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Downregulation of survivin expression and enhanced chemosensitivity of MCF-7 cells to adriamycin by PDMAE/survivin shRNA complex nanoparticles

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ABSTRACT

Gene silencing mediated by RNA interference (RNAi) presents a promising strategy for gene therapy. The aim of this work is to evaluate a new gene delivery system for downregulation of survivin expression and enhanced chemosensitivity of MCF-7 cells to adriamycin (ADR). A new cationic poly(2-dimethylaminoethylamine/2-(2-aminoethyoxy)ethoxy)phosphazene (PDMAE) with multiple amino groups was synthesized through Michael addition for survivin shRNA (shSur) delivery in MCF-7 cells. PDMAE51/shSur complex nanoparticles with the size of 190 nm and zeta potential of +15 mV achieved maximal suppression of survivin, even superior to PEI25K or poly(2-(2-aminoethyoxy)ethoxy)phosphazene (PAEP) based complex nanoparticles. The significant downregulation of survivin expression was achieved by PDMAE51/shSur nanoparticles. The nuclear localization by confocal laser scanning microscopy (CLSM) and apparent apoptosis peak of cell cycle in MCF-7 cells were observed when transfected by PDMAE51/shSur nanoparticles The combined use of PDMAE51/shSur and ADR enhanced the sensitivity of MCF-7 cells to ADR at a larger extent than that of PEI or PAEP based complex nanoparticles. These results suggested that PDMAE51 could be potential as an efficient and safe gene carrier in RNAi therapeutics and tumor chemotherapy.

1. Introduction

Gene silencing mediated by RNA interference (RNAi) shows a tremendous therapeutic potential on treating various diseases including genetic disorders and cancer. RNAi represents a natural endogenous mechanism that cells utilize to regulate RNA expression (Novina and Sharp, 2004; Rahman et al., 2008). Now, there are two types of RNAi-based therapeutics: DNA-based RNAi and RNA-based RNAi, namely, a plasmid DNA encodes for a short hairpin RNA (shRNA) and a chemically synthesized small interference RNA (siRNA). Synthetic siRNA brings about the specific and transient effect, while more durable knock-down is achieved on using shRNA expressing vector. Despite the specific and effective gene silencing action, the major hurdle for delivering RNA effectively to the target cell still remains. Naked siRNA exhibits very low gene inhibition effect in vitro and in vivo due to their poor intracellular uptake and rapid enzymatic degradation in blood. To overcome these problems, siRNA or shRNA is often complexed with cationic polymers or liposome to form nano-sized complex nanoparticles for facilitating intracellular delivery as well as enhancing stability from enzymatic attack (Tanabe et al., 2004; Yamashita et al., 2007; Altieri, 2008). For example, synthetic vectors based on polycations, such as polyamidoamine (PAMAM), polyethylenimine (PEI), chitosan and cyclodextrin, commonly used for plasmid DNA delivery, have recently been investigated to transfer siRNA to cells (Chakravarti et al., 2004; Dean et al., 2007; Stauber et al., 2007; Pennati et al., 2008). Nevertheless more efforts are still made to find the optimum vectors for more efficiently knocking down the target gene in the target organ with lower toxicity.

In order to achieve high efficacy in RNAi-mediated therapy, it is critical to synthesize the correct target gene for the success of gene silencing. Recently, survivin has drawn much attention due to its up-regulation in the majority of malignancies, but rarely expressing in normal differentiated tissues (Tan et al., 2009; Suh et al., 2009; Takahashi et al., 2009). Survivin, a novel member of the inhibitors of apoptosis (IAP) family, plays a dual role in suppressing apoptosis and regulating cell division, and is predisposed as a potential new target for cancer treatment (Tsutsumi et al., 2007). In addition, survivin also was a cellular factor potentially involved in the chemo-resistant and radio-resistant phenotypes of human tumor cells, and the inhibition of survivin expression could lead to human tumor sensitization to chemical and physical agents (Bolcato-Bellemin et al., 2007; Liu et al., 2007; Shim and Kwon, 2009). So, the downregulation of survivin gene expression might be an efficient strategy for anti-cancer therapy in term of gene silencing and enhanced chemosensitivity.

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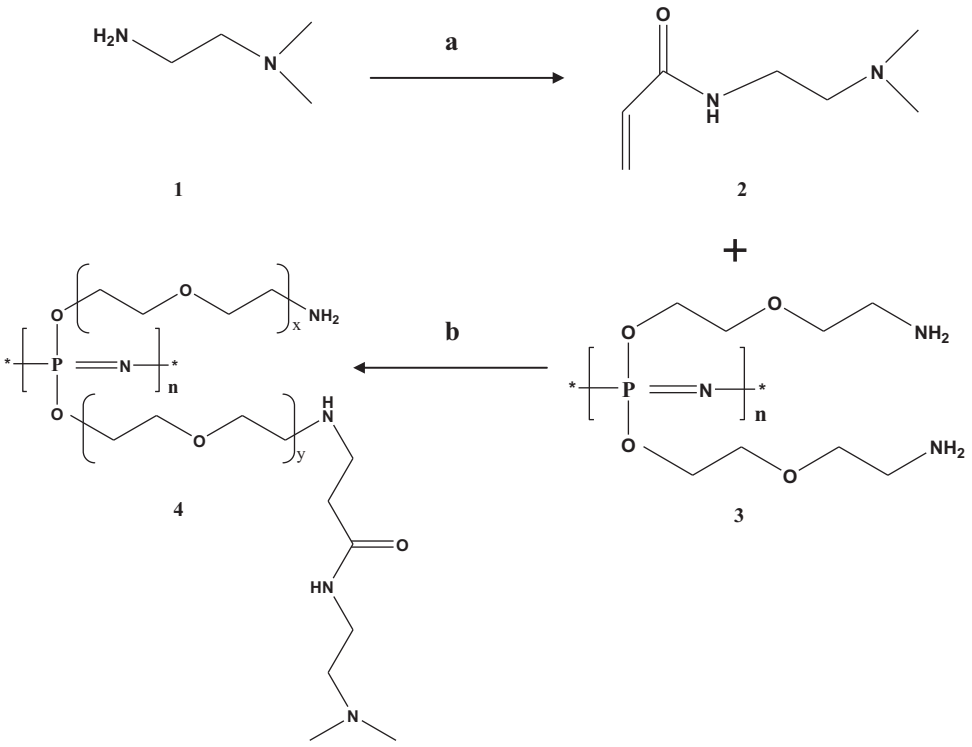

Fig. 1. Synthetic scheme of poly(DMAEA/2-(2-aminoethyoxy)ethoxy)phosphazene (PDMAE).

Polyphosphazene, a well tolerated biodegradable polymer, shows the good potential for plasmid DNA delivery in recent years (Luten et al., 2003; Yang et al., 2008, 2010a,b). However, its potential application in gene silencing has not been explored until today. In this work, a novel cationic polyphosphazene derivative called poly(2-dimethylaminoethy-lamine/2-(2-aminoethyoxy)ethoxy)phosphazene (PDMAE) with multiple amino groups were synthesized and investigated for RNAi-based therapeutics. To achieve a stable and prolonged gene silencing, shRNA targeting survivin was employed. Survivin shRNA (shSur) delivery systems based on PDMAEs carriers were characterized, evaluated in vitro for their gene silencing effect. In addition, the chemosensitivity of MCF-7 to adriamycin (ADR) in or without the presence of polymer/shSur delivery system was investigated as well.

2. Materials and methods

Poly(2-(2-aminoethyoxy)ethoxy)phosphazene (PAEP) was synthesized and characterized as described previously (Yang et al., 2010a,b). 2-Dimethylaminoethylamine (DMAEA), acryloyl chloride, adriamycin (ADR) and branched polyethylenimine (PEI 25K) were purchased from Aldrich. YOYO-1, Hoechst 33258 and Lysotracker Red were obtained from Invitrogen. Trypan Blue was purchased from TianGen (Beijing, China). Propidium iodide (PI) and RNase A were purchased from Sigma. FIX & PERM Kit were purchased from Multiscience (Shanghai, China).

Plasmid expressing small hairpin RNA against survivin (survivin shRNA, shSur), survivin shRNA-EGFP (shSur-E) and scrambled shRNA (scrshRNA) were synthesized by GenePharma Co. Ltd. (Shanghai, China) and amplified in DH5a strain of *Escherichia coli* and prepared by EndoFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). The targeted survivin mRNA sequence is GAATTAACCCTTGGTGAAT (Xu et al., 2010). Anti-human survivin-fluorescein monoclonal antibody and fluorescein isotype control were purchased from R & D systems China Co. Ltd. (Shanghai, China).

2.1. Cell culture

MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in DMEM containing 10% fetal bovine serum (FBS), streptomycin (40 μg/ml) and ampicillin (40 U/ml). Cells were maintained at 37 °C in a humidified and 5% CO_2 incubator.

2.2. Synthesis and characterization of PDMAE

PDMAE, a new cationic polyphosphazene with multiple amino groups, was synthesized via Michael addition between PAEP and DMAEA as shown in Fig. 1. In brief, acryloyl chloride solution (1.5 ml, 10.84 mmol) in 10 ml of dioxane was slowly added to the mixture solution of DMAEA (0.807 g, 9.15 mmol), compound **1** and triethylamine (5 ml, 36 mmol), which was dissolved in 10 ml of dioxane in an ice bath. The reaction mixture was stirred for 10 min, and the product was filtrated. After the filtrate was concentrated under reduced pressure, the product was isolated by column chromatography on silica gel using methanol/dichloromethane (1/1, v/v) to obtain the compound **2** as a yellow oil. Then, a variety of compound **2** in 10 ml of DMF with equivalent triethylamine was added to PAEP solution (compound **3**), which was dissolved in 2 ml of methanol and diluted with 5 ml of dimethylformamide (DMF). The resulting mixture was stirred for 24 h at 25 °C, and dialyzed using cellulose membrane (MW cutoff 6000–8000) against distilled water for 3 days. The resulting solution was lyophilized overnight to obtain PDMAE (compound **4**). ^{1}H NMR and ^{31}P NMR spectra of PDMAE were determined from Varian Mercury Plus-400 NMR spectrometer (Varian, USA). The chemical shifts were given relative to tetramethylsilane or 85% H_3PO_4 as an external standard. The molecular weight and distribution were determined by gel permeation chromatography (GPC, Waters 600) with acetonitrile/water/trifluoro acetic acid (10/90/0.05, v/v/v) as the eluent with flow rate (0.5 ml/min). The calibrations were peptide standards.

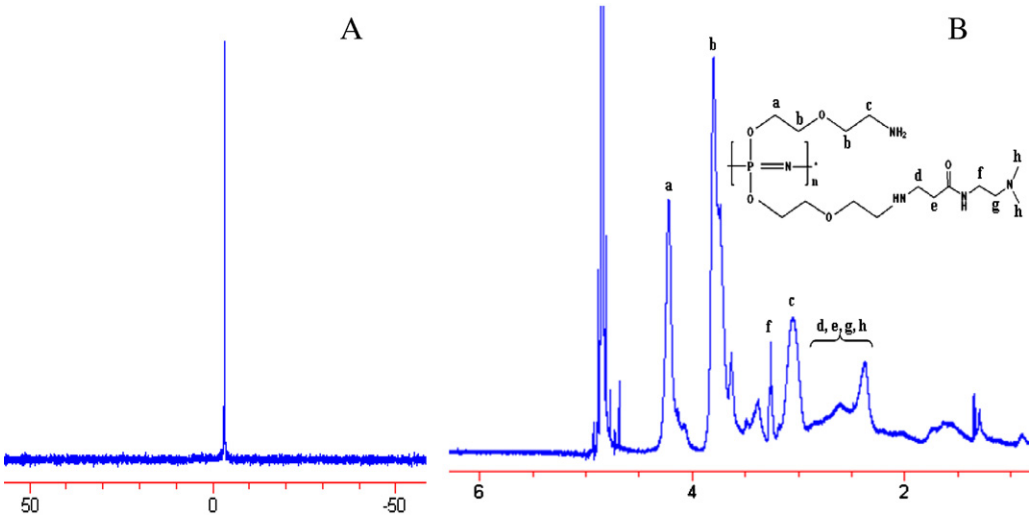

Fig. 2. ^{31}P NMR (A) and ^{1}H NMR (B) spectra of PDMAE34.

2.3. Preparation and characterization of PDMAE/shSur complex nanoparticles

To prepare complex nanoparticles between PDMAE and survivin shRNA expressing plasmid (shSur), the solution of shSur with concentration of 0.2 mg/ml was added to polymer solution with various N/P ratios. The mixture was gently vortexed for 20 s and allowed to stand for 30 min incubation. Then, the complexes were confirmed by electrophoresis on a 1% agarose gel with Tris-acetate–EDTA buffer system (pH 8.0) at 110 V/cm for 45 min. DNA was visualized using ethidium bromide staining. The sizes and ζ potentials of nanoparticles were measured by laser light scattering following their dilution with water by a Nicomp 380/ZLS zeta potential analyzer (Santa California, USA). The N/P ratio was defined as the ratio between the moles of the amine groups of polymers to those of the phosphate ones of DNA.

2.4. EGFP expression of MCF-7 cells transfected by PDMAE/shSur-E complex nanoparticles

In vitro transfection mediated by PDMAE/shSur-E nanoparticles was evaluated on MCF-7 cells at a DNA dose of 3 μg/well. 24 h prior to transfection, MCF-7 cells were seeded in 24-well plates at a density of 1×10^5 cells per well in 500 μl complete medium. Freshly prepared nanoparticles suspension was added to each well and incubated with cells for 3 h at 37 °C. Then, the medium was replaced with fresh growth medium with additional 45 h incubation. After that, the cells were washed three times with cold PBS for directly observing under microscopy or harvested for determining the transfection efficiency (fluorescence intensity of green fluorescent protein) with a flow cytofluorometer (Becton Dickinson, USA) equipped with an argon ion laser. PEI and PAEP/shSur-E complex nanoparticles were used as control.

2.5. Survivin silencing experiment using polymer/shSur complex nanoparticles

Plasmid expressing small hairpin RNA against survivin (shSur) was used to silence survivin expression in MCF-7 cells with PEI and PAEP/ complex nanoparticles as control. As survivin is not a cell surface protein, the cells must be first fixed and permeabilized for intracellular staining. After transfected for 48 h in 24-well plates with the procedure as above description, MCF-7 cells were gathered and suspended in 100 μl of fixation medium, and incubated at room temperature for 15 min. Then, the cells were washed by PBS with 5% FBS twice and fully resuspended in 100 μl of permeabilization medium with gently vortexing. Added 5 μl of anti-human survivin-fluorescein antibody, the cell resuspension were vortexed 1–2 s and incubated for 20 min at dark. Then, the cells were washed twice and resuspended in each cube with 400 μl of PBS for final flow cytometric analysis. The silent percent of survivin protein was calculated after normalizing the results with untransfected cells. All experiments were performed in triplicates.

2.6. Cellular uptake and confocal laser scanning microscopy (CLSM)

For cellular uptake, 200 μl of shSur (0.2 μg/ml) was mixed with 10 μl of 50 μM YOYO-1 and incubated at room temperature for 1 h in the dark in advance. The cellular uptake experiment was performed as transfection procedure described above with minor modification. After incubation with transfection agent in 24-well plates for 3 h at 37 °C, the cells were rinsed with cold PBS and harvested by trypsinization. To quench the extracellular fluorescence, the cell suspension was mixed with 25 μl of a 0.4% trypan blue (TB) solution in PBS. The mean fluorescence intensity (MFI) of the cells was measured with a flow cytofluorometer. MFI of PEI/shSur complex nanoparticles was normalized to 100.

The intracellular tracking of nanoparticles with YOYO-1-labled shSur was visualized via CLSM. The cells were seeded on 10 mm^2 glass coverslips coated with a cell-adhesion polymer (poly(L-lysine), PLL) in 24-well plates (1×10^5 cells/well), and incubated for 24 h. 60 μl of nanoparticles suspension containing 3 μg of shSur in distilled water was added into each well. After 3 h of incubation, the cells were stained by treating 10 mM nuclear dye Hoechst 33258 and 2 μM Lysotracker Red in complete medium for 20 min, then washed with PBS and incubated with 25 μl trypan blue (0.4% in PBS 7.4) for 2 min to quench the extracellular fluorescence. After the cells were washed twice with cold PBS and fixed with 4% paraformaldehyde for 20 min in dark, they were mounted on glass slides with 3 μl of MobiGlow (MoBiTec, Goettingen, Germany) and visualized by confocal microscopy (Leica Microsystems).

2.7. Cell cycle analysis

MCF-7 cells were seeded in 24-well plates at a density of 1×10^5 cells per well in 500 μl complete medium in 24 h prior to transfection. The complex nanoparticles loading shSur or scrshRNA were

added to each well, respectively, and incubated with cells for 3 h at 37 °C. Then, the medium was replaced, and the cell was incubated for additional 45 h. After washed with cold PBS twice and gathered, the cells were fixed with 70% ethanol overnight at 4 °C and resuspended in 100 μl of PBS with 50 μg/ml RNase A for 30 min at room temperature. Finally, stained with 20 μg/ml PI for 20 min, the cells were analyzed by flow cytofluorometer (Becton Dickinson, USA).

2.8. Cell viability and chemosensitivity of MCF-7 cells to ADR

The cytotoxicity of polymer/shSur complex nanoparticles was assessed by MTT assay on MCF-7 cells in 96-well culture plate at a density of 0.5×10^4 cells/well. At 24 h later, polymer/shSur nanoparticles were added to the cells in fresh complete medium for 3 h. Then, the medium was replaced with fresh complete medium, and the cells were incubated for additional 45 h. The medium was refreshed and MTT solution (5 mg/ml) was added. After the cells were incubated for 4 h at 37 °C in 5% CO_2, the medium containing MTT was removed, and 150 μl of DMSO was added to dissolve the crystals formed by living cells. The absorbance was measured at 490 nm (Bio-Rad Model 550, Hercules, CA, USA).

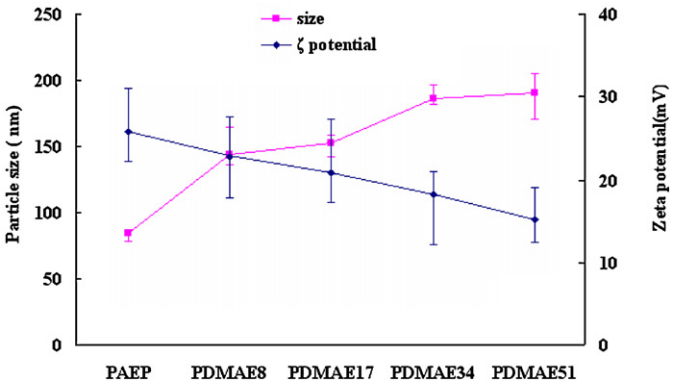

Fig. 3. The particle size and zeta potential of PAEP and PDMAEs/shSur complex nanoparticles at the N/P ratio of 35 in distilled water. Data represented the means of triploid sample ± SD.

It has been reported that RNAi-mediated downregulation of survivin could lead to augment the sensitivity of cancer cell lines to anti-tumor drugs (Huynh et al., 2006; Yonesaka et al., 2006). So, the similar procedure was performed as described above with minor modifications to investigate whether blocking survivin action could

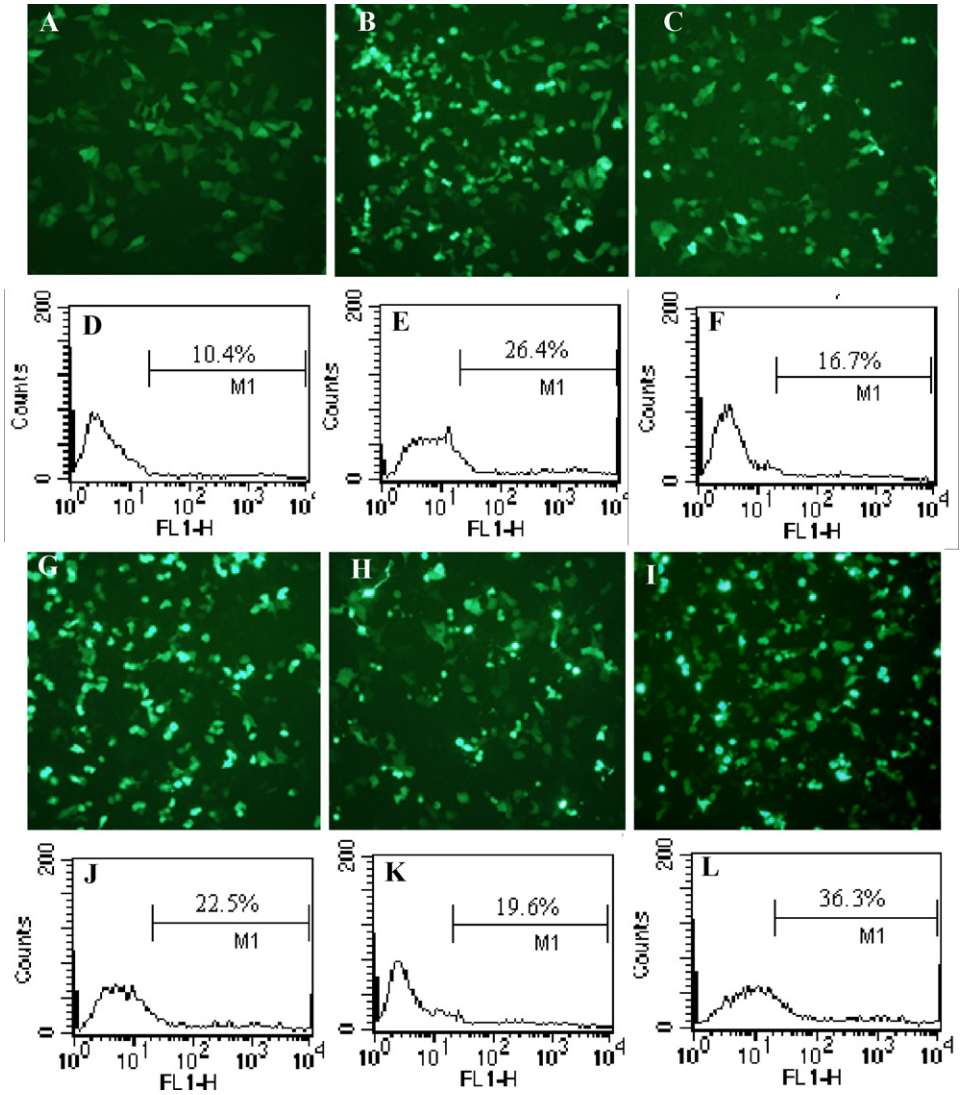

Fig. 4. The EGFP expression of MCF-7 cells mediated by PEI (A and D), PAEP (B and E), PDMAE17 (C and F), PDMAE8 (G and J), PDMAE34 (H and K), PDMAE51 (I and L) carriers by fluorescent microscopy (A–C and G–I, 20× magnification) and flow cytometry (D–J, K and L) after 48 h transfection. The N/P ratio of nanoparticles was 35 except that of PEI carrier was 20.

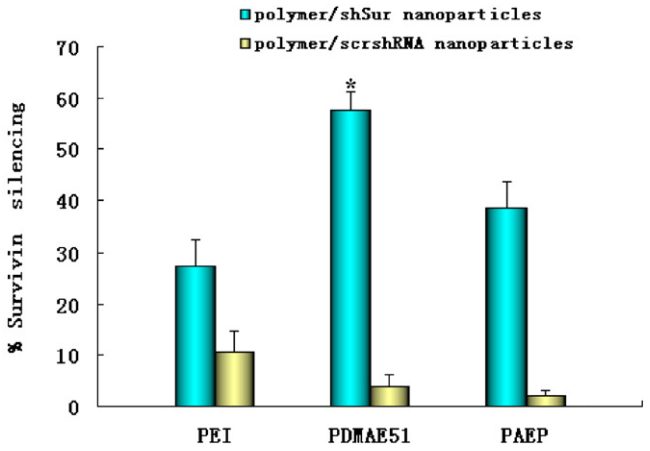

Fig. 5. Survivin silencing efficiency when delivered by polymer/shSur nanoparticles in MCF-7 cells by FACS analysis with polymer/scrshRNA nanoparticles as control. The percentage of survivin silencing was normalized with untransfected cells (error bar represents standard deviation, $n = 3$). $^{*}p < 0.05$ compared with the percentage of survivin silencing by treatment of PEI or PAEP carriers, respectively.

increase the sensitivity of MCF-7 cells to ADR. After polymer/shSur or polymer/scrshRNA complex nanoparticles were incubated with cells for 3 h, the medium was aspirated. ADR was added at a concentration of 10^{-4} mg/ml each well in fresh growth medium and incubated for another 45 h at 37 °C in 5% CO_2. The cells were treated with free ADR alone at a concentration of 10^{-4} mg/ml each well for 48 h as control.

2.9. Statistical analysis

Statistical analysis was performed using a Student's *t*-test. The differences were considered significant for $p < 0.05$ and $p < 0.01$ indicative of a very significant difference.

3. Results and discussion

3.1. Synthesis and characterization of PDMAEs

The delivery of plasmid EGFP based on PAEP carrier with primary amino groups has been investigated in our preceding work (Yang et al., 2010a,b). In this study, the higher transfection activity is supposed to be achieved at the coexistence of secondary (2°) and tertiary (3°) amino groups with PAEP carrier. The primary amino groups of PAEP facilitate the conjugation of function groups to develop more efficient gene carrier with less cytotoxicity. Thus, acrylated DMAEA monomer with 3° amino groups was conjugated to the primary amines of linear PAEP via a Michael addition reaction resulting in the introduction of 2° and 3°amino groups. Several polymers with different substituent degrees of DMAEA were synthesized and characterized by means of multinuclear (^{1}H, ^{31}P) NMR spectroscopy. A sharp single peak in ^{31}P NMR (Fig. 2A) and a wide peak at 2.28–3.0 ppm (i.e., protons of DMAEA) in the ^{1}H NMR spectrum of PDMAE (Fig. 2B) indicated successful conjugation of DMAEA monomer. The substituent degree of DMAEA was calculated from the integration ratio of DMAEA to 2-(2-aminoethyoxy)ethanol. In this work, four polymers named PDMAE8, PDMAE17, PDMAE34 and PDMAE51 were synthesized with DMAEA substituent degree of 8%, 17%, 34% and 51%, respectively. The molecular weight of PDMAE8, PDMAE17, PDMAE34 and PDMAE51 determined by GPC was 1.86×10^4 Da, 1.98×10^4 Da, 2.22×10^4 Da and 2.45×10^4 Da, respectively.

3.2. Characterization of PDMAEs/shSur complex nanoparticles

It is necessary to form stable and compact complex nanoparticles for efficient shRNA delivery using PDMAEs. All of PDMAEs

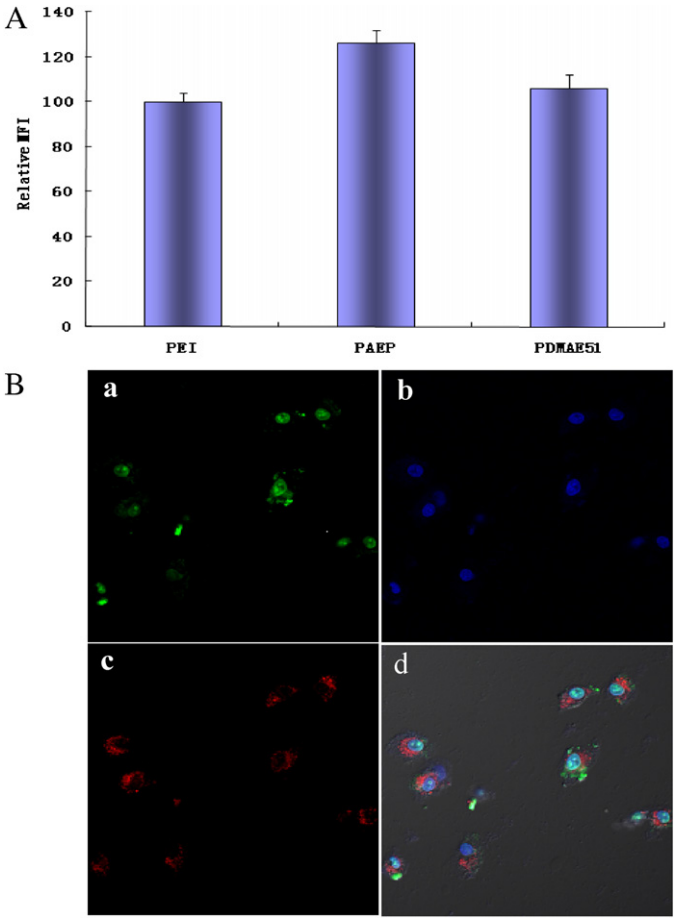

Fig. 6. (A) Cellular uptake efficiency of YOYO-1 labeled polymer/shSur nanoparticles at the N/P ratio of 35 after incubation with MCF-7 cells for 3 h at 37 °C in growth medium. MFI of PEI/shSur nanoparticles prepared at the N/P ratio of 20 was normalized to 100 ($n = 3$, error bars represent standard deviation). (B) CLSM-study of MCF-7 cells upon incubation with PDMAE51/shSur nanoparticles for 3 h at 37 °C in growth medium (magnification 40×). (a) YOYO-1 labeled shSur (green). (b) The nuclei stained by Hoechst 33258 (blue). (c) The lysosomes stained by lysotracker RED (red). (d) The image obtained after overlapping of a, b and c images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

with different substituent degree of DMAEA presented the efficient binding capacity with shSur at the N/P ratio of 5:1 by gel retardation assay (data not shown). The particle size and potential of PDMAEs/shSur complex nanoparticles at the N/P ratio of 35 were shown in Fig. 3. The PDMAEs could condense shSur into nanoparticles with the size of 140–190 nm, which was much larger than PAEP/shSur complex nanoparticles with the size of 84 nm. On the contrary, the potential of complex nanoparticles based PDMAE slightly decreased from +22.9 mV to +15.2 mV when the substituent degree of DMAEA increased from 8 to 51. The particle sizes increased, and zeta potentials of PDMAEs/shSur complex nanoparticles slightly decreased with the substituent degree of DMAEA increasing, which could be due to diminished positive charges from the conjugation of DMAEA with PAEP. In line with this, PDMAE51 based nanoparticles exhibited lower cytotoxicity than PAEP ones.

3.3. EGFP expression of MCF-7 cells transfected by PDMAEs/shSur-E complex nanoparticles

EGFP expression of MCF-7 cells mediated by PDMAEs/shSur-E complex nanoparticles at the N/P ratio of 35 was evaluated using

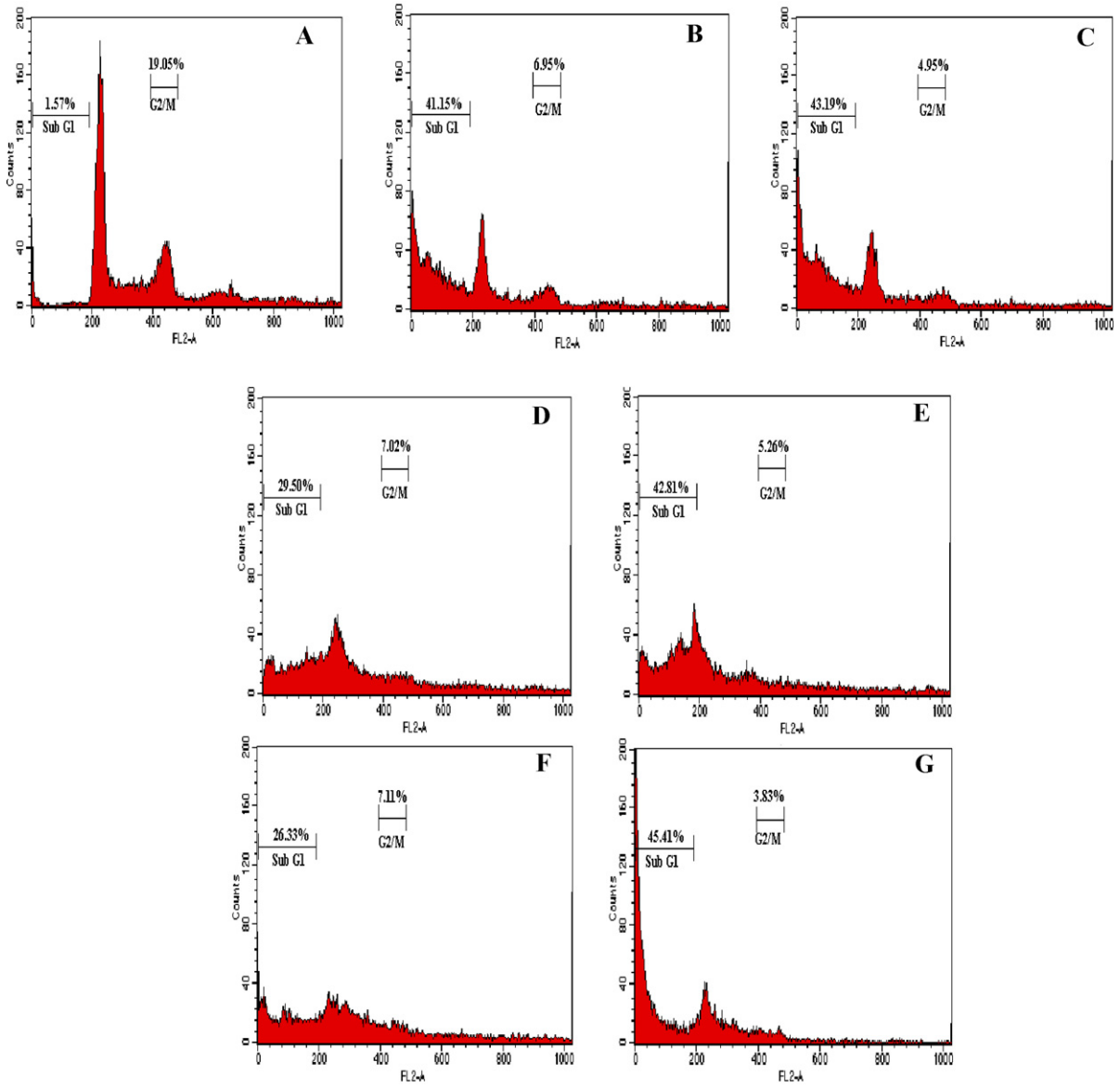

Fig. 7. Changes of cell cycle distribution analysis using flow cytometry in MCF-7 cells (A: untransfected cells) or after transfected by polymer/scrshRNA nanoparticles (B: PEI; D: PAEP; F: PDMAE51), polymer/shSur nanoparticles (C: PEI; E: PAEP; G: PDMAE51) for 48 h, respectively. The MCF-7 cells were fixed with 70% ethanol overnight at 4 °C, then washed and resuspended in 100 μl of PBS with 50 μg/ml RNase A for 30 min at room temperature. Finally, the cells were stained with 20 μg/ml propidium iodide (PI) for 20 min to analyze.

plasmid shSur-E encoding enhanced green fluorescence protein. The highest green fluorescence intensity and transfection efficiency were found for PDMAE51/shSur-E nanoparticles (Fig. 4). PAEP/shSur-E nanoparticles mediated higher transfection activity than other PDMAE carriers. The results suggested that PDMAE51 with the introduction of 2° and 3° amino groups at an appreciate ratio could achieve higher gene expression than PAEP with only 1° amino groups. With superior transfection efficiency, PDMAE51 was used as positive group for following experiments with PEI and PAEP as control. The optimal N/P ratio was fixed at 20 for PEI, which was based on the maximum transfection level. With the introduction of DMAEA monomer at the substituent degree of 51%, PDMAE51 contained 1°, 2° and 3° amino groups simultaneously. The 1° amines within PDMAE51 play an essential role in condensing pDNA into small and compact nanoparticles. Furthermore, newly added 2° and 3° amino groups could be more protonatable at lower pH thus facilitating shSur escaping from endosomal into nucleus, which was shown as CLSM pictures (Fig. 6B). So the coexistence of 2° and 3° amino groups with the 1° amino groups at an appreciate ratio could account for the superior transfection activity of PDMAE51 carrier.

3.4. Down-regulating effects of RNAi on survivin expression

As gene silencing mediated by synthetic siRNA is transiently effective, shRNA is preferred to achieve a stable and extended gene silencing in RNAi for anti-cancer treatment (Takeshita and Ochiya, 2006; Jere et al., 2008). To expand the application of PDMAE for RNAi, anti-human survivin–fluorescein monoclonal antibody designed for multiparameter flow cytometric was employed as intracellular staining reagent to determine the expression level of survivin protein quantitatively. Cells were initially fixed in order to minimize the leakage of proteins out of the cell. After the fixed cells were permeabilized, the conjugated antibody was allowed to penetrate and bind to its target within the cell. Following a final wash, the cells are analyzed on a flow cytometer. Flow cytometric analysis of fluorescent conjugates will generate a signal which can be

detected in the FITC signal detector. Compared with untransfected MCF-7 cells, the maximal survivin gene silencing was obtained with 57% for PDMAE51/shSur nanoparticles (Fig. 5). The silencing percents of survivin expression were 38.2% and 27.3% for PAEP/shSur and PEI/shSur complex nanoparticles, respectively. However, there was no significant survivin suppression for cells by scrshRNA treatment. All the above evidences meant that PDMAE51/shSur complex nanoparticles had excellent and more specific interfering effect on survivin expression than PAEP and PEI carriers in MCF-7 cells.

3.5. Cell uptake and CLSM experiment

The internalization of PDMAE51/shSur nanoparticles was investigated by CLSM and flow cytometer. The amount of YOYO-1 labeled shSur in MCF-7 cells was estimated by flow cytometer after quenching the fluorescence of nanoparticles adsorbed on the cell surface by adding trypan blue to the cell suspension in PBS. As shown in Fig. 6A, the cellular uptake efficiency mediated by PDMAE51/shSur nanoparticles was slightly lower than that of PAEP/shSur nanoparticles. The inferior cellular uptake of PDMAE51/shSur complex nanoparticles to that of PAEP ones could ascribe to less positive charges of PDMAE51 ones resulting in less adsorption onto the cells. However, no significant difference of uptake efficiency between PDMAE51 and PEI carriers was observed. Minor difference of cell uptake efficiency between PDMAE51 and PAEP or PEI/shSur nanoparticles was not helpful for explaining the significant reduction of survivin expression of the former. While CLSM showed that most fluorescence was observed in the nucleus for YOYO-1 labeled PDMAE51/shSur nanoparticles, which indicated the rapid endosomal release and entry into nucleus (Fig. 6B). As for PAEP or PEI based nanoparticles, the most fluorescence was observed in cytosol and around the cell membrane, but rarely in nucleus (data not shown). The results suggested that the entry into nucleus is the limited-step of transfection for PAEP and PEI carriers rather than for PDMAE51 carrier. The data from CLSM might be an explanation for the excellent suppression of survivin achieved by PDMAE51 based nanoparticles.

3.6. Changes in cell cycle by survivin gene RNAi

As we know, the ability of survivin to counteract apoptotic stimuli enhances cell survival, which in turn facilitates cell proliferation including the proliferation of mutant cells. Consistent with this, survivin is regulated in highly cell cycle-dependent manner with a marked increase in G2/M phase (Li and Altieri, 1999). Cell cycle distribution analysis by flow cytometry indicated that there were great changes compared with untransfected MCF-7 cells (Fig. 7). The apoptosis peak before the G1 phase, which consists of apoptosis and necrosis, increased from 1.57% to 45.41% and reduced sharply in G2/M phase from 19.05% to 3.8% for MCF-7 cells transfected by PDMAE51/shSur nanoparticles, compared with untransfected cells. The obvious apoptosis and sharply decreased G2/M phase might ascribe to the significant downregulation of survivin expression. So the role of survivin in cell survival and apoptosis is well established by the efficient survivin gene silencing. Similar cell cycle distribution was observed for PAEP/shSur nanoparticles. However, the apoptosis peaks of MCF-7 cells with scrshRNA treatment were just up to 26.33% and 29.50% for PDMAE51 and PAEP carriers, respectively. The results suggested that apart from the cell toxicity of nanoparticles, PDMAE51/shSur and PAEP/shSur nanoparticles could induce cell apoptosis significantly and block the cells for entry into the G0/G1 phase. The apoptosis peak of MCF-7 cells also increased to 43.19% after treatment by PEI/shSur nanoparticles, but there was no obvious difference between shSur and scrshRNA treatment groups, which indicated that cell apoptosis increase might attribute to necrosis induced by the cytotoxicity of PEI/shSur nanoparticles other than survivin gene silencing.

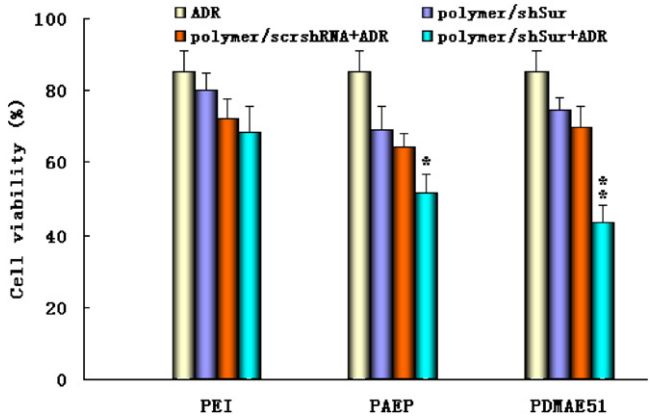

Fig. 8. The cell viability measured by MTT assay after the MCF-7 cells were treated by ADR; polymer/shSur nanoparticles; codelivery of ADR and polymer/shSur nanoparticles; codelivery of ADR and polymer/scrshRNA nanoparticles, respectively. All of the nanoparticles were prepared at the N/P ratio of 35 except PEI nanoparticles at the ratio of 20. ADR was added at a concentration of 10^{-4} mg/ml each well ($n = 3$, error bars represent standard deviation). $^{*}p < 0.05$ compared with the cytotoxicity of ADR; $^{**}p < 0.01$ compared with the cytotoxicity of ADR, polymer/shSur or codelivery of ADR and polymer/scrishRNA, respectively.

3.7. Cell viability and chemosensitivity of MCF-7 cells to ADR

As high levels of survivin in tumor cells confer resistance to a range of anticancer drugs, it is believed that downregulation of survivin expression in cancer cells could be useful to enhance the response to multiple types of conventional cancer therapies. ADR at a concentration of 10^{-4} mg/ml for each well was almost ineffective in inducing cytotoxicity, and PDMAE51/shSur nanoparticles showed slightly higher cell viability of 74% compared with 69% cells survival when treated with PAEP/shSur nanoparticles in MTT assay (Fig. 8). Meanwhile, the cytotoxicity of dual agent (ADR–shSur) was investigated in MCF-7 cells compared with control groups only treated by nanoparticles or ADR. The codelivery of PDMAE51/shSur nanoparticles and ADR greatly decreased the cell viability with 41% compared with 89% for free ADR or 74% for PDMAE51/shSur nanoparticles alone. However, there was no difference in cytotoxicity for the addition of PEI/shSur nanoparticles. Furthermore, the combination treatment of ADR and scrshRNA nanoparticles did not exert an obvious effect on the cell viability compared to ADR group. The results showed that the sensitivity of MCF-7 cells to ADR was obviously enhanced by survivin gene silencing mediated by PDMAE51/shSur nanoparticles, and resulted in decreased administration of antitumor drugs thus reduced side effects eventually. Further investigation is necessary to clarify the mechanism enhancing the chemosensitivity from PDMAE51/shSur nanoparticles.

4. Conclusions

PDMAE, a new cationic polyphosphazene with multiple amino groups derivatized from PAEP showed a great potential in gene expression and gene silencing. The level of EGFP expression and survivin gene silencing mediated by PDMAE51 carrier were more evident than PAEP and PEI carriers in MCF-7 cells. PDMAE51/shSur nanoparticles induced an apparent apoptosis in cell cycle as well as greatly enhanced the sensitivity of MCF-7 cells to ADR compared with PAEP and PEI based nanoparticles. The evidence so far accumulated suggested that PDMAE51 could emerge as an efficient and safe gene carrier in RNAi therapeutics for cancer treatment.

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