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Size-dependent properties of M-PEIs nanogels for gene delivery in cancer cells

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Abstract

Polyethyleneimine nanogels (named as M-PEIs) with different sizes were prepared by photo-Fenton reaction in aqueous solution from which samples of 38, 75, 87, 121, 132 and 167 nm were selected for *in vitro* transfection. The homogeneous structure and the same component made it possible to study the size effect of M-PEIs nanogels on gene transfection efficiency when loading the same quantity of plasmid DNA (pLEGFP-C1) into A549, Bel7402, BGC-823 and Hela cells. M-PEIs and its DNA complexes were characterized by photo correlation spectroscopy and atomic force microscopy. The protein expression was observed by flow cytometry and fluorescence microscopy. All of the DNA complexes had no obvious cytotoxicity and the surface charges were positive charged at the optimum weight ratio. Therefore, the expressed protein was affected by the size of M-PEIs when the same quantity of DNA was used to transfect cells. In addition, the samples of 75 and 87 nm yielded the highest transfection efficiency about 30% in all of the four cell lines which were also cell line independent.

Keywords: Polyethyleneimine (PEI); Gene delivery; Size effect; Nanogels; Photochemistry

1. Introduction

Cationic liposomes and polycations of nanometer-dimension had been accepted as effective non-viral gene delivery vectors with low immunogenicity. They could be prepared more easily than viral vectors. The subcellular size facilitated the process in which nanoparticles were endocytosed by the cells, and this could increase cellular uptake of the protected gene (Davda and Labhasetwar, 2002). It was found that cationic polymers of different structural elements could self-assemble with DNA *via* electrostatic interaction to form complexes of different properties. The transfection efficiency of polycations were affected by many factors, such as particle size (Rudolph et al., 2003), morphology (Boffi et al., 2002), surface charge (Pang et al., 2002; Weecharangsan et al., 2006), ability to condense DNA (Nimesh et al., 2006), stability of DNA complexes (Rudolph et al., 2004), cytotoxicity (Florea et al., 2002) and transfected cell lines (cell line-dependent), etc. The particle size also affected the other factors significantly.

Nano-sized particles would be taken up easily by the cell via endocytosis, resulting in very high transfection efficiency. It was thought that particles smaller than 150 nm in size were more suitable for gene delivery because of greater numbers of smaller-sized nanoparticles taken up by the cells (Cherng et al., 1998; Nimesh et al., 2006). Poly(D,L-lactide-*co*-glycolide) nanoparticles of 148.7 nm were found to have higher transfection than those of 298.2 nm in HEK-293 and COS-7 cell lines under almost the same conditions of surface charge, cellular uptake and DNA release (Prabha, 2002). In addition, gene delivery vectors below size of 150 nm were usually of lower cytotoxicity, which was very important to the transfection efficiency (Cherng et al., 1998; Nimesh et al., 2006).

In contrast with much smaller nano-particles, sediment onto cell surface of the suitably large nano-particles would be more rapid which made the membrane favor to take up nano-particles into cells (Ogris et al., 1999; Tang and Szoka, 1997; Godbey et al., 1999). On the other hand, the suitably large nano-particles

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offered more protection to the plasmid DNA carried by them (Godbey et al., 1999).

Polyethyleneimine (PEI), as a nonviral gene delivery vectors, differs from the others in that every third atom in its backbone is a protonable nitrogen atom, which makes it easier to buffer endosomes. It was suggested that PEI might be a potential gene delivery vector as "proton-spone", with higher transfection efficiency than PLL and naked DNA (Boussif et al., 1995). Since then, many efforts had been made in studying PEI as gene delivery vectors and its size effects on the transfection efficiency. Godbey found that PEI molecules of Mw = 70,000 produced higher transfection efficiency than 10,000 (Godbey et al., 1999). In order to improve transfection efficiency, some groups also modified PEI and studied the size effect. It was found that the modified PEI had improved biocompatibility and hydrophilicity. For example, the protein expression levels of DNA loaded by glycosylated PEI were related more likely to the particle sizes but less to the surface charges (Bettinger et al., 1999).

Different gene delivery vectors of PEI had been synthesized using different methods, such as jet PEI, linear PEI (22 kDa), block PEI (800 Da and 25 kDa), and much more. However, little has been reported on spherical PEI nanogels. And because the difference existing in these PEI products, size effect of PEI particles on transfection efficiency were still ambiguous.

We had prepared a series of spherical PEI nanogels (named as M-PEIs) with size ranging from 38 to 168 nm and studied the size effect on transfection efficiency. Morphologies and sizes of the M-PEIs before and after DNA condensing were characterized by atomic force microscopy (AFM) and photo correlation spectroscopy (PCS), respectively. The samples used in this study had the same chemical structure and physical morphology. Biological tests were against four cell lines, i.e. human lung cancer A549, human liver cancer Bel7402, human cervix cancer Hela and human gastric cancer BGC-823 cell lines. Expressed protein was investigated by flow cytometry and fluorescence microscopy after plasmid DNA was transferred into the four kinds of cancer cell lines.

2. Materials and methods

2.1. Materials

PEI prepolymer (CAS 9002-98-6, as a mixture the molecular weight was not available) was purchased from Tokyo Chemical Industry. Roswell Park Memorial Institute (RPMI) 1640 (Giboco) medium was used as cell culture medium. Phosphate buffer solution (PBS) was prepared with 137 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, 0.4 mM KH_2PO_4 and 4.7 mM $NaHCO_3$. Ethylenediamine tetraacetic acid (EDTA) and tris(hydroxymethyl)aminomethane (Tris) was supplied by Shanghai Yito Enterprise Co., Ltd. Fetal bovine serum (FBS) was bought from Gibco BRL Co., Ltd. MTT, i.e. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was purchased from Sino-American Biotechnology Co., Ltd. The reporter gene for determining gene transfection efficiency was pLEGFP-C1 (6.9 kb). The plasmid DNA was amplified in *E. coli* DH5α competent cells, isolated and purified with QIA filter Mega Kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration and purity of the resulted DNA in a buffer (pH 7.5) was determined by measuring the absorbance at 260 and 280 nm respectively with a ratio of $OD_{260\,nm}/OD_{280\,nm} = 1.63$. Other reagents were of analytical grade. Double distilled water was used.

2.2. Preparation of M-PEIs with different sizes

In a three-necked quartz flask, PEI prepolymer was diluted in water with pH value being adjusted by 1 mol/l HCl. The solution was added with aqueous solution of $FeCl_2$ and bubbled with pure N_2 for 30 min to exclude O_2. H_2O_2 was added to the mixture under vigorous stirring before being irradiated by 253.7 nm UV light of 18 mW/cm^2 from low-pressure Hg lamps. The M-PEIs products were filtrated with membrane filters of 0.45 μm and treated further by dialysis bags with size exclusion below 10 kDa. The pH values of the solutions were adjusted to seven by 0.5 mol/l HCl before the size distributions and zeta potentials of M-PEIs nanogels were characterized by photo correlation spectroscopy (PCS, Zetasizer Nano ZS, Malvern Instruments, UK).

Changing the reaction conditions, spherical M-PEIs of different sizes and narrow size distributions were prepared. Detailed preparation of M-PEIs could be referred elsewhere (Yao et al., 2006a,b). As the vector's size should be below 150 nm to ensure ideal transfection efficiency (Cherng et al., 1998; Prabha et al., 2002; Nimesh et al., 2006), six samples in mean sizes of 38–168 nm (*Z* average size detected by PCS in aqueous solution, Table 1) were used for *in vitro* gene transfection.

Table 1
Mean particle size and zeta potential of M-PEIs nanogels

Sample no.	PEI prepolymer (weight ratio)	H_2O_2 (volume ratio)	pH	Sizes[a] (nm)	Poly·Index	Zeta potentials (mV)
1	6.30%	1.36%	8.23	38	0.272	2.12
2	5.90%	1.25%	8.71	75	0.346	8.29
3	3.33%	0.22%	8.45	87	0.269	10.90
4	5.90%	1.25%	8.12	121	0.526	48.28
5	6.00%	0.36%	8.52	132	0.441	33.30
6	4.55%	1.36%	8.65	168	0.206	24.20

Data are presented as the mean value of three measurement. Poly·Index is the polydispersity value.
[a] *Z* average value by PCS.

2.3. Preparation of M-PEIs/DNA complexes

M-PEIs samples were diluted with H_2O to appropriate concentrations, and the DNA solutions were diluted to 40 μg/ml. According to different weight ratios, 50 μl of PEI dilutions were mixed with 50 μl of DNA in Eppendorf tubes. The mixtures were then incubated for 10 min at room temperature before transfection. AFM and PCS were used respectively for measuring morphologies and sizes of M-PEIs and M-PEIs/DNA complexes.

2.4. In vitro cytotoxicity assay

A549, Bel7402, Hela and BGC-823 cells were obtained from Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. Cytotoxicity of the M-PEIs/DNA complexes was evaluated by means of MTT assay. Cells were seeded in 96-well plates (1×10^5 cells/well) with 200 μl RPMI-1640 in each well and incubated at 37 °C in a humidified atmosphere (5% CO_2) for 24 h. After removing culture medium, the control groups were treated with 100 μl normal saline while the experimental groups were treated with 100 μl M-PEIs/DNA complexes. Each treatment was done three times. All the cell samples were incubated again for another 24 h before 20 μl of 5 mg/ml MTT solution in PBS was added to each well. The plates were incubated once more for 4 h. Then, the MTT containing medium was removed and 150 μl dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals produced from the reduction of MTT by viable cells. The optical density was measured by ELISA reader (Microplate Reader, Model 550, Bio Rad, USA) at 570 nm. The cell viability was calculated by the following equation:

$$\text{cell viability} = \frac{\text{OD}_{\text{experiment}}}{\text{OD}_{\text{control}}} \times 100\% \quad (1)$$

2.5. In vitro transfection assay

Prior to transfection, the cells were seeded in 24-well plates at a density of 5×10^4 cells/well and cultured in RPMI-1640 containing 10% FBS at 37 °C in humidified 5% CO_2 atmosphere until 50–80% confluent was reached. Then the cells were washed once with PBS after RPMI-1640 was removed. After adding 100 μl of complex solutions to corresponding wells, the cells were cultured for 2 h at 37 °C in 5% CO_2 incubator. The cells were washed twice by PBS, and then the media was replaced by fresh RPMI-1640 containing 10% FBS and antibiotics PS before they were incubated again for 24 h at 37 °C. The proportion of positive cells was measured by fluorescence microscope to determine the transfection efficiency. At the same time, cells were harvested, washed twice with PBS, and resuspended in 300 μl PBS. The fluorescence intensities of 10,000 cells were examined by flow cytometry with a FACStar Plus cytometer (Becton Dickinson, San Jose, CA, USA) using 400 mW of 488 nm light from an argon ion laser. Sort windows were used on forward and side scatter to eliminate debris. Size and fluorescence emission at 507 nm were recorded at a rate of 800 cells/s. The data were analyzed using Cells Quest software (Becton Dickinson) plotting forward scatter versus GFP fluorescence.

2.6. Statistical analysis

Statistical analysis was performed using OriginPro. 7.0. A p-value of <0.05 was considered to be significant.

3. Results and discussion

3.1. Formation of M-PEIs/DNA complexes

All of the six M-PEIs samples were almost spherical in morphology and the AFM image of 75 nm M-PEIs as an example was revealed in Fig. 1a. Fig. 1b showed the AFM image of DNA complexes formed by 75 nm M-PEIs. It had been thought that incorporation of M-PEIs and DNA would be induced by both complex formation and physical entrapment (Jang et al., 2006). Fig. 1b showed that some DNA molecules were adhered to the surface of M-PEIs (due to probably electrostatic interaction) whiles others was embedded into the nanogels owing to the loose structure. One could be sure that in the surface of M-PEI nanogels there were many PEI branches that embedded and protected DNA from enzyme degradation. The branched morphology and the slightly positive surface made M-PEIs/DNA complexes dispersed very well in aqueous solution as revealed

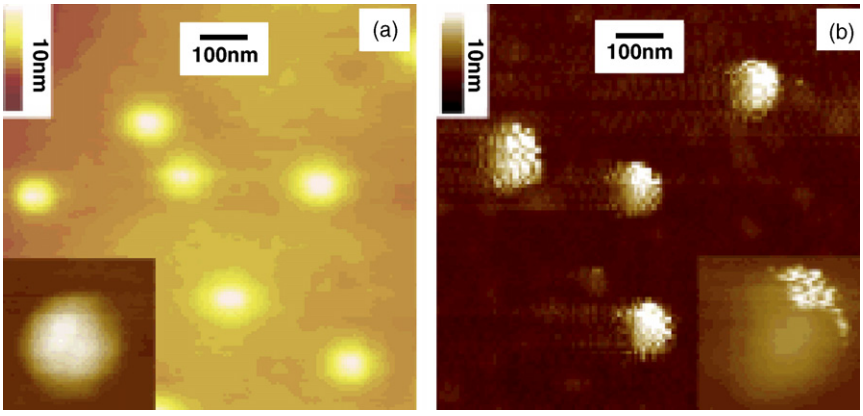

Fig. 1. AFM images of (a) M-PEIs and (b) M-PEIs/DNA complexes.

Table 2
Mean particle size and zeta potential of M-PEIs/DNA complexes

	Sample					
	38 nm	75 nm	87 nm	122 nm	132 nm	168 nm
M-PEIs nanogels (μg):DNA (μg)	20:2	6:2	4:2	0.4:2	1:2	1:2
Size (nm)	47	86	98	167	171	260
Zeta (mV)	0.519	4.06	3.22	2.12	4.15	6.87

Particle size (nm) and zeta potential (mV) were detected in water. Twenty micrograms of DNA was complexed with M-PEIs firstly and diluted to 1 ml.

in Fig. 1b. On the other hand, M-PEIs/DNA complexes could be aggregated on the negative surface of cell *via* electrostatic interaction. Nevertheless, it was still difficult for M-PEIs to entrap all DNA into their inside.

Usually, plasmid DNA incubated with low molecular weight vectors formed large complexes (in size of approximately 1.0 μm) and high molecular weight polymers were able to condense DNA effectively, hence the formation of smaller complex particles of 0.17–0.21 μm (Wetering et al., 1997). In this study, the sizes of M-PEIs/DNA complexes had no obvious difference comparing with original M-PEIs (data not shown). The reason might be that the molecular weight of these six samples was moderate. The zeta potentials of M-PEIs/DNA complexes were positive charged at the optimum weight ratios listed in Table 2.

3.2. Cytotoxicity study

Cytotoxicity was always the most important problem for gene delivery. Polycations could induce cytotoxicity due to electrostatic interactions with negatively charged cell membranes depending on the number, density and arrangement of cationic charges in the three-dimensional structure of the polymer (Fischer et al., 2003). But almost no toxicity was found with these slightly positive charged M-PEIs/DNA complexes. The cell viability of Hela cells treated by M-PEIs/DNA complexes was shown in Fig. 2. The low toxicity was probably related with two aspects. The first was that the M-PEIs could degrade into small molecules and excrete after transfecting DNA into nucleolus because the C=N bonds in the M-PEIs molecules (Yao et al., 2006a) might be degradable at pH 7.4 (Kim et al., 2005). It also might because the size of 30–170 nm was small enough to be excreted by these four kinds of cancer cells. Secondly, surface charge of the complexes at these M-PEIs/DNA ratios was close to neutrality which caused no obvious damage to the cell membranes at the selected ratios. In a word, M-PEIs in sizes of 30–170 nm could be used in *in vitro* transfection assays safely.

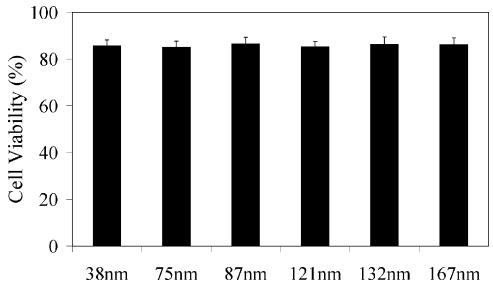

Fig. 2. Cytotoxicity of M-PEIs to cultured Hela cells.

3.3. In vitro transfection

Because the surface charges of all M-PEIs/DNA complexes were similar, the cells in every well were incubated with the same quantity of DNA containing complexes and the M-PEIs samples of 30–170 nm in size were noncytotoxic, the differences in protein expression could be related to the sizes of M-PEIs. From Fig. 3, one would agree that the DNA complexes combined with M-PEIs of 75 and 87 nm had higher transfection efficiency than the other samples when loaded DNA into A549, Bel7402, Hela and BGC-823 cells. M-PEIs of 75 and 87 nm made their DNA complexes sedimentate on negative membrane of cancer cell and licked up by the cancer cell more easily than the others. On the other hand, the suitable larger-sized nanoparticles might have slightly higher extent of DNA release than equal weight of the smaller-sized nanoparticles (Prabha, 2002). Therefore, the M-PEIs of 75 and 87 nm released greater quantities of DNA than 38 nm M-PEIs. In addition, larger M-PEIs could condense DNA to form more stable complexes that would delay the expression (Koping et al., 2001; Lavertul et al., 2006) which might be the other cause of higher transfection efficiency of 75 and 87 nm than the larger nanoparticles (121, 132, 167 nm) at 24 h. The critical change of GFP expression between 87 and 121 nm should be due to the sharp variation in size of their DNA complexes from 98 to 167 nm which indicated that the DNA complexes with size below 150 nm were more suitable for transfection.

From Fig. 3, it also could be seen that M-PEIs was cell line-independent for cancer cells which was unlike other gene transfection mediated by nanoparticles. The most suitable size range of M-PEIs for transfection into the four cancer cell lines was 70–90 nm, with maximum transfection efficiencies being about 30% at 24 h. Fig. 4 showed the protein expression for which DNA were transfected by 87 nm observed by fluorescence microscope at 24 h.

All above results demonstrated that the size of gene delivery vectors affected the transfection efficiency and a given range of the size would favor the particle entry into different cell lines. However, other cell lines, might have different capability to take up nanoparticles and request different size of M-PEIs to achieve the maximum expression.

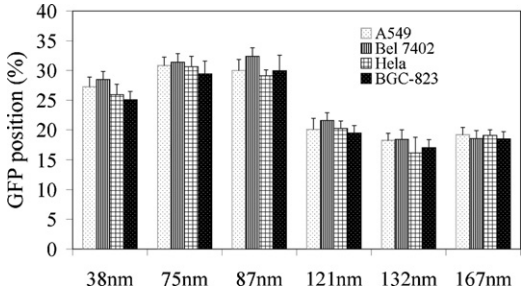

Fig. 3. Gene transfection efficiency by use of M-PEIs with different sizes transferred pLEGFP-C1 into A549, Bel7402, Hela and BGC-823 cells in method of flow cytometric assay. The maximum transfection efficiencies were 30.8%, 32.4%, 30.7%, 30.0%, respectively, after 24 h, $p < 0.01$.

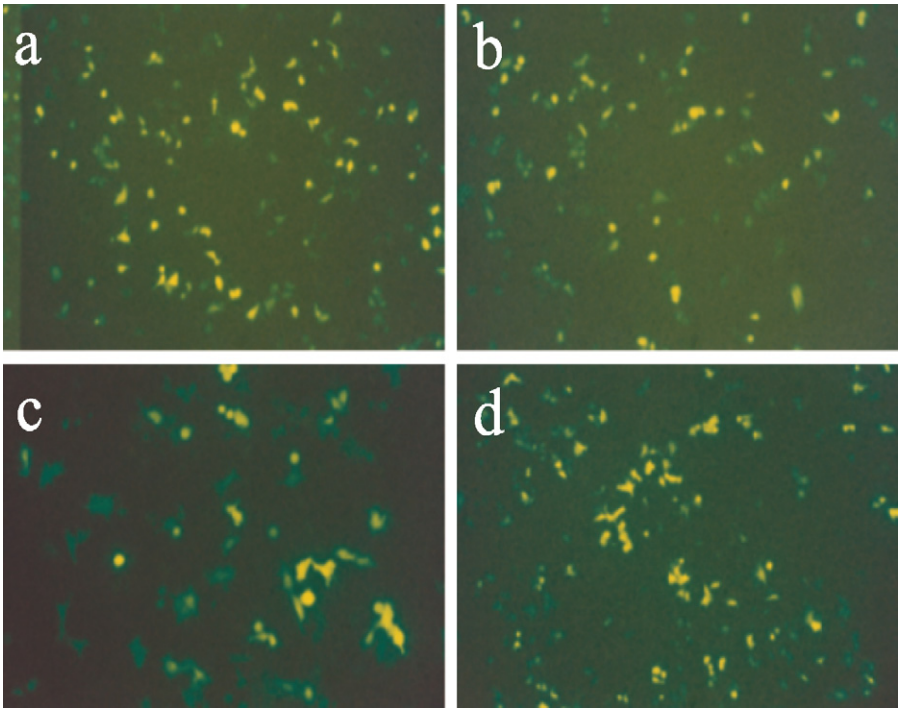

Fig. 4. Protein expressed in (a) A549, (b) Bel7402, (c) Hela, (d) BGC-823 transfected by M-PEIs nanogels (75 nm)/DNA after 24 h and the images were recorded at (a) 10×, (b) 10×, (c) 20× and (d) 10× magnification, respectively.

4. Conclusion

In this study, a series of M-PEIs nanogels with different sizes were prepared by photo-Fenton reaction to examine the size effect on transfection efficiency. M-PEIs had good performance as vectors to transfer DNA into cancer cell lines. GFP transferred into human lung, liver, ovary and esophagus cancer cell lines was performed by use of 38, 75, 87, 121, 132 and 167 nm M-PEIs, respectively. DNA complexes formed by 75 and 87 nm M-PEIs were more efficient than the others when the low cytotoxicity, zeta potential and the quantity of DNA used to transfect cells were almost same.

Suicide gene hTERT-CD-TK delivered by M-PEIs nanostructured hydrogels was practiced for therapy of lung cancer on the back of lotus mice by injection in abdomen, and encouraging results have been obtained (Jin et al., 2005). However, considerable efforts would have to be made before we could have a better understanding about gene therapy with M-PEIs.

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