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Guanidinylated allylamine-*N*-isopropylacrylamide copolymer nonviral transgene vectors

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Abstract

N-Isopropylacrylamide and allylamine copolymers (PNIALM) were prepared by radical polymerization method. To endow them with arginine-like cell membrane penetrating function, the aminos in PNIALMs were transformed to guanidinium groups by chemical modification. The formation and guanidinylation PNIALM were confirmed by NMR; the composition of copolymers and the degree of substitution of guanidino in modified copolymer (PNIALM-G) were estimated as well. The electrophoretic assay revealed that PNIALM-G was capable of condensing DNA in spite of lower binding affinity compared to its parent copolymers. The results of particle size analyzer and TEM indicated that at higher copolymer/DNA weight ratios, the copolymer/DNA complexes were condensed to nanoparticles. PNIALM-G1-3 was shown to be very efficient in mediating plasmid DNA transfection to COS-1 cells both in the presence and absence of serum, even superior to PEI.

Keywords: *N*-Isopropylacrylamide; Allylamine; Guanidinylation; Nonviral vector

1. Introduction

In gene delivery systems, amine-carrying cationic nonviral vectors such as PAMAM dendrimer (Kono et al., 2005), chitosan (Liu et al., 2003), PEI (Weiss et al., 2006), polypeptide (Emi et al., 1997) and amino-modified nanotube (Pantarotto et al., 2004), have been extensively investigated in the past decade. A common feature of these vectors is that the protonated aminos are able to effectively condense DNA into narrow globular or toroidal chambers, thereby protecting gene from enzyme degradation. However, their applications have been greatly hindered by low transfection efficiency or high cytotoxicity (Forrest et al., 2005; Boussif et al., 1999). Recently, arginine-rich peptides and oligoarginines were reportedly shown to facilitate the cellular internalization of DNA and proteins though the mechanism underlying is still not very clear (van Rossenberg et al., 2004; Rudolph et al., 2003). Nonetheless, it has been widely evidenced that guanidinium group in arginine peptides plays an important role in cell penetrating function (Vives, 2003; Futaki, 2005). Lehn and co-workers summarized several features of guanidinium group, among which the interesting two are: (1) highly basic, insensitive to pH variations; (2) possible hydrogen bonding with DNA phosphate anions provides additional strength (Aissaoui et al., 2003). More recently, an attempt has been made to incorporate guanidinium group to synthetic polymer (Funhoff et al., 2004), cationic lipids (Sen and Chaudhuri, 2005) and natural products (Luedtke et al., 2003) to improve cellular uptake and ultimate transfection level. Due to the simplicity and flexibility of converting amines to guanidinium group in chemistry, it is a facile route to endow common polymer vectors bearing amines with transmembrane function by guanidinylation modification.

Previously, our group reported a thermoresponsive chitosan/*N*-isopropylacrylamide-vinyl laurate copolymer vector which was capable of tuning DNA packing and unpacking by varying dehydration and rehydration of macromolecular chains at a temperature above or below the critical solution temperature (LCST) of PNIPAAm (Sun et al., 2005). But the transfection efficiency was less satisfied presumably due to the limited transfection ability of chitosan. Enlightened by the uniqueness of guanidinium group in gene delivery, we synthesized a temperature sensitive NIPAAm-allylamine copolymer where part of allymine moieties were transformed to guanidinium group. It was anticipated that guanidine-containing copolymer vectors could increase the transfection efficiency.

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Herein, we reported the preliminary results of guanidinylated allylamine-*N*-isopropylacrylamide copolymer-mediated transfection of luciferase reporter gene into COS-1 cells.

2. Materials and methods

2.1. Materials

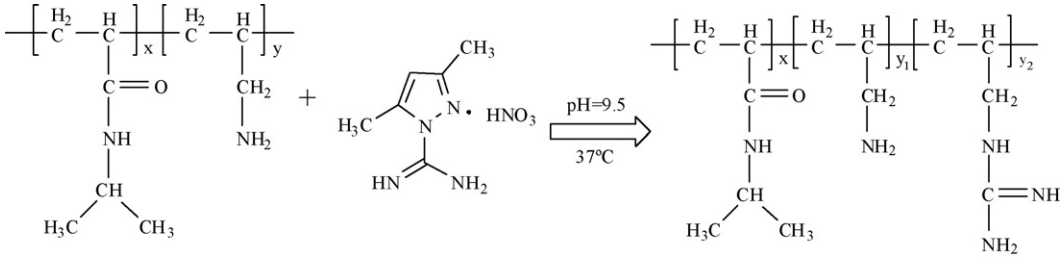
N-Isoacrylpropylamide (NIPAAm, Aldrich Co.) was purified by recrystallization in hexane and dried in vacuo at 25 °C. Allylamine (≥99%) was supplied by Aldrich Co. The initiator ammonium persulfate (APS), activator *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED, Fluka Co.) and guanidinylating agent 1-amidino-3,5-dimethylpyrazole nitrate salt (Aldrich Co.) were used without further purification. Cell culture reagents were purchased from Invitrogen. Plasmid PGL3-control with SV40 promoter and enhancer sequences encoding luciferase was obtained from Promega. Ethidium bromide (Fluka Co.) was used as received. 3-(4,5-Dimethyl-2-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma Co. All other reagents used were of analytical grade.

2.2. Synthesis of poly(NIPAAm-co-allylamine) (PNIALM)

Poly(NIPAAm-co-allylamine) was prepared by random radical polymerization. Appropriate amounts of NIPAAm and allylamine were dissolved in deionized water and stirred for 30 min with continuous N_2 purging. Then APS and TEMED were added successively both at the weight ratio of 1:20 to reacting monomers. The reaction was carried out at 20 °C for 24 h. Herein, copolymers with molar feed ratios of 1:1 and 1:3 (NIPAAm to allylamine) were synthesized. Each resultant mixture was dialyzed against water at 4 °C using Cellu SepH1 membrane (MWCO of 3500) for 5 days with refresh water replacement every 12 h. The purified copolymers were harvested after lyophilization. For description simplicity, the copolymers with feed ratios of 1:1 and 1:3 were coded as PNIALM1-1 and PNIALM1-3, respectively. ^{1}H NMR spectra were recorded on a Varian UNITY plus-500 NMR spectrometer using D_2O as a solvent: for PNIALM1-1, δ (ppm) 3.79 (1H, $-CH(CH_3)_2$), 2.60 (2H, $-CH_2NH_2$) (Hollas et al.,1998), 1.00 (6H, $-(CH_3)_2$); for PNIALM1-3, δ (ppm) 3.76 (1H, $-CH(CH_3)_2$), 2.64 (2H, $-CH_2NH_2$), 0.99 (6H, $-(CH_3)_2$). The copolymerization ratio of NIPAAm to allylamine in PNIALM1-1, estimated by calculating the ratio of peak area at 3.79 ppm to that at 2.60 ppm, was 9.48. Likewise, the copolymerization ratio was estimated to be 3.24 for PNIALM1-3. This result implied that only a small part of allylamine actually participated in the polymerization, which should be attributed to the low reactivity of allylamine relative to that of NIPAAm in the aqueous system. Elemental analysis calculated for PNIALM1-1: C, 63.64; N, 13.00; H, 9.86%. Found: C, 59.77; N, 12.58; H, 10.24%. The values calculated for PNIALM1-3: C, 63.64; N, 10.08; H, 14.03%. Found: C, 60.95; N, 13.29; H, 10.07%.

2.3. PNIALM guanidinylation

PNIALM was guanidinylzated in terms of the method reported by Morpurgo et al. (2004). Briefly, PNIALM1-1 (0.1 g) and 1-amidino-3,5-dimethylpyrazole nitrate salt (0.2 g) were dissolved in 10 ml phosphate buffer (50 mM, pH 7.4) and then NaOH was added to adjust the pH to 9.5. The guanidinylation reaction was performed under gently stirring at 37 °C for 48 h. PNIALM1-3 was modified in the same way. After dialysis against water (MWCO = 3500) for 5 days, the lyophilized products were finally collected. The guanidinylated PNIALM1-1 and PNIALM1-3 were coded as PNIALM-G1-1 and PNIALM-G1-3, respectively. The reaction scheme is depicted as follows:



^{1}H NMR (D_2O): for PNIALM-G1-1, δ (ppm) 3.79 (1H, $-CH(CH_3)_2$), 3.00 (2H, $-CH_2NH_2$); for PNIALM-G1-3, 3.76 (1H, $-CH(CH_3)_2$), 2.99 (2H, $-CH_2NH_2$). ^{13}C NMR (D_2O): for PNIALM-G1-1, δ (ppm) 175.6 (–NHCO), 157.2 ($-CH_2NHC(NH)NH_2$); for PNIALM-G 1-3, 175.7 (–NHCO), 157.1 ($-CH_2NHC(NH)NH_2$). The characteristic absorption of guanidino at 155–160 ppm (Tolman and Sedmera, 1988) further confirmed the successful introduction of guanidino to PNIALM copolymer.

The results of elemental analysis for PNIALM-G1-1: C, 55.02; N, 13.54; H, 9.91%. For PNIALM-G1-3: C, 59.46; N, 16.20; H, 9.96%. Based on the content of N-atom in PNIALM-G and PNIALM, the percentage of amino transformed to guanidine was estimated. The substitution degree of guanidino for PNIALM-G1-3 was about 65.24%, and PNIALM-G1-1 about 62.14%, indicating that more guanidinos were formed at higher allylamine content in copolymer.

2.4. Preparation of polymer/DNA complexes

PNIALM and PNIALM-G were separately dissolved in sodium acetate/acetic acid buffer (50 mM, pH 5.4) to ensure all amino and guanidino groups to be fully charged. Polymer/DNA complexes at various weight ratios were formulated by adding polymer of desired concentrations to aqueous solutions of plasmid DNA (0.2 mg/ml) at equal volume. The mixture was briefly

vortexed and then incubated at room temperature for 30 min to allow complexes formation.

2.5. Gel retardation assay

Polymer/DNA complexes were formed as described above. A 5 μl of each complex solution was loaded in a well for electrophoresis assay on a 1% agarose gel with Tris-acetate (TAE) running buffer at 90 V for 30 min. DNA bands were visualized with ethidium bromide staining.

2.6. Measurement of particle size

Complexes were prepared in the same manner as described above. The volume of the samples was 1 ml containing a final DNA concentration of 0.01 wt.%. The particle sizes were determined on BI-FOQELS particle size analyzer.

2.7. Transmission electron microcopy

For TEM measurement, the final concentration of DNA in complex solutions was 20 μg/ml. A 5 μl of each sample was carefully dropped onto clean copper grids and negatively stained with 1.5 wt.% phosphotungstic acid (pH 6.7). The copper grids surface was dried at room temperature before imaging on JEOL JEM-100CXII TEM.

2.8. Cell line

COS-1 cells (African green monkey kidney cells) were purchased form Peking Union Medical College). The cells were grown in Dulbecco's Modified Eagles Medium (DMEM, Sigma), supplemented with l-glutamine, penicillin/streptomycin, and 10% fetal bovine serum (FBS, heat-inactivated at 56 °C for 30 min, GIBCO) at 37 °C under a humidified atmosphere containing 5% CO_2.

2.9. Cytotoxicity assay

COS-1 cells were seeded into a 96-well plate at a density of 3×10^4 cells per well in 180 μl of growth medium and incubated for 4 h (37 °C, 5% CO_2) until adhesion to the plate surface. A 20 μl solution containing polymer vectors with concentration ranging from 0.01 to 0.3 mg/ml was applied to each well. After additional incubation for 48 h, 20 μl/well MTT (5 mg/ml in PBS) was added to each well, and the plate was further incubated for 4 h (37 °C, 5% CO_2). Then all media were discarded carefully and 150 μl of DMSO was added to each well. The plate was gently shaken for 5 min to dissolve the blue formazan crystals. The absorbance was measured at 570 nm on a ∑960 plate-reader (Metertech). Non-treated cell (in DMEM) was used as a control and the relative cell viability (mean% ± S.D., $n = 3$) was calculated according to the formula:

$$\text{cell viability}\,(\%) = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}} \times 100.$$

The absorbance measured at 570 nm with pure DMSO was used as a blank.

2.10. In vitro transfection

COS-1 cells were seeded in a 24 well plate at an initial density of 2×10^5 cells per well and incubated for 18–24 h to 60–70% confluence. Complexes were formed by adding 20 μl of polymer with different concentrations to 20 μl of DNA (50 μg/ml). Each sample of complex containing 1 μg of DNA was incubated at room temperature for 30 min. ExGen 500 (Fermentas), a sterile solution of linear 22 kDa polyethylenimine (PEI), was used as positive control. PEI/DNA complexes were prepared according to the recommended protocols. The cell growth medium was removed and replaced with 600 μl fresh serum-free DMEM or DMEM with 10% serum (both without antibiotics) followed by addition of the complexes. After incubating at 37 °C in 5% CO_2 for 16 h, the complex-containing medium was replaced with 1 ml cell-growth medium. Following 32 h of additional incubation, the growth medium was removed, and each well was washed with PBS twice and 150 μl of reporter lysis buffer (RLB, Promega) was added, and then the cells were frozen at −80 °C until luciferase activity analysis. When testing, a total of 100 μl of supernatant was analyzed using luciferase assay system in 96 multiwell plates according to the manufacture's instruction. The amount of protein was determined by BCA protein assay kit. Each transfection was done in triplicate. Values are given as mean relative light units (RLUs) per milligram of cell protein.

3. Results and discussion

3.1. Gel retardation assay

As a potential transgene vector, it should first interact with DNA to form a stable complex to prevent gene from DNase degradation, which is critical for the DNA transportation and the final gene expression. In this study, we used gel electrophoresis to investigate the physical stability of polymer–DNA complexes.

Fig. 1 shows the gel electrophoresis of PNIALM1-1/1-3–DNA and PNIALM-G 1-1/1-3–DNA complexes at room temperature. As a whole, both of these two types of polymers electrostatically neutralize plasmid DNA and deter electrophoretic mobility. The complex ratio where the migration of DNA is completely retarded decreases as the content of allylamine increases from 9.54% in PNIALM1-1 to 23.58% in PNIALM1-3 (top A and B), implying allylamine has a good DNA condensing capability. On the other hand, guanidinylated copolymers exhibit a weaker DNA-binding strength relative to their non-guanidinylated counterparts, which means guanidine has an attenuated interaction with DNA than amine does as reported previously (Morpurgo et al., 2004).

3.2. Size and morphology of polymer/DNA complexes

The particle sizes of complex are very important for gene expression especially in physiological conditions. It is already

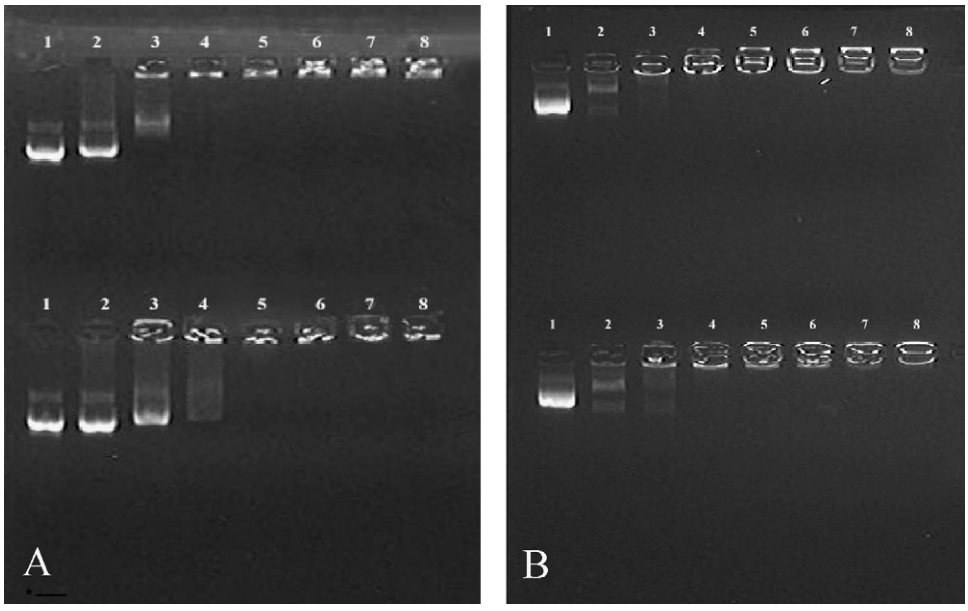


Fig. 1. Agarose gel electrophoresis of PNIALM1-1 (top of A), PNIALM-G1-1 (bottom of A), PNIALM1-3 (top of B) and PNIALM-G1-3 (bottom of B). Lane 1: plasmid DNA; lanes 2–8 represent the complexes with the weight ratio (polymer:DNA) of 2:1, 4:1, 6:1, 8:1, 10:1, 15:1 and 20:1, respectively.

reported that endocytosis by many types of mammalian cells is limited to the particles less than about 150 nm in diameter (Guy et al., 1995). Fig. 2 shows the particle sizes of the complexes measured by particle size analyzer (Brookhaven Instruments Corp.) at varying weight ratios. The particle sizes show a tendency to decrease with the increment in weight ratio and level off at higher ratios. A common feature of these complexes is the dimension of particle becomes stable with size around 150–250 nm at weight ratio of 16:1. It is necessary to note that guanidinylation leads to a mild increase of particle size compared to its parent copolymer, which is in agreement with the results of electropheretic retardation assay. Additionally, one can also see that the particle sizes of complexes are smaller under higher allylamine contents in copolymers. The above result means that the compaction of DNA is highly dependent on the charge ratios, and high charge ratio is considered to be required for satisfactory transfection efficiency.

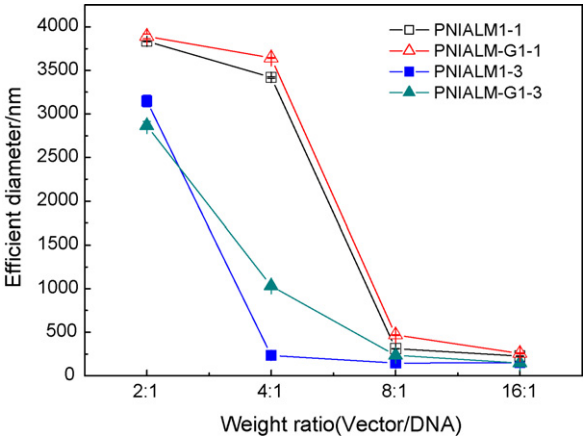


Fig. 2. Particle sizes of polymer/DNA complexes at various weight ratios.

The TEM images of polymer/DNA complexes at weight ratios of 8:1 (Fig. 3) and 20:1 (Fig. 3) demonstrate the relatively homogenous complex particles with spherical shape and compacted structure. From the particle sizes obtained from TEM images (Table 1), we conclude that the higher DNA condensation capability, the smaller DNA condensates, and the descending order of particle sizes of complexes at the fixed weight ratio is: PNIALM-G1-1/DNA > PNIALM1-1/DNA > PNIALM-G1-3/DNA > PNIALM1-3/DNA. The result from TEM agrees well with that of Particle Size Analyzer. The series of 1–3 polymer/DNA complexes is likely to be more appropriate for gene transfection with diameters below 100 nm at weight ratio of 20:1.

3.3. In vitro transfection and cytotoxicity

The copolymers in this study showed a strong binding affinity to DNA as evidenced by complete gel retardation, and were able to condense DNA into particles of suitable size. In the preliminary experiments, in order to obtain the appropriate weight ratios of each type complex for transfection studies, we conducted the transfection on COS-1 cells in serum using complexes at weight ratios of 8:1, 15:1, 20:1 and 30:1. The results indicated that no evident gene expression was achieved with all polymer vectors except for PNIALM-G1-3 and this situation did not change

Table 1
Particle sizes of complexes obtained from TEM images

	Average diameter (nm)	
	8:1	20:1
PNIALM1-1/DNA	248 ± 29	117 ± 27
PNIALM-G1-1/DNA	354 ± 65	132 ± 21
PNIALM1-3/DNA	165 ± 51	76 ± 7
PNIALM-G1-3/DNA	205 ± 30	56 ± 8

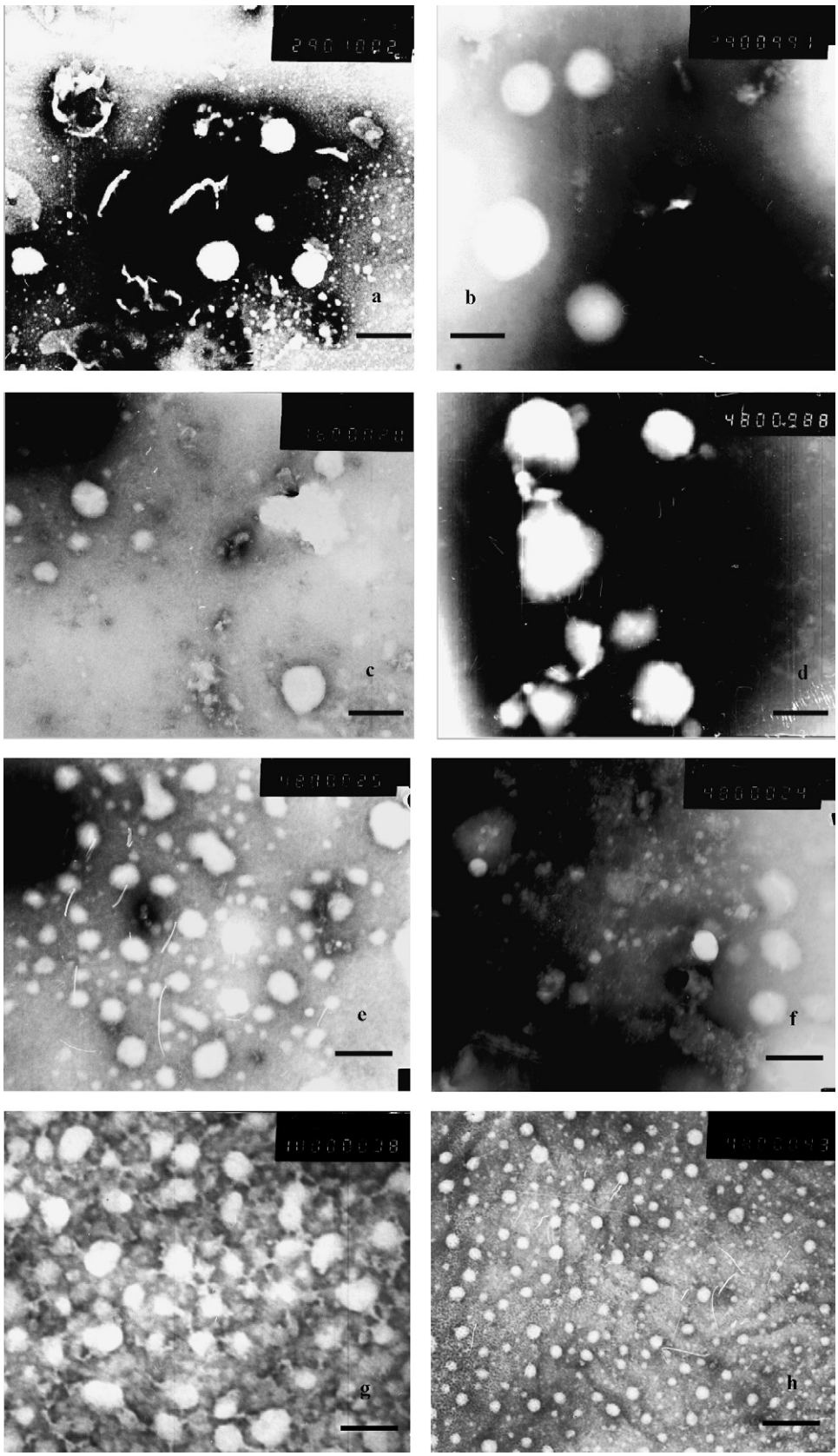


Fig. 3. TEM images of PNIALM1-1/DNA (a), PNIALM-G1-1/DNA (b), PNIALM1-3/DNA (c) and PNIALM-G1-3/DNA (d) at weight ratio 8:1; PNIALM1-1/DNA (e), PNIALM-G1-1/DNA (f), PNIALM1-3/DNA (g) and PNIALM-G1-3/DNA (h) at weight ratio 20:1. The scale bars are 345 nm (a and b), 278 nm (c), 200 nm (d–f and h) and 100 nm (g). Phosphotungstic acid was used as a negative staining agent.

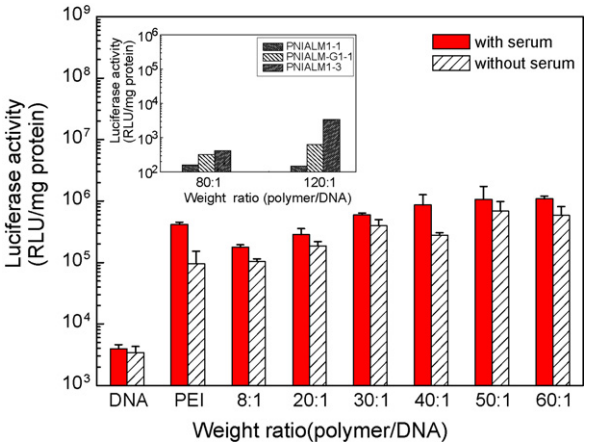


Fig. 4. Transfection of COS-1 cells by PNIALM-G1-3/DNA complexes at various weight ratios with or without serum, using PEI as a positive control. The inset is the transfection levels of PNIALM1-1, PNIALM-G1-1 and PNIALM-1-3 obtained in serum at polymer/DNA weight ratio of 80:1 and 120:1. Luciferase activities were measured as described in Section 2 and expressed as the relative light unit (RLU) per mg of protein ($n = 3$, mean ± S.D.).

at all when weight ratios were further increased to 40:1, 60:1. We tried to increase the ratios of vector up to 80:1 and 120:1 and recorded the transfection results (inset in Fig. 4). It is seen that at very high vector/DNA ratios, the transfection levels of PNIALM1-1, PNIALM-G1-1 and PNIALM1-3 are still very low in spite of a marginal improvement with PNIALM1-3 relative to naked DNA at the ratio of 120:1. It is well known that polycations that exhibit tight binding to nucleic acids and that can be complexed with DNA in sub-100 nm particles can effectively transport DNA across cellular membrane, but it seems not true in our studies. For the cationic copolymers of PNIALM1-1, PNIALM-G1-1 and particularly PNIALM1-3 which has the strongest DNA condensing capability among the copolymer vectors used in this work, tight DNA binding and suitable particle size cannot necessarily ensure a successful gene delivery and gene expression. Therefore, the great difference of transfection efficiency between PNIALM-G1-3/DNA and other three copolymer–DNA complexes has to be explained by some factors other than the binding ability and particle size. It is surmised that the PNIALM copolymer vectors may be just limited by the difficulty in moving across the cell membrane, thus suffering from the inefficacy in transfection. Besides, the over tight association between vectors and DNA may also result in a low transfection efficiency by blocking the release of DNA from complexes (Pichon et al., 2002). On the other hand, the guanidine was reported to form hydrogen bonding with the phospholipids present in the lipid bilayers and play a great role in facilitating cellular uptake (Wender et al., 2000; Pujals et al., 2006). With the aid of guanidine, PNIALM-G1-3 could transport the DNA across the cell membrane more easily, and owing to concerted electrostatic and hydrogen bonding interactions of guanidine with DNA, this copolymer could unload the DNA more easily too. Thus, all of these factors above make PNIALM-G1-3 efficient in gene transfection. But PNIALM-G1-1 failed to benefit from the advantage of guanidine because of the insufficient amount it contains.

Fig. 4 displays the luciferase expression levels mediated by copolymer PNIALM-G1-3 in the presence or absence of serum in COS-1 cell line. As a whole, the gene luciferase expression of PNIALM-G1-3/DNA complexes far exceeds that of naked DNA by 100- to 400-fold. What is more, PNIALM-G1-3 exhibits an equivalent or better transfection activity compared to PEI. Importantly, the activity of PNIALM-G1-3 is not negatively affected by serum as reported previously that serum protein could interact with cationic polyplexes to form aggregates and deteriorate the transfection effect (Funhoff et al., 2004). Instead, the transfection efficiency is raised to a different extent in the presence of serum compared to the case when serum is absent, which reveals the good serum resistance of PNIALM-G1-3 as a promising vector. We can also see that in the presence of serum the transfection efficiency increases with increasing copolymer concentration until a maximum is reached at a weight ratio of 50:1; while in the absence of serum there was no definite correlation between weight ratio and transfection efficiency. As we know, high polymer concentration will result in high cytotoxicity, thus decreasing the transfection efficiency. The initial increase of transfection efficiency in the presence of serum should be ascribed to the dominance of stronger DNA condensing capability over higher cytotoxicity, but the increase is halted and a maximum is reached when these two sides come to a balance. In the absence of serum, underfed cells become even more vulnerable to the cytotoxicity caused by the presence of an increasing amount of polymer, accordingly rendering the transfection results uncertain to some extent.

In view of its potential transfection capability, we determined the cytotoxicity of PNIALM-G1-3 at varied concentrations, and meanwhile compared with that of PNIALM1-3 (Fig. 5). For PNIALM-G1-3 below 0.09 mg/ml, the cell viability is around 100%; while in the same range of vector concentration, the viability of cells decreases to 89% for PNIALM1-3. With increasing concentration up to 0.3 mg/ml, 30 and 58% cell viability loss is observed for PNIALM-G1-3 and PNIALM1-3, respectively. It is noted that in our transfection experiment, the vector concentration fell in the range of 0.19–0.01 mg/ml, so PNIALM-G1-3 was low toxic as a vector. Interestingly, in the selected range of

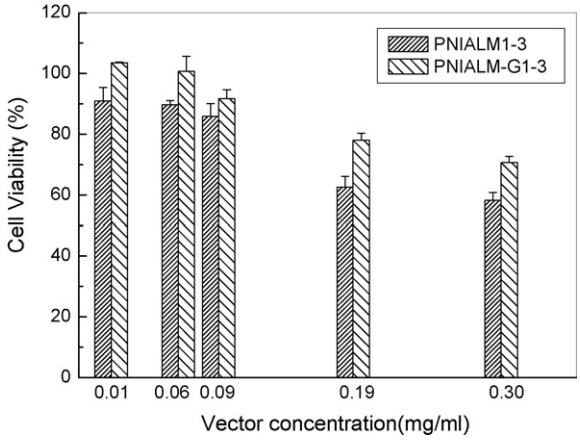


Fig. 5. Viability of COS-1 cells vs. the concentration of PNIALM-G1-3 and PNIALM1-3.

concentration, the cytotoxicity of PNIALM-G1-3 is lower than that of PNIALM1-3. As mentioned above, after amino groups in PNIALM1-3 were converted to guanidines, the electrostatic force was weakened due to partial role of hydrogen bonding of guanidine in binding cell membrane. It is deemed that the depressed electrostatic interaction is beneficial to the improvement of biocompatibility of vector. A similar result has been reported in our previous work (Cheng et al., 2006).

It is necessary to point out that all LCST values of PNIALM and its guanidinylated derivatives are over 38 °C, higher than body temperature; hence we did not examine the effect of temperature on gene expression in this preliminary experiment. Further work will be done to decrease the LCST of copolymers by introducing hydrophobic moieties.

4. Conclusion

N-Isopropylacrylamide-allylamine copolymers could be successfully modified with 1-amidino-3,5-dimethylpyrazole nitrate to prepare guanidinylated derivatives. The guanidinylated copolymer was capable of condensing DNA to form nanoparticles at an appropriate complex ratio though it binding affinity with DNA was slightly decreased relative to its parent compolymers. PNIALM-G1-3 showed higher transfection efficiency than PEI in mediating plasmid DNA to COS-1 cells. What's more, it exhibited particularly good serum resistance. It is envisioned that PNIAL-G copolymers have a potential not only as an effective nonviral vector, but also as a thermoresponsive gene delivery system by lowering LCST.

Acknowledgments

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