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Biocleavable Polycationic Micelles as Highly Efficient Gene Delivery Vectors

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Abstract An amphiphilic disulfide-containing polyamidoamine was synthesized by Michael-type polyaddition reaction of piperazine to equimolar N, N′-bis(acryloyl)cystamine with 90% yield. The polycationic micelles (198 nm, 32.5 mV), prepared from the amphiphilic polyamidoamine by dialysis method, can condense foreign plasmid DNA to form nanosized polycationic micelles/DNA polyelectrolyte complexes with positive charges, which transfected 293T cells with high efficiency. Under optimized conditions, the transfection efficiencies of polycationic micelles/DNA complexes are comparable to, or even higher than that of commercially available branched PEI (Mw 25 kDa).

Keywords Gene delivery · Polycationic micelles · Biocleavable

Introduction

Polycationic gene delivery vectors have attracted more and more attention in the past decade because of their versatile chemical structures, ease of preparation, lack of immune response in vivo, etc. [1–4]. Various cationic polymers have been applied in in vitro and in vivo gene delivery, including polylysine (PLL) [5, 6], polyethylenimine (PEI) [7, 8], polyamidoamine dendrimer [9–12], cationic polyester [13, 14], poly(beta-aminoester) [15, 16], polyphosphoester and polyphosphoramidate [17–21]. In general, water-soluble polycations were directly used to condense negatively charged DNA or RNA to from nanosized polyelectrolyte complexes (polyplexes), which can associate with the negatively charged surface of the cells and facilitate the cells uptake of polycation/DNA (or RNA) complexes. Recently, cationic polymeric micelles, prepared from amphiphilic cationic graft or block polymers, were reported as alternative gene delivery vectors [22–31]. The used cationic micelles have core–shell structures composed of hydrophobic segments as core and cationic hydrophilic segments as shell. The cationic polymeric micelles can condense DNA and efficiently deliver foreign DNA into various cell lines. In some cases, the transfection efficiencies of cationic micelles were comparable to polyethylenimine (PEI), even higher than PEI. The significant advantages of cationic polymeric micelles as gene carriers include low cytotoxicity and high transfection efficiency. The usage of polycationic micelles as gene delivery vectors represents the new advances in polymer-based gene delivery. However, most reported cationic polymeric micelles for gene delivery were prepared from block or graft polymer containing hydrophobic polyester segments and hydrophilic polyamine segments. These cationic polymers are often easy to slowly degrade in the process of synthesis and storage, which may limit their practical applications in gene delivery. The goal of this work is to develop novel cationic polymeric micelles for gene delivery, which are stable and nondegradable during the polymer synthesis, preparation and storage of micelles and cationic micelles/DNA complexes. After the uptake of cationic polymeric micelles/DNA complexes by cells, cationic polymers degrade and release DNA under cellular reductive conditions. For this goal, we designed and synthesized an amphiphilic polyamidoamine containing disulfide bonds in the backbone (polymer 1) and investigated the self-assembly

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of polymer 1 into micelles as efficient gene delivery vectors. Although polymers containing reducible disulfide bonds have been widely applied in biomedical field, such as drug delivery [32–34], gene delivery [35–46] and layer-by-layer assembly [47–49], we here report the first example of the preparation and application of disulfide-containing cationic polymeric micelles in nonviral gene delivery.

Experiments

Materials and Methods

Cystamine dihydrochloride (97%) was purchased from Fluka and used as received. Dithiothreitol (DTT) was purchased from Merck (Darmstadt, Germany). Other reagents and solvents were of analytical grade obtained from suppliers and used without purification. N,N′-bis(acryloyl) cystamine(BAC) was synthesized from cystamine hydrochloride and acryloyl chloride according to the literature method [50]. FTIR spectra were measured on a Perkin–Elmer Spectrum one spectrometer. ^{1}HNMR spectra were recorded on a Mercury VX-300 MHz instrument. Gel permeation chromatography (GPC) measurement was carried out by using a Waters-2690D HPLC equipped with Shodex K802.5 and K805 columns. Sample was detected with a Wyatt multiangle light scattering detector and a Waters 2,100 differential refractive index detector. Chloroform was used as the mobile phase at a flow rate of 1.0 ml min^{-1}. The green fluorescent protein encoding plasmid pEGFP-C1 was transformed in *E. coli* DH5α and isolated and purified using Qiagen endotoxin-free plasmid Gigaprep kits according to the supplier's protocol. The quantity and quality of plasmid DNA was analyzed by spectrophotometric analysis at 260 and 280 nm and by electrophoresis in 0.8% agarose gel. Purified plasmid DNA was diluted in TE buffer and stored at −20°C.

Synthesis and Characterization of Disulfide-Containing Polyamidoamine (Polymer 1)

N,N′-bis(acryloyl)cystamine (BAC) (1.190 g, 4.6 mmol) and piperazine (0.394 g, 4.6 mmol) were mixed in a two-neck flask with a magnetic stirrer under a nitrogen atmosphere. The temperature of the reaction mixture was gradually raised to 100°C and maintained at this temperature for 4 h, and then cooled to room temperature to form slightly yellow solid. The solid was dissolved in 10 ml of methanol and precipitated in a 15-fold excess of acetone. The obtained white powder was dried in vacuum to a constant weight. Yield: 1.432 g (90.4%). FTIR (KBr, cm^{-1}): 3,434, 3,305, 2,940, 2,819, 1,644, 1,548, 1,438, 1,302, 1,131 cm^{-1}. ^{1}H NMR ($CDCl_3$, ppm) : 1.92 (4H, s, CH_2CO); 2.45 (4H, t, CH_2N); 2.60–2.70 (8H, m, CH_2N); 2.85 (4H, t, CH_2S); 3.58 (4H, t, CH_2); 8.55 (1H, broad, CONH). Mw = 2.17 × 10^4, Mw/Mn = 1.40.

CMC Measurement

To a vial, 0.1 ml of pyrene solution in chloroform was added and the solvent was allowed to evaporate to form a thin film at the bottom of the vial. Polymer solutions (5.0 ml) at different concentrations were added to the vials, and the final pyrene concentration was 6 × 10^{-7} mol l^{-1} in water. The concentrations of polymer solutions varied from 0.5 to 350 mg l^{-1}. The solutions were kept on a shaker at room temperature for 24 h to reach equilibrium prior to fluorescence runs. The fluorescence spectra were recorded on a Shimadzu RF-5301PC spectrophotometer (Shimadzu, Japan). The emission spectra were scanned from 350 to 450 nm at the excitation wavelength of 333 nm. The slit setting was 7.5 nm. The intensity at 374 nm was analyzed as a function of polymer concentration.

Preparation and Characterization of Disulfide-Containing Polycationic Micelles

Polymer 1 was dissolved in methanol at different concentrations ranging from 0.1 to 5.0 mg ml^{-1} and then separately dialyzed against ultrapure water using a dialysis tube (MW cut off 8,000–10,000) for 24 h. The water was changed every 4 h. The obtained polymeric micelles solution was directly used for the measurement of size and zeta potential.

Particle Size and Zeta Potential Measurement

The particle size and zeta potential of the freshly prepared polymeric micelles or polymeric micelles/pEGFP-C1 complexes were measured using a Zetasizer (Nano ZS, Malvern Instruments Ltd., UK).

In Vitro Cytotoxicity Assay

The cytotoxicity of disulfide-containing polycationic micelles against 293T cells was evaluated using MTT assay. Before testing, cells in 100 μl of complete DMEM were seeded into 96-well plates at a density of 7,000 cells/well. After 24 h incubation, 100 μl solutions of polycationic micelles in ultrapure water at different concentrations in the range from 0.005 to 2 mg ml^{-1} were separately added into the wells. The control wells were added with ultrapure water without polycationic micelles. Treated cells were incubated for further 24 h at 37°C. MTT solution (5 mg ml^{-1}, 20 μl) in PBS (0.1 mol l^{-1}, pH 7.4) was added into each well except that PBS (20 μl) was added into the background wells, and

the cells were incubated at 37°C for 4 h. After removal of the medium in each well, 150 μl of DMSO was added to each well and the plates was shaken until all the formed formazan blue crystal inside the wells dissolved. The absorbance of the solution in each well at 570 nm was measured using a microplate reader (Bio-Rad 550, Hercules, CA, USA). The percent-relative viability in reference to control wells containing complete DMEM without polymer was calculated by the following equation (*A:* absorbance at 570 nm, A_{blank} is the absorbance of the solution containing cells and complete DMEM without MTT and polymer): Relative cell viability (%) = 100 × $(A_{test}-A_{blank})/(A_{control}-A_{blank})$.

Preparation of Polymeric Micelles/DNA Complexes

Polymeric micelles/pEGFP-C1 complexes at various N/P ratios were separately prepared by rapidly adding calculated amount of polymeric micelles solution to calculated amount of 150 mmol l^{-1} sodium chloride containing 1 μg of pEGFP-C1 with gentle vortexing and allowed to incubate at room temperature for half an hour. The total volume of polymeric micelles solution and DNA solution is 100 μl.

Agarose Gel Electrophoresis

Polymeric micelles/DNA complexes at various N/P ratios, ranging from 1 to 40, were separately electrophoresed on 0.7% (W/V) agarose gel containing 0.1% GelRed (V/V) with Tris–acetate–EDTA (TAE) running buffer (pH 8) at 80 V for 60 min. Then, the gel was illuminated on a UV illuminator to show the mobility of DNA. Naked DNA was used as a reference.

DTT Reduction

Polymeric micelles/pEGFP-C1 complexes at N/P ratio of 20 were selected to study the effect of DTT on the stability of polyplex. DTT solution in 0.15 M NaCl was added to the polyplex solutions to get a final concentration of DTT from 1 to 10 mM, while equal volume of 0.15 M of NaCl without DTT was added to the polyplex solutions as a control. The mixture was incubated at 37°C. At predetermined intervals, the mixture was taken out and electrophoresed on 0.7% (W/V) agarose gel containing 0.1% GelRed (V/V) with Tris–acetate–EDTA (TAE) running buffer (pH 8) at 80 V for 60 min.

In Vitro Transfection and Green Fluorescent Protein Assay

At the density of 5 × 10^4 cells/well, 293T cells were seeded into a 24-well plate and incubated in 1 ml of complete DMEM for 24 h prior to transfection. The medium was removed and washed gently with PBS (pH 7.4) when the cells were cultured to 40–50% confluency in the 24-well plate. One microliters of serum-free DMEM and 100 μl of 1 μg DNA-containing polymeric micelles/DNA complexes in saline were added into each well. After 4 h incubation at 37°C, the serum-free DMEM was replaced by 1 ml of complete DMEM. After 44 h incubation at 37°C, the cells that expressed green fluorescent proteins were directly observed by inverted fluorescence microscope.

Results and Discussion

Synthesis and Characterization of Disulfide-Containing Polyamidoamine (Polymer 1)

Polymer 1 was synthesized by a simple Michael-type polyaddition reaction, which was shown in Fig. 1. The bulk polyaddition of piperazine to equimolar N,N′-bis(acryloyl)cystamine was carried out at 100°C for 4 h under Ar atmosphere. The cooled crude product was dissolved in methanol, precipitated in acetone, and dried in vacuum to give polymer 1 with 90% yield. Polymer 1 was characterized by FTIR and ^{1}HNMR. The spectra of IR and ^{1}HNMR were shown in Figs. 2 and 3, respectively. The molecular weight of polymer 1 was measured by size-exclusion chromatography combined with multiangle laser light scattering (SEC-LLS), using chloroform as eluent. The weight average molecular weight (Mw) of polymer 1 is 2.17 × 10^4 with a polydispersity index of 1.40. Polymer 1 is soluble in organic solvents such as methanol, however insoluble in water.

CMC Measurement

The critical micelle concentration (CMC) of amphiphilic polymer is an important evidence of forming micelles via the self-assembly. The CMC of polymer 1 in ultrapure water, measured by fluorescence spectroscopy using pyrene as a probe, is 49.4 mg l^{-1}, which is lower than surfactants. The results are shown in Fig. 4. Amphiphilic

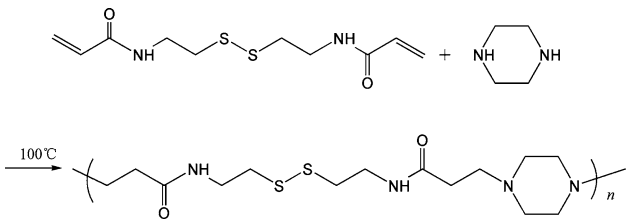

Fig. 1 Synthesis of disulfide-containing polyamidoamine (polymer 1)

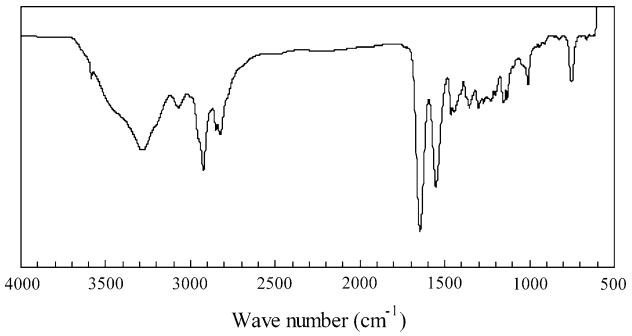

Fig. 2 The IR spectrum of polymer 1

block or graft polymers have been widely used to prepare polymeric micelles; however, amphiphilic homopolymers were seldom reported to from micelles. Polymer 1 is a homopolymer with both hydrophobic part (CH_2CH_2SS CH_2CH_2) and hydrophilic part (amide and tert-amine) in each repeating unit. Although the chemical structure of amphiphilic homopolymer is greatly different from amphiphilic block and graft polymers, we found that polymer 1 can easily form polymeric micelles via self-assembly in ultrapure water at relatively low concentration.

Preparation and Characterization of Polycationic Micelles

Cationic micelles were prepared from polymer 1 by dialysis of a solution of polymer 1 in methanol against pure water. It was found that the concentration of the solution of polymer 1 in methanol for dialysis may significantly affect the size and zeta potential of the formed micelles. The results are summarized in Table 1. In this work, we chose the micelles, prepared from a solution of polymer 1 in methanol at 2 mg ml^{-1} by dialysis, for DNA binding and gene transfection. The mean size and zeta potential of the micelles were 198 nm and 32.5 mV, respectively, which were measured by a Zetasizer (Nano ZS, Malvern Instrument, United kingdom). The morphology of the micelles, analyzed by TEM is shown in Fig. 5, indicated that the size of the dry micelles was relatively smaller than that measured by dynamic light scattering in solution. This implied that the micelles shrank after the removal of water in dry state.

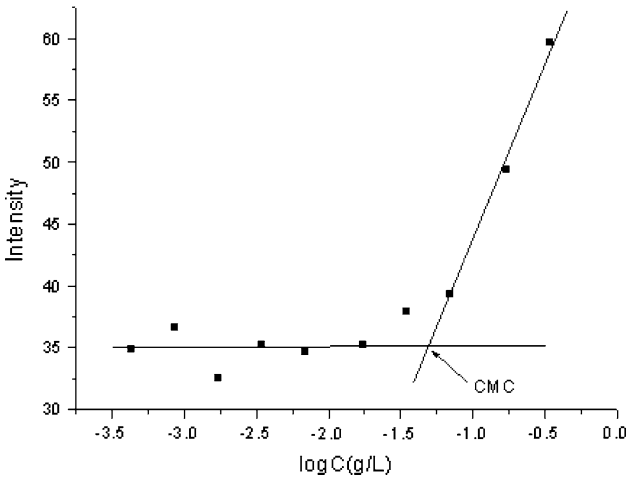

Fig. 4 *Plots* of fluorescence intensity versus log C of polymer 1

Table 1 Average sizes and zeta potentials of polymeric micelles formed from a solution of polymer 1 in methanol at different concentrations

Concentration (mg ml^{-1})	Size(nm)	Poly. index	Zeta potential (mV)
0.1	209.2	0.089	+6.7
0.5	237.4	0.0504	+13.3
1	225.5	0.281	+24.5
2	197.6	0.168	+32.5
3	222.3	0.316	+40.1
5	293.9	0.459	+46.1

Cytotoxicity

Good biocompatibility is necessary for the application of polymers in gene delivery. The cytotoxicity of the polycationic micelles was evaluated in 293T cells by MTT assay using bPEI (*Mw* 25 kDa) as a control. As shown in

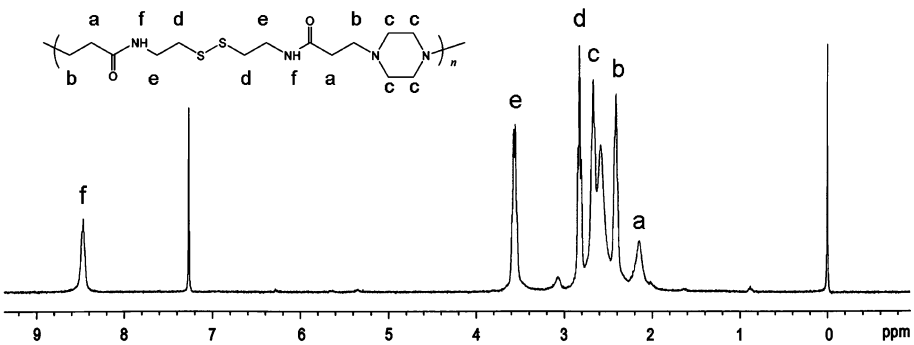

Fig. 3 The ^{1}HNMR spectrum of polymer 1

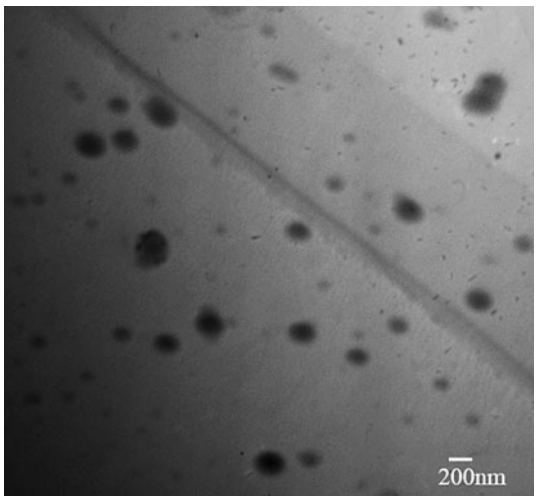

Fig. 5 TEM image of micelles prepared from amphiphilic disulfide-containing polyamidoamine by dialysis of polymer solution in methanol (2 mg ml^{-1}) against pure water

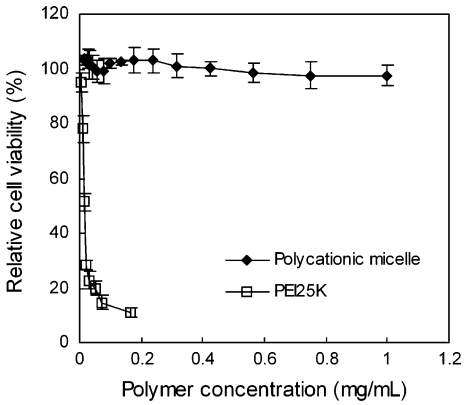

Fig. 6 Cytotoxicity of polycationic micelles against 293T cells using branched PEI (*Mw* 25 kDa) as a reference. *Data* represent the mean standard deviation ($n = 4$)

Fig. 6, polycationic micelles exhibited almost no cytotoxicity. Almost 100% of cells retained their metabolic ability even at the polycationic micelles concentration of 1 mg ml^{-1}. In contrast, in the case of bPEI 25 kDa, only 50% of cells retained their metabolic ability at the concentration of 15 μg ml^{-1}. The nontoxicity of disulfide-containing polycationic micelles encouraged us to study their application in gene delivery.

DNA-binding Ability of Polycationic Micelles

The DNA-binding ability of the micelles (198 nm and 32.5 mV), prepared by dialysis of a solution of polymer 1 in methanol at 2 mg ml^{-1} against pure water, was evaluated by agarose gel electrophoresis. The N/P ratio was calculated by relating the number of tert-amino groups in polymer 1 and the number of phosphate groups in DNA.

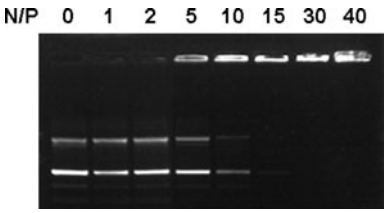

Fig. 7 Agarose gel electrophoresis images of polycationic micelles/pEGFP-C1 complexes. From *Left* to *right*, naked DNA; N/P ratio: 1, 2, 5, 10, 15, 30, 40

The mixture of cationic polymeric micelles and pEGFP-C1 at different N/P ratios, including 0:1, 1:1, 2:1, 5:1, 10:1, 15:1, 30:1, 40:1, were separately electrophoresised in 0.8% agarose gel. As shown in Fig. 7, cationic polymer micelles are able to fully retard the mobility of plasmid DNA in agarose gel when the N/P ratio is 15:1 or higher than 15:1. When N/P is lower than 15:1, the retardation is not full. The results indicated that the polyelectrolyte complexes formed by simply mixing the solution of cationic polymer micelles and DNA solution.

Size and Zeta Potential of Polycationic Micelles/DNA Complexes

The mean size and zeta potential of cationic micelles/DNA complexes were measured by Zetasizer and shown in Figs. 8 and 9. The size of micelles/DNA complexes (450–550 nm) is higher than cationic micelles (198 nm) and slightly increased with increasing the N/P ratio at the range of 2–60. The polycationic micelles/DNA complexes have negative zeta potential when N/P is lower than 5:1. However, the zeta potential became positive when N/P is 10:1 and remained constant ($\sim$10 mV) over a range of N/P ratios from 20 to 60.

Reductive Dissociation of Polymeric Micelles/pEGFP-C1 Complexes and Responsive Release of DNA

Release of foreign DNA from polymeric micelles/DNA complexes into the nucleus of the cells is important for protein expression in the transfected cells. Disulfide linkages in the polycations can be biocleaved by the reductants inside the cells, such as glutathione at a concentration of 0.5–10 mM, which will cause the dissociation of the polyplexes and promote the release of DNA into the nucleus and enhance the level of gene expression. DTT reduction experiments were conducted to mimic the degradation of disulfide bonds in the main chains of polycations under reductive intracellular conditions. Polymeric micelles/DNA complexes at N/P ratios of 20 were incubated in 1–10 mM DTT solutions at 37°C. The migration of naked DNA and treated polyplexes on agarose gel was

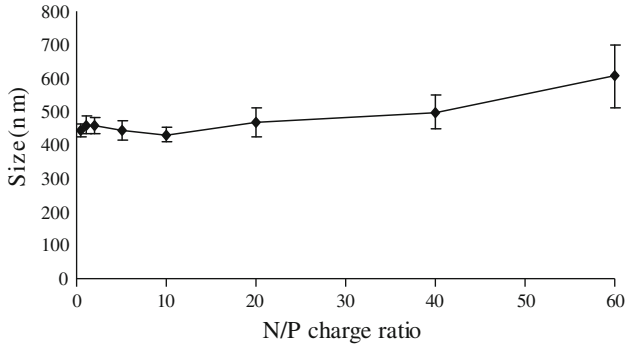

Fig. 8 Average sizes of polycationic micelles/pEGFP-C1 complexes as a function of N/P ratios

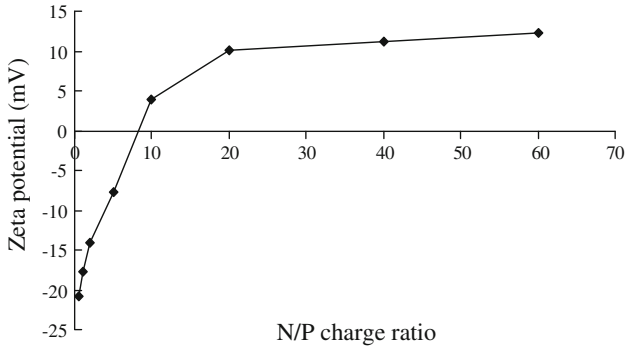

Fig. 9 Zeta potential of polycationic micelles/pEGFP-C1 complexes as a function of N/P ratios

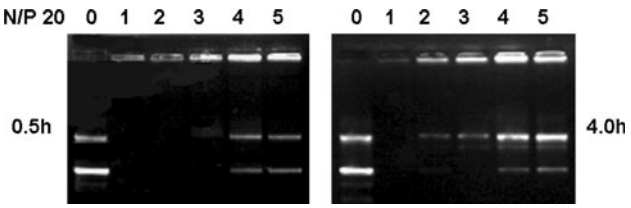

Fig. 10 Agarose gel electrophoresis images of polycationic micelles/pEGFP-C1 complexes pretreated with various concentrations of dithiothreitol (DTT) solutions for different time. Polyplexes at N/P ratio of 20. Lane 0: naked DNA; lane 1–5: DTT concentration of 0, 1.0, 2.5, 5.0, 10 mM

shown in Fig. 10. No free DNA released from polymeric micelles/DNA complexes at N/P ratios of 20 after 0.5- and 4-h incubation in the solution of 0.15 M NaCl at 37°C. In contrast, small amount of free DNA released from polymeric micelles/DNA complexes at N/P of 20 when separately incubated in 5 mM and 10 mM of DTT solution for only half an hour, which implied that the dissociation of polymeric micelles/DNA complexes occurred after 0.5 h incubation in NaCl solution containing DTT at relatively high concentration (5–10 mM). After 4 h incubation in DTT solution (5 and 10 mM), more free DNA released out. However, in the case of low concentration DTT (1 and 2.5 mM), no free DNA was observed to migrate when incubated for only half an hour. Only small amount of free DNA released out even incubated for 4 h. As a reference, the incubation of PEI 25 kDa/DNA complexes at N/P ratio of 10 in DTT solution at various concentrations resulted in no release of DNA (figure not shown), because there is no cleavable disulfide bond in PEI. From these results, we can conclude that the release of incorporated DNA from polymeric micelles/DNA complexes is triggered by the reductive cleavage of disulfide bond in the polymer main chain and the rate of DNA release is dependent on the DTT concentration.

In Vitro Transfection of 293T Cells With Polycationic Micelles/DNA Complexes

To evaluate the potential application of cationic micelles in gene delivery, the in vitro gene transfection experiments were conducted on human embryonic kidney (293T) cell line using pEGFP-C1 plasmid. Naked DNA and commercial branched PEI (Mw 25 kDa) were used as negative control and positive control, respectively. The cells were incubated in the presence of DNA alone or DNA complexes for 4 h and further 44 h after the removal of DNA or DNA complexes, and then observed by fluorescence microscope. As shown in Fig. 11, branched PEI (Mw 25 kDa), at optimized N/P ratio of 10:1, induced highly efficient transfection of 293T cells with pEFGP-C1. In contrast, almost no transfected 293T cells were observed using pEGFP-C1 alone. For cationic micelles, efficient expression of GFP in 293T cells was observed. At N/P ratio of 20:1, the transfection efficiency of micelles/pEGFP-C1 complexes was much lower than that of PEI/pEGFP-C1 complexes. The transfection efficiencies of micelles/pEGFP-C1 increased with increasing N/P ratio of micelles to DNA from 20:1 to 40:1, which remained the high level up to N/P ratio of 60:1. The transfection efficiencies of 293T cells using micelles/pEGFP-C1 complexes, at N/P ratios of 40:1 and 60:1, were as high as that of branched PEI (Mw 25 kDa) at optimized N/P ratio (10:1).

Conclusions

In summary, an amphiphilic disulfide-containing polyamidoamine homopolymer was synthesized and characterized. The polymer could form cationic micelles with nanosize and moderate positive zeta potential via self-assembly in aqueous solution at low concentration. The formed cationic micelles could condense foreign DNA to form nanosized micelles/DNA complexes and mediate high efficient protein expression in cells at relatively high N/P ratio. Our results indicated that the novel cationic polymeric micelles

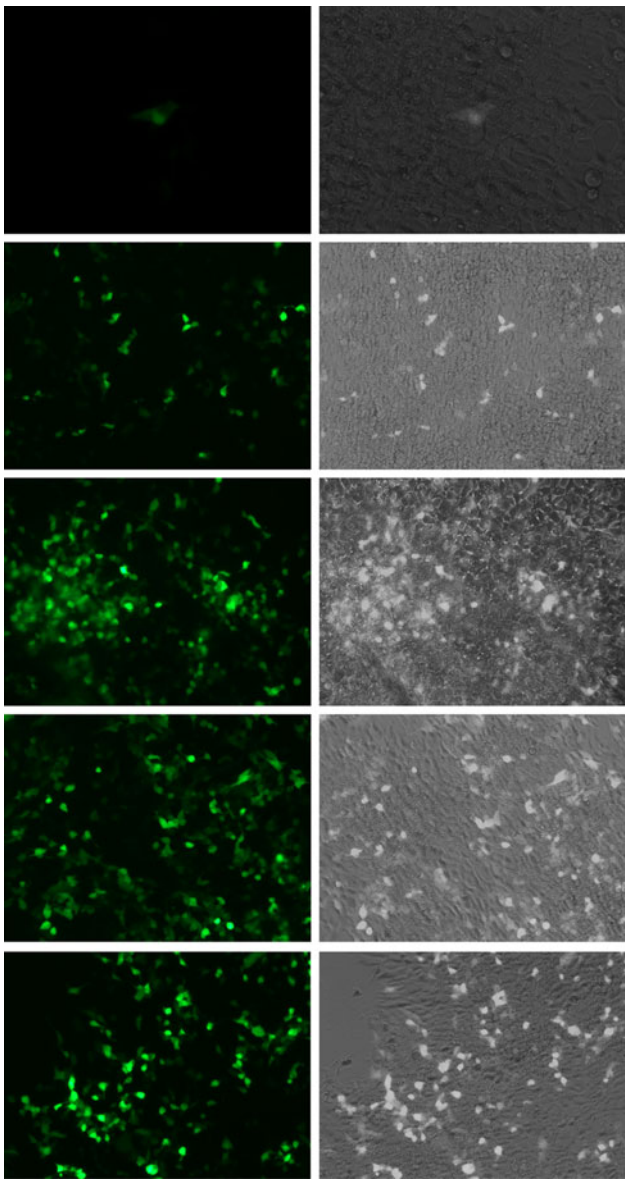

Fig. 11 GFP expression in 293T cells transfected with polycationic micelles/pEGFP-C1 complexes. Naked pEGFP-C1 and branched PEI 25 kDa/pEGFP-C1 complexes (N/P = 10) were used as controls. From *top* to *down*: naked DNA; N/P of polycationic micelles/DNA at 20:1, 40:1, 60:1 and PEI 25 kDa (N/P = 10:1)

would be a promising choice in the future nonviral gene delivery.

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