity limits its applications in bio-related research. To solve such an efficiency-versus-cytotoxicity catch-22 problem, the disulfide bond has been previously used to link less toxic short PEI chains (2 kD), but previous literature results are controversial. Recently, we found that it is vitally important to remove both carbon dioxide and water in the linking reaction as well as to control the structure of the resultant chains linked by dithiobis(succinimidyl propionate) (DSP). Under a programmable mixing of PEI and DSP, we can use laser light scattering (LLS) to in-situ monitor the linking reaction kinetics in DMSO in terms of the change of the average molar mass (M_w). Therefore, we were able to withdraw a series of linked PEI chains with different molar masses from one reaction mixture. Two such linked PEI samples (M_w ~7 kD, PEI-7K-L and ~400 kD, PEI-400K-L) were used to illustrate the effect of the sample preparation and the chain structure on the *in vitro* gene transfection and cytotoxicity. Our results reveal that PEI-7K-L is less cytotoxic and more effective in the gene transfection than both PEI-25K and Lipofectamine 2000 in the *in vitro* gene transfection. However, PEI-400K-L has no gene transfection efficiency even though it is non-toxic.

1. Introduction

The development of non-viral vectors for gene transfection has attracted more and more interest in recent years because of their enhanced safety, biocompatibility, facile preparation and high flexibility to accommodate different sizes of DNAs in comparison with viral vectors [1–4]. Non-viral vectors are mostly made of cationic surfactants or polymers. Hundreds, if not thousands, of synthetic polymers or copolymers have been prepared and tested in the last few decades, but polyethyleneimine (PEI) is still regarded as one of the most effective non-viral vectors. Its high gene transfection efficiency is believed to originate from the so-called intrinsic "proton sponge" effect [5–8]; namely, partially protonated PEI absorbs more protons inside endocytic vesicles embedded with some ATPase proton pumps, accompanied by an influx of chloride counter-ions, ultimately rupturing endocytic vesicles due to a higher osmotic pressure.

It has been known that long PEI chains are highly effective in gene transfection, but more cytotoxic [9–11]. To circumvent such a catch-22 problem, short PEI chains are linked by some degradable linkers, i.e. ester, β-aminoester and disulfide, for the development of higher efficient and lower cytotoxic non-viral vectors in recent years [10,12–18]. The former two linkers have a hydrolysis half-life time ranging from hours to days. In contrast, the reduction of a disulfide is fast in the presence of glutathione (GSH) in cytosol [19–21]. Disulfide linkers can react with primary amine groups on PEI with a neutral or a preserved cationic linkage. It has been reported that dithiobis(succinimidyl propionate) (DSP), yielding a neutral amide inking bond, leads to a higher gene transfection efficiency than those with some charge-preserved linkages [14,22]. It was also reported that some DSP-linked branched PEI chains are highly effective and nearly non-toxic [13,14].

However, Kloeckner et al. [14] found that conditions of the linking reaction strongly affect the final molar mass and chain structure. Even for a given linker/PEI molar ratio, different groups obtained products with significantly different molar masses [12,13]. Most of the previous studies only listed the linker/PEI ratio instead of the actual average molar mass. To develop a useful macromolecular biomaterial, it is important to control its molar mass and structure before we study how its biomedical properties are affected by the chain length and structure. Previously, a mixture of DMSO and water was always used to dissolve PEI [12] because it is apparently insoluble in pure DMSO. The shortcoming of such a mixture solvent is that water inactivates

* Corresponding author.
E-mail address: dengruidr@gmail.com (R. Deng).

0168-3659/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2009.07.009

DSP because it hydrolyses both active ester terminus in the reaction mixture [23] or losses one proton-capture amine group on PEI by inactivating only one termini on DSP.

In the current study, we found that it is carbon dioxide that ionizes PEI and makes PEI insoluble in pure DMSO. Therefore, after the complete removal of water and carbon dioxide, PEI becomes soluble in pure DMSO so that we can avoid the side reaction. Further, we developed an in-situ laser light scattering (LLS) method to in-situ monitor the linking reaction in terms of the increase of the chain length. In this way, we can repeatedly obtain linked PEI chains with a desired molar mass. Armed with these well-defined PEI samples made of short PEI-2K chains, we studied the effects of the chain length and structure on their cytotoxicity and *in vitro* gene transfection efficiency.

2. Materials and methods

2.1. Materials and cell lines

Two branched PEIs (M_w = 2000 g/mol, PEI-2K; and M_w = 25,000 g/mol, PEI-25K), dithiobis(succinimidyl propionate) (DSP) and D,L-dithiothreitol (DTT) were purchased from Sigma-Aldrich and used without further purification. Dimethylsulphoxide (DMSO) was freshly dried under a reduced pressure before use. The PEI/DMSO solution was clarified by a 0.2-μm filter under the protection of N_2. Plasmid DNA pGL3 with a SV40 promoter and an enhancer sequences encoding luciferase was purchased from Promega (Madison, USA). Plasmid DNA pLUNIG-LIGL, a lentivirus vector with an enhanced green fluorescent protein (GFP) and a luciferase reporter gene was constructed in house. Lipofectamine 2000 was purchased from Invitrogen. Fetal bovine serum (FBS), phosphate buffered saline (PBS) and Dulbecco's modified Eagle's medium (DMEM) were products of GIBCO (NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Deutschland). Bright-Glo assay kit and DNaseI kit were purchased from Promega (Madison, USA), respectively. 293T cells were grown in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin in a humidified environment with 5% CO_2 at 37 °C.

2.2. Linking of short PEI-2K chains with DSP

As shown in Fig. 1, we developed a laser light scattering (LLS) device to in-situ monitor the linking reaction. The dust-free DSP/DMSO solution with a concentration of 1.0×10^{-2} g/mL was slowly injected into a PEI-2K/DMSO solution (4.6×10^{-2} g/mL) with a rate of 0.1 or 0.5 mL/h by a programmable syringe pump (Kd Scientific). The solution mixture was vigorously mixed. A commercial LLS spectrometer (ALV/DLS/SLS-5022F) was used to monitor the scattering intensity change during the linking reaction. Note that during each LLS measurement, the stirring was stopped. A container filled with NaOH was used to keep the solution mixture away from both water and carbon dioxide. A fraction of the reaction mixture was withdrawn by a syringe at different times, i.e., different DSP/PEI ratios. Each extraction was diluted with deionized water to stop the linking reaction and then dialyzed in deionized water, where a membrane with a cut-off molar mass of 500 was used.

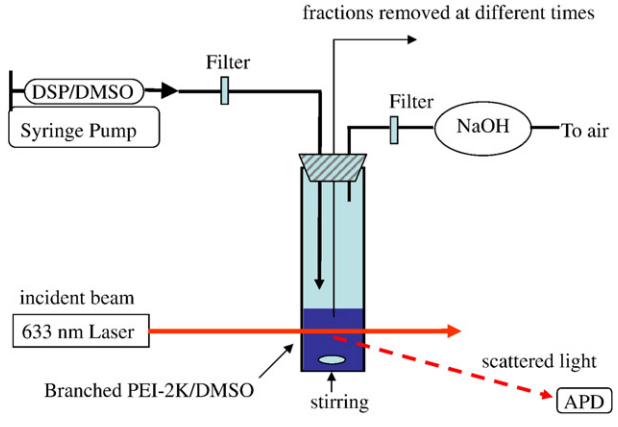

Fig. 1. Schematic of a recently developed reaction device to link polyethyleneimine (PEI) with dithiobis(succinimidyl propionate) (DSP) with an in-situ laser light scattering monitor.

2.3. Transmission electron microscopy

The samples for the transmission electron microscopy (TEM) studies were prepared using negative staining with 1% uranyl acetate. Briefly, a drop of the sample solution was allowed to settle on a carbon film-coated copper grid for 1 min, the excess sample was wicked away with filter paper and a drop of staining solution was allowed to contact the sample for 1 min. The samples were analyzed using a FEI CM120 electron microscope.

2.4. Cytotoxicity assay

The cytotoxicity assessment was carried out on 293T cells by using the MTT assay. 293T cells were seeded in a 96-well plate at an initial density of ca. 5000 cells/well in 100 μL of the DMEM complete medium. After 24 h, the cells were treated with polymers at different chosen concentrations. 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 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 100 μL DMSO. The plate was gently agitated for 15 min before the absorbance (A) at 490 nm was recorded by a microplate reader (Bio-Rad). The cell viability was calculated by

$$\text{Cell viability}(\%) = (A_{\text{treated}} / A_{\text{control}}) \times 100\% \quad (1)$$

where A_{treated} and A_{control} are the absorbance values of the cells cultured with and without PEI. Each experiment condition was done in quadruple. The data are shown as the mean value plus a standard deviation (±SD).

2.5. Formation of polyplexes

The DNA/PEI polyplexes were prepared with the different desired N:P ratios (ratios of nitrogen atoms on PEI to phosphates on plasmid DNA) by adding an appropriate amount of PEI to 2 μg of plasmid DNA (plasmid pGL3, 5 kbp) in PBS. The resultant polyplexes were incubated for 10 min at the room temperature before use.

Such prepared polyplexes were analyzed by the gel retardation assay, in which the polyplexes were mixed with a buffer and then loaded on a 0.8% agarose gel containing EtBr in TBE buffer. The gel electrophoresis was run at 80 V for 1 h and then photographed under UV. The DNA/Lipofectamine 2000 complexes were prepared according to the supplier's protocol [24]. In DNase assay, 2 units of DNaseI was added respectively to the plasmid DNA and the polyplexes, containing 1 mg of DNA, and incubated for 1 h at 37 °C. Before conducting the gel electrophoresis, 1 mL stop buffer was added and incubated at 65 °C for 15 min to stop the reaction.

A commercial laser light scattering (LLS) instrument (ALV5000) with a vertically polarized 22 mW He–Ne laser head (632.8 nm, Uniphase) was used to determine the particle size. In dynamic LLS (DLS), 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 $G^{(2)}$. For a diffusive relaxation, Γ is related to the translational diffusion coefficient D by $(\Gamma/q^2)_{(C\to 0,q\to 0)} = D$, so that $G(\Gamma)$ can be converted into a transitional diffusion coefficient distribution $G(D)$ or a hydrodynamic radius distribution $f(R_h)$ via 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.

The zeta-potential of the polyplexes dispersed in the PBS (1×10^{-2} μg/mL pDNA) was measured by a commercial Brookhaven Zeta Plus spectrometer with two platinum-coated electrodes and one He–Ne laser as the light source. The current was fixed at 10 mA and at least 30 cycles were measured for each polyplexes dispersion.

2.6. 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 report gene. 293T cells were plated in a 48-well plate at an initial density of ~50,000 cells/well, 24 h prior to the gene transfection. The polyplexes were further diluted in a serum-free medium and then added to each well. The final plasmid DNA concentration is 0.4 μg/well in a total volume of 300 μL. The complete DMEM medium (1 mL for each well) was added 4 h after the transfection. The gene expression was determined after 48 h by using a GloMax 96 Microplate Luminometer (Promega, USA). Meanwhile, the protein concentration in each well was determined by the Bio-Rad protein assay reagent (Bio-Rad, USA). The transfection efficiency is expressed as relative light units (RLU) per mg protein in each well (mean ± SD, triplicates). The transfection efficiency can also be directly visualized by a fluorescent microscope when the plasmid pLUNIG-LIGL (10 kbp) is used. The transfection procedure of using the two different plasmids was identical.

3. Results and discussion

After trying to use some previously reported procedures of linking short PEI chains with DSP [13,14], we found that PEI is insoluble in DMSO if there exists carbon dioxide (see Fig. S1 of Appendix A) because it reacts with PEI to form ammonium carbonate in the presence of a trace amount of H_2O. To the best of our knowledge, such a phenomenon has not been reported before. In the presence of CO_2 and a trace amount of H_2O, the reaction mixture is inhomogeneous. The addition of H_2O into DMSO can dissolve the ionized PEI chains [12], but some side reactions become inevitable. Therefore, we found that in order to make the linking reaction controllable, one has to completely remove CO_2 and H_2O from the reaction mixture.

Since branched PEI chains have more than two $-NH_2$ groups, the linking reaction can easily lead to the formation of microgels if it is not done properly. Previous work reported by Gosselin et al. [13] and Kloeckner et al. [14] used an equal or excessive amount of DSP so that their reaction mixture has to be purified by a column to remove microgels or even macrogels, which makes the process more complicated and expensive with a poorly controlled molar mass and a low yield. Particularly, for a given [linker]:[PEI] ratio, they obtained two linked PEI samples with different molar masses (2.3×10^4 and 8.0×10^3 g/mol). It is well known that a controllable molar mass is an essential requirement for a FDA approval. Furthermore, we found that after the removal of carbon dioxide and water, it is also vitally important to control how fast DSP is added into the PEI/DMSO solution. Namely, a quick addition of DSP leads to the formation of microgels so that the reaction mixture becomes turbid even at a low [DSP]:[PEI] ratio. In contrast, if DSP is extremely slowly introduced, the reaction mixture remains transparent even when [DSP]:[PEI] approaches 1. To make the reaction controllable and repeatable, we used a computer-controlled syringe pump to inject DSP into the PEI/DMSO solution (Fig. 1). During the linking reaction, LLS was used to in-situ monitor the change of the scattering intensities, i.e., the weight average molar mass (M_w) of the linked PEI chains. M_w can be calculated using:

$$M_w = M_{w,\text{PEI-2K}} \times \frac{\langle I_0\rangle_{\text{PEI}}}{C_{\text{PEI}}} \Big/ \frac{\langle I_0\rangle_{\text{PEI-2K}}}{C_{\text{PEI-2K}}} \quad (2)$$

where C_{PEI} and $C_{\text{PEI-2K}}$ are the concentrations of resultant linked PEI and initial short PEI-2K chains, respectively; $\langle I_0\rangle_{\text{PEI}}$ and $\langle I_0\rangle_{\text{PEI-2K}}$ are the time-average scattering intensities of resultant linked PEI and initial short PEI-2K chains at the zero scattering angle (θ), respectively. Assuming that

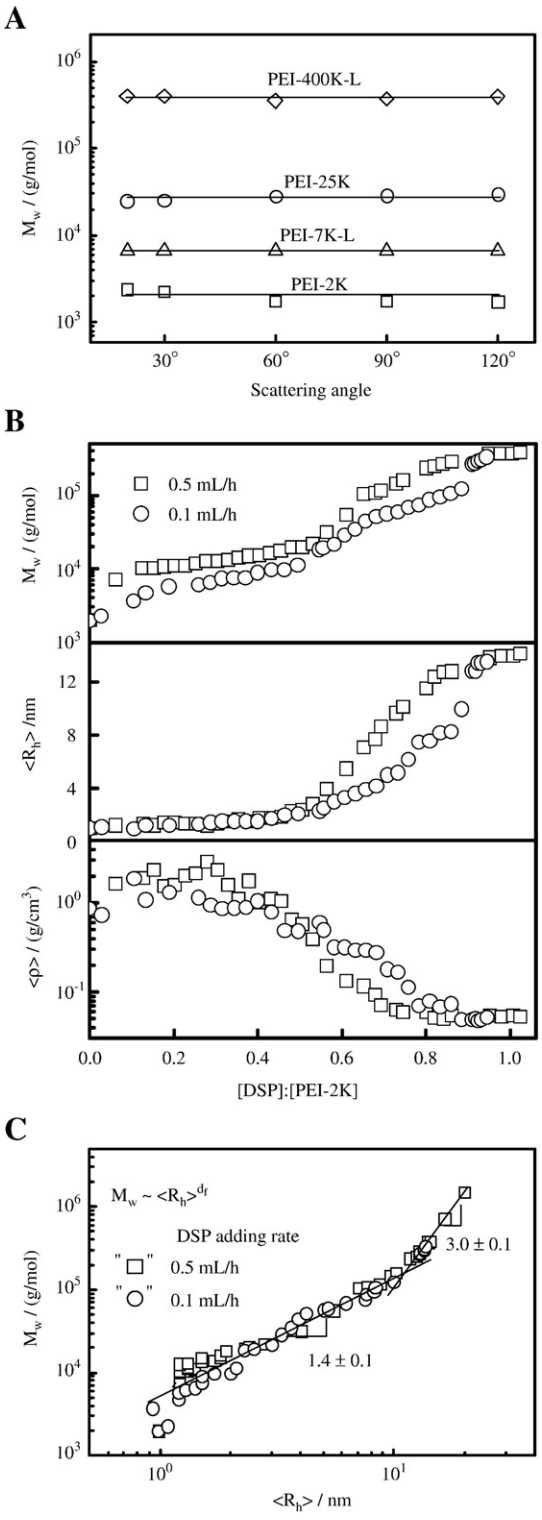

Fig. 2. A. Angular dependence of weight average molar mass (M_w) of different polyethyleneimine (PEI) polymers in water. B. Linking reagent (DSP) content dependence of weight average molar mass (M_w), average hydrodynamic radius ($\langle R_h\rangle$) and average chain density ($\langle\rho\rangle$) of resultant linked PEI-2K chains, where $\langle\rho\rangle = M_w/[(4/3)\pi\langle R_h\rangle^3]$. C. Double-logarithmic plots of weight average molar mass (M_w) vs average hydrodynamic radius ($\langle R_h\rangle$) of resultant linked PEI-2K chains.

most of PEI-2K chains are linked, we have $C_{PEI} \approx C_{PEI\text{-}2K}$ so that Eq. (2) can be rewritten as $M_w \approx M_{w,PEI\text{-}2K} \langle I_0 \rangle_{PEI} / \langle I_0 \rangle_{PEI\text{-}2K}$. Fig. 2A shows that $\langle I \rangle_{PEI}$ at each [DSP]:[PEI-2K] ratio is independent of the scattering angle, even for PEI-400K-L with the highest molar mass, indicating that the radius of gyration ($\langle R_g \rangle$) of these linked PEI chains must be smaller than 15 nm. In other words, we have $\langle R_g \rangle / \langle R_h \rangle < 1$ because $\langle R_h \rangle = 22$ nm for PEI-400K-L in H_2O. Therefore, PEI-400K-L has a spherical microgel conformation with a more uniform chain density [25–27]. To further testify the microgel conformation of PEI-400K-L, TEM images are taken and shown in Fig. S2 of Appendix A.

Fig. 2B shows how the DSP adding rate affects the weight average molar mass (M_w), the average hydrodynamic radius ($\langle R_h \rangle$) and the average chain density ($\langle \rho \rangle$) of resultant linked PEI chains, where $\langle \rho \rangle$ is defined as $M_w / [(4/3)\pi \langle R_h \rangle^3]$. The addition of DSP at a rate of 0.5 mL/h leads to a sharp initial increase of M_w from 2×10^3 to ~10^4 g/mol even when [DSP]:[PEI-2K] is only 0.1, indicating the formation of a small amount of large clusters at this initial stage. Further addition DSP results in a gradual increase of M_w before another sharp increase of M_w from 2×10^4 to 3.8×10^5 g/mol at [DSP]:[PEI-2K] ~1.0. Using a lower DSP adding rate of 0.1 mL/h, M_w is only 3.7×10^3 g/mol when [DSP]:[PEI-2K] = 0.1, revealing that on average only two chains are linked together. In contrast, the initial increase of $\langle R_h \rangle$ is less affected by the DSP adding rate.

Fig. 2C shows a double-logarithmic plot of M_w vs $\langle R_h \rangle$ for the linked PEI chains in DMSO. In the range of $1 < R_h < 10$ nm, $M_w \propto \langle R_h \rangle^{1.4 \pm 0.1}$, indicating that these linked PEI chains have a more extended linear structure with some inevitable branches. The scaling exponent (d_f) becomes 3.0 ± 0.1 when $R_h > 10$ nm, revealing that the linked PEI chains have a uniform chain density, presumably due to the formation of spherical microgels. A combination of Fig. 2B and C shows that when [DSP]:[PEI-2K] ~0.9 spherical microgels are formed in spite of different DSP adding rates. It is worth noting that the higher DSP adding rate always results in a broader molar mass distribution. Therefore, it is better to add DSP slowly into the PEI/DMSO solution in order to obtain more linearly linked PEI chains with less branches structure, as schematically shown in Fig. 3.

Fig. 4A shows that both M_w and $\langle R_h \rangle$ of PEI-7K-L and PEI-400K-L remain constants in the range of pH = 7.8–4.5, revealing that disulfide bond in an acidic environment is stable, which is important since the linked PEI chains should provide the same protection as long PEI-25K chains in the endosomal and lysosomal compartments. In contrast, the disulfide bond can be quickly reduced by DTT under a neutral or alkaline condition. Fig. 4B shows the reducing kinetics of the disulfide bond inside PEI-7K-L and PEI-400K-L in the presence of DTT at pH = 5 in terms of the decrease of M_w. It is clear that the breakage of the disulfide bond takes ~10 h in both cases, revealing that the initial reduction of the disulfide bond after the rupture of endolysosome should be a slow process because of its acidic micro-environment. Fig. 4C shows a better view of the break of the disulfide bond in PEI-7K-L after the addition of a reduce agent (DTT) because both M_w and $f(R_h)$ quickly decrease to their respective values of PEI-2K.

Armed with these well-characterized linked PEI chains, we studied the effects of the chain length and structure on their cytotoxicity and gene transfection efficiency. Two such linked PEI samples, respectively with $M_w = 7 \times 10^3$ and 4×10^5 g/mol, denoted as PEI-7K-L and PEI-400K-L, obtained from one reaction at two different [DSP]:[PEI-2K] ratios were used. Their molecular characterizations are summarized in Table 1. The cytotoxicity was tested by using the MTT assay. Fig. 5 shows that both PEI-7K-L and PEI-400K-L exhibit a significantly lower cytotoxicity in comparison with long PEI-25K chains. In the working concentration range, i.e., N:P ⩽ 80, corresponding to $C_{PEI} \leqslant 14$ μg/mL, PEI-7K-L and PEI-400K-L are even less cytotoxic than or comparable to a commercial vector, Lipofectamine 2000, at its optimal condition based on the supplier's protocol. It is worth noting that PEI-400K-L has a much higher molar mass than PEI-25K, but less cytotoxic, clearly indicating that linking short PEI chains into larger PEI molecules with disulfide bond can indeed reduce its cytotoxicity. However, it is strange that PEI-400K-L is even less cytotoxic than PEI-7K-L even its molar mass is higher. We will come back to this point later. Further, using the gel retardation assay, we studied the condensation of DNA with different PEIs.

Fig. 6A shows that PEI-25K can retard DNA at N:P = 3 and efficiently condense DNA at N:P = 5, reflecting in the disappearance of the two DNA strips. The complete complexation of DNA with PEI-7K-L occurs only when N:P >10, indicating that PEI-7K-L is a less effective condensing agent than PEI-25K. Surprisingly, PEI-400K-L is completely ineffective because the retardation appears only when N:P >60. Fig. 6B shows that the hydrodynamic radius ($\langle R_h \rangle$) of the polyplexes for each vectors measured in 30 mM NaCl. The $\langle R_h \rangle$ values of PEI-25K, PEI-7K-L and PEI-400K-L after the retardation occurs are ca. 50, 60 and 80 nm, respectively. All of the $\langle R_h \rangle$ increases slightly but not significantly with increasing of N:P ratio in a wide range. On the other hand, Fig. 6C shows that as expected, the zeta-potential of the polyplexes in PBS changes from −55 mV to +25 mV as the N:P ratio increases. Consisting with those gel retardation results, the addition of PEI-25K or PEI-7K-L inverses the zeta-potential at N:P ~3 and the zeta-potential reaches a plateau at N:P ~5. In contrast, when PEI-400K-L is used, a much higher ratio of N:P = 30 is needed to reverse the zeta-potential. The low complexation efficiency of DNA with PEI-400K-L can be attributed to its microgel nature. It is supposed that, as schematically shown in Fig. 3, DNA only wraps on the surface of the microgel, and such a DNA-winding complex is formed, resembling with the DNA chains winding around histone octamer to form nucleosome in the chromatin. With such a structure of PEI-400K-L/

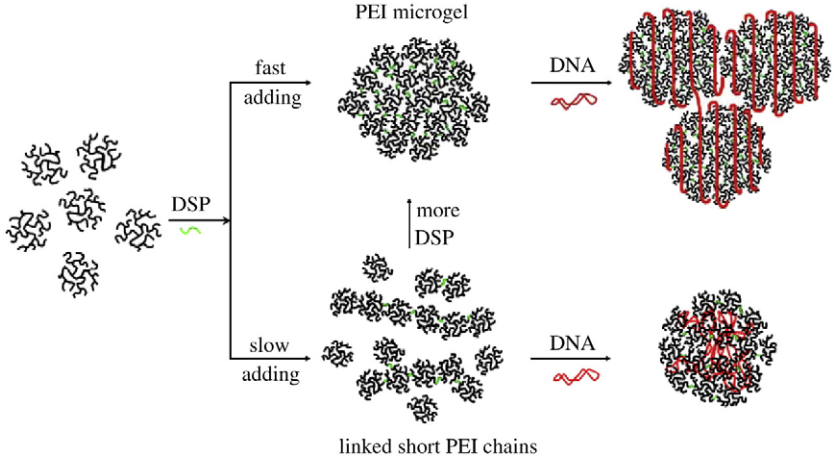

Fig. 3. Schematic of effect of different DSP adding rates on resultant linked PEI-2K chains and their corresponding complexation with plasmid DNA.

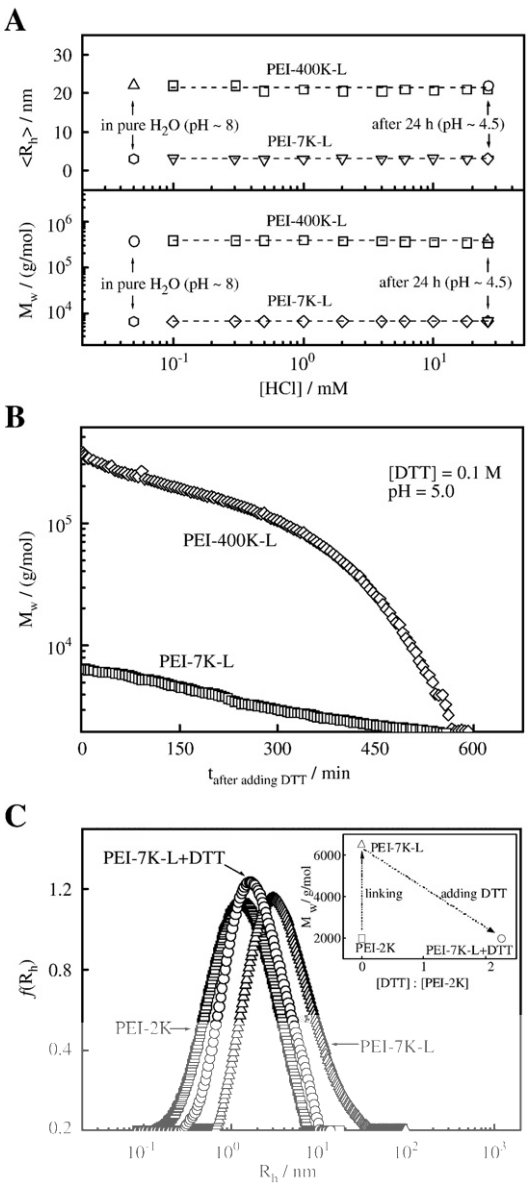

Fig. 4. A. HCl concentration-dependence of stability of linked PEI-2K chains (PEI-7K-L and PEI-400K-L) in acidic solutions in terms of changes of weight average molar mass (M_w) and average hydrodynamic radius $<R_h>$. B. Time dependence of M_w of PEI-7K-L and PEI-400K-L ($C = 1\times10^{-2}$ g/mL) with 0.1 M added D,L-dithiothreitol (DTT). C. Effect of adding a reducing agent, D,L-dithiothreitol (DTT, $C = 0.1$ M) on hydrodynamic radius distribution ($f(R_h)$) of resultant linked PEI chains (PEI-7K-L) in water at 37 °C. For comparison, we also plot $f(R_h)$ of initial PEI-2K.

DNA complexes, DNA is more accessible by EtBr molecule to emit stronger fluorescence even after the DNA is retarded in gel electrophoresis test (ca. N:P >40), whilst the fluorescence intensity is very weak for PEI25K or PEI-7K-L after the DNA is complexed and retarded, because the DNA is embedded by the smaller cationic PEI molecules which prevent the intercalating of EtBr into DNA helix. Such a DNA-winding structure is also proposed by some research groups through

Table 1
Molecular characterization of two linked PEI-2K samples in different solvents.

Sample	$\frac{n_{DSP}}{n_{PEI-2K}}$	M_w	$<R_h>$/nm	
		g/mol	in DMSO	in H_2O
PEI-7K-L	2.86×10^{-1}	6.5×10^3	1.4	3.0
PEI-400K-L	1.02	3.8×10^5	14	22

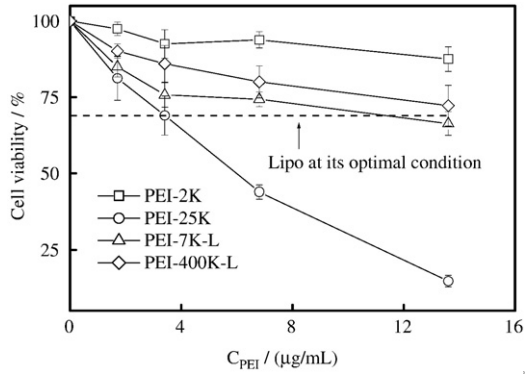

Fig. 5. Comparison of 293T cell viability of four different PEI samples.

theoretic calculation and experimental results [28,29]. To evaluate the protection effect of the vectors against enzyme degradation, the DNaseI was added to the complexes. As shown in Fig. 7, lane 3 shows that PEI-400K-L retards DNA at N:P = 60 and shows strong fluorescence in the slot. Lane 4 shows that after the addition of 2 M NaCl to the PEI-400K-L/DNA complexes, the DNA can be released again exhibiting near the same bands with naked DNA (lane 2). After the treatment of DNaseI, the naked DNA was degraded completely (lane 5). The PEI-400K-L/DNA complexes before and after the addition of salt also do not show any fluorescence anymore (lane 6 and 7), which means the poor DNA protection effect of PEI-400K-L comparing with PEI-25K and PEI-7K-L (data not shown). Such result may prove our proposed structure of the PEI-400K-L/DNA complexes, since the DNA chains wrapping around the positively charged microgel can be easily attacked by the enzyme, in contrast, the PEI-25K and PEI-7K-L can embed and protect DNA sufficiently.

Fig. 8A shows a comparison of *in vitro* transfection efficiencies of different PEI chains, where pGL3 is used as the report gene. PEI-25K shows an optimal efficiency at N:P ~10, consistent with previous reports [13,15,30]. Lipofectamine 2000 is ~6 times less efficient than PEI-25K in the transfection of 293T cells. The gene transfection efficiency of PEI-7K-L is 2–10 times higher than that of PEI-25K in the range of N:P = 10–60 and reaches its maximum at N:P = 30. The decrease of the transfection efficiency at higher N:P ratios can be attributed to the higher cytotoxicity. Again, the transfection efficiency of the DNA/PEI-400K-L polyplexes is even surprisingly lower than the naked DNA without any PEI.

The transfection efficiency can also be directly visualized with a fluorescent microscope when pLUNIG-LIGL is used as the report gene. Fig. 8B shows that the cells transfected by the DNA/PEI-7K-L polyplexes with N:P ~30 express more green fluorescent proteins than those transfected by the DNA/Lipofectamine 2000 and the DNA/PEI-25K polyplexes, respectively, at their optimal conditions. It reveals that PEI-7K-L as an effective vector to deliver larger plasmids is much better than those reported vectors [13,14]. We have to reconsider previous assumption that PEI with a higher molar mass are more effective in condensing, protecting and delivering genes into a cell [9]. Our results reveal that it is important to properly control the linking reaction to avoid the formation of a microgel structure.

4. Conclusion

Using a recently developed reaction device, we are able to in-situ monitor the linking reaction by using in-situ laser light scattering to control the molecular parameters of the linked PEI chains. We have found that in the reaction of using dithiobis(succinimidyl propionate) (DSP) to link short polyethyleneimine (PEI) chains (M_w = 2000 g/mol), it is vitally important to remove both a trace amount of water and CO_2. The DSP adding rate as well as the amount of DSP is also vitally important for the preparation of low cytotoxic and high efficient non-

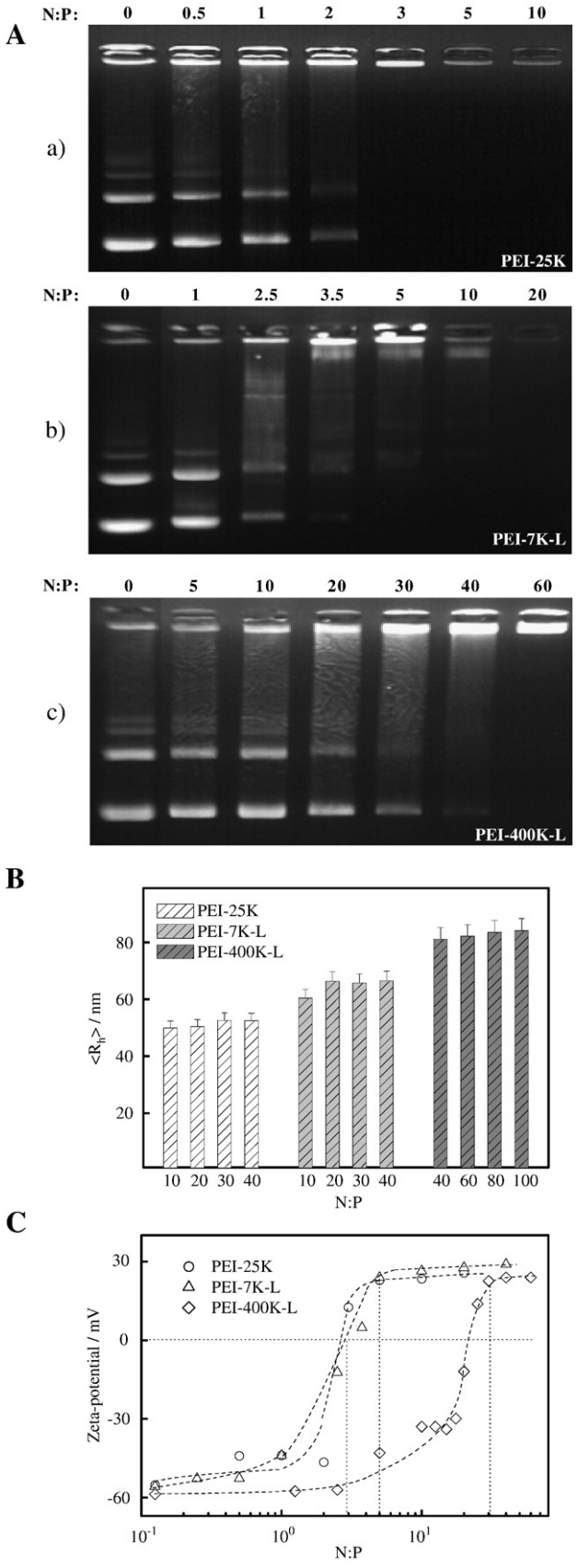

Fig. 6. A. N:P ratio dependence of gel retardation assay tests of three different PEI samples: a) PEI-25K, b) PEI-7K-L and c) PEI-400K-L. B. N:P ratio dependence of hydrodynamic radius (<R_h>) of different PEI/DNA polyplexes in 30 mM NaCl solution. C. N:P ratio dependence of zeta-potential of different PEI/DNA polyplexes in PBS buffer.

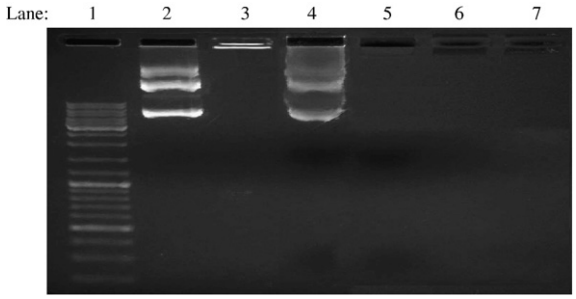

Fig. 7. DNase assay of DNA/PEI-400K-L complexes. Lane 1 is the DNA marker; lane 2 is naked pGL3; lane 3 is PEI-400K-L/DNA complex at N:P 60; lane 4 is 2 M NaCl treated PEI-400K-L/DNA complex at N:P 60; lane 5 is digested naked pGL3 by DNaseI; lane 6 is PEI-400K-L/DNA complex at N:P 60 treated with DNaseI; and lane 7 is 2 M NaCl treated PEI-400K-L/DNA complex at N:P 60 after digested with DNaseI.

viral gene transfection vectors because they affect not only the linked PEI chain length, but also the chain structure. A comparative study of the gene transfection efficiency and cytotoxicity of two such linked PEI samples (PEI-7K-L and PEI-400K-L, respectively with $M_w = 6.5 \times 10^3$

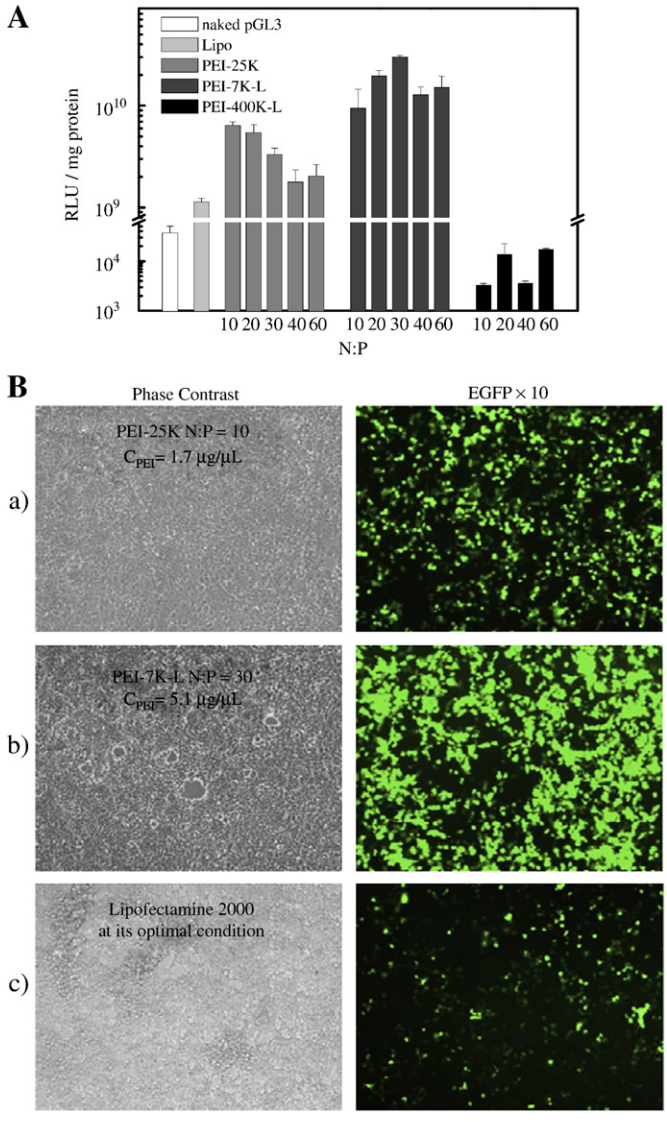

Fig. 8. A. N:P ratio dependence of *in vitro* gene transfection efficiency of naked pGL3 and four different non-viral vectors. B. Fluorescent microscopic images of 293T cells transfected with plasmid DNA (pLUNIG-LIGL) in the presence of different PEI vectors.

and 3.8×10^5 g/mol) reveals that PEI-7K-L with an extend linear chain structure is even less cytotoxic and 2–10 times more effective in the gene transfection of 293T cells than both the "golden standard" PEI-25K and the commercially widely used Lipofectamine 2000. On the other hand, PEI-400K-L with a spherical microgel structure is ineffective in spite that it is much non-toxic. Our current study clearly demonstrates that a proper control of the chain structure is more important than that of the overall molar mass; namely, one should try to avoid the microgel formation in the linking reaction.

Acknowledgements

The financial support of the Chinese Academy of Sciences (CAS) Special Grant (KJCX2-SW-H14), the National Natural Scientific Foundation of China (NNSFC) Projects (20534020 and 20574065) and the Hong Kong Special Administration Region (HKSAR) Earmarked Project (CUHK4036/05P, 2160269) is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2009.07.009.

References

[1] A.G. Schatzlein, Non-viral vectors in cancer gene therapy: principles and progress, Anti-Cancer Drugs 12 (4) (2001) 275–304.
[2] A.G. Schatzlein, Targeting of synthetic gene delivery systems, J. Biomed. Biotechnol. 2003 (2) (2003) 149–158.
[3] D.W. Pack, A.S. Hoffman, S. Pun, P.S. Stayton, Design and development of polymers for gene delivery, Nat. Rev. Drug Discov. 4 (7) (2005) 581–593.
[4] D.A. Jackson, S. Juranek, H.J. Lipps, Designing nonviral vectors for efficient gene transfer and long-term gene expression, Mol. Ther. 14 (5) (2006) 613–626.
[5] O. Boussif, M.A. Zanta, J.P. Behr, Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold, Gene Ther. 3 (12) (1996) 1074–1080.
[6] A. Baker, M. Saltik, H. Lehrmann, I. Killisch, V. Mautner, G. Lamm, G. Christofori, M. Cotten, Polyethylenimine (PEI) is a simple, inexpensive and effective reagent for condensing and linking plasmid DNA to adenovirus for gene delivery, Gene Ther. 4 (8) (1997) 773–782.
[7] W.T. Godbey, K.K. Wu, A.G. Mikos, Poly(ethylenimine) and its role in gene delivery, J. Control. Release 60 (2–3) (1999) 149–160.
[8] A.S. Verkman, N.D. Sonawane, F.C. Szoka, Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine–DNA polyplexes, J. Biol. Chem. 278 (45) (2003) 44826–44831.
[9] W.T. Godbey, K.K. Wu, A.G. Mikos, Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle, J. Biomed. Mater. Res. 45 (3) (1999) 268–275.
[10] D. Fischer, T. Bieber, Y.X. Li, H.P. Elsasser, T. Kissel, A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity, Pharm. Res. 16 (8) (1999) 1273–1279.
[11] A. von Harpe, H. Petersen, Y.X. Li, T. Kissel, Characterization of commercially available and synthesized polyethylenimines for gene delivery, J. Control. Release 69 (2) (2000) 309–322.
[12] M. Thomas, Q. Ge, J.J. Lu, J.Z. Chen, A.M. Klibanov, Cross-linked small polyethylenimines: while still nontoxic, deliver DNA efficiently to mammalian cells in vitro and in vivo, Pharm. Res. 22 (3) (2005) 373–380.
[13] M.A. Gosselin, W.J. Guo, R.J. Lee, Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine, Bioconjugate Chem. 12 (6) (2001) 989–994.
[14] J. Kloeckner, E. Wagner, M. Ogris, Degradable gene carriers based on oligomerized polyamines, Eur. J. Pharm. Sci. 29 (5) (2006) 414–425.
[15] Q. Peng, Z.L. Zhong, R.X. Zhuo, Disulfide cross-linked polyethylenimines (PEI) prepared via thiolation of low molecular weight PEI as highly efficient gene vectors, Bioconjugate Chem. 19 (2) (2008) 499–506.
[16] M. Breunig, U. Lungwitz, R. Liebl, A. Goepferich, Breaking up the correlation between efficacy and toxicity for nonviral gene delivery, Proc. Natl. Acad. Sci. U. S. A. 104 (36) (2007) 14454–14459.
[17] M. Ou, X.L. Wang, R.Z. Xu, C.W. Chang, D.A. Bull, S.W. Kim, Novel biodegradable poly(disulfide amine)s for gene delivery with high efficiency and low cytotoxicity, Bioconjugate Chem. 19 (3) (2008) 626–633.
[18] C. Lin, C.J. Blaauboer, M.M. Timoneda, M.C. Lok, M. van Steenbergen, W.E. Hennink, Z.Y. Zhong, J. Feijen, J.F.J. Engbersen, Bioreducible poly(amido amine)s with oligoamine side chains: synthesis, characterization, and structural effects on gene delivery, J. Control. Release 126 (2) (2008) 166–174.
[19] G. Saito, J.A. Swanson, K.D. Lee, Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities, Adv. Drug Deliv. Rev. 55 (2) (2003) 199–215.
[20] F.Q. Schafer, G.R. Buettner, Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple, Free Radic. Biol. Med. 30 (11) (2001) 1191–1212.
[21] Y. Lee, H. Mo, H. Koo, J.Y. Park, M.Y. Cho, G.W. Jin, J.S. Park, Visualization of the degradation of a disulfide polymer, linear poly(ethylenimine sulfide), for gene delivery, Bioconjugate Chem. 18 (1) (2007) 13–18.
[22] M.A. Gosselin, W.J. Guo, R.J. Lee, Incorporation of reversibly cross-linked polyplexes into LPDII vectors for gene delivery, Bioconjugate Chem. 13 (5) (2002) 1044–1053.
[23] A.J. Lomant, G. Fairbanks, Chemical probes of extended biological structures — synthesis and properties of cleavable protein cross-linking reagent [dithiobis (succinimidyl-S-35 propionate)], J. Mol. Biol. 104 (1) (1976) 243–261.
[24] B. Dalby, S. Cates, A. Harris, E.C. Ohki, M.L. Tilkins, P.J. Price, V.C. Ciccarone, Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications, Methods 33 (2) (2004) 95–103.
[25] W. Burchard, M. Schmidt, W.H. Stockmayer, Information on polydispersity and branching from combined quasi-elastic and integrated scattering, Macromolecules 13 (5) (1980) 1265–1272.
[26] A.Z. Akcasu, C.C. Han, Molecular-weight and temperature-dependence of polymer dimensions in solution, Macromolecules 12 (2) (1979) 276–280.
[27] W. Burchard, Light Scattering — Principles and Development, Clarendon Press, Oxford, 1996.
[28] T.T. Nguyen, B.I. Shklovskii, Complexation of DNA with positive spheres: phase diagram of charge inversion and reentrant condensation, J. Chem. Phys. 115 (15) (2001) 7298–7308.
[29] V.A. Kabanov, V.G. Sergeyev, O.A. Pyshkina, A.A. Zinchenko, A.B. Zezin, J.G.H. Joosten, J. Brackman, K. Yoshikawa, Interpolyelectrolyte complexes formed by DNA and astramol poly(propylene imine) dendrimers, Macromolecules 33 (26) (2000) 9587–9593.
[30] D.S. Manickam, D. Oupicky, Multiblock reducible copolypeptides containing histidine-rich and nuclear localization sequences for gene delivery, Bioconjugate Chem. 17 (6) (2006) 1395–1403.