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Apoptosis of A549 cells by small interfering RNA targeting survivin delivery using poly- β -amino ester/guanidinylated O-carboxymethyl chitosan nanoparticles



Yue Tang*, Yun Liu, Yuwen Xie, Jing Chen, Yushun Dou

Department of Pharmacy, China Pharmaceutical University, Nanjing 211198, China

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ABSTRACT

Gene-based therapeutics has emerged as a promising approach for human cancer therapy. Among a variety of non-viral vectors, polymer vectors are particularly attractive due to their safety and multivalent groups on their surface. This study focuses on guanidinylated O-carboxymethyl chitosan (GOCMCS) along with poly- β -amino ester (PBAE) for siRNA delivery. Binding efficiency of PBAE/siRNA/GOCMCS nanoparticles were characterized by gel electrophoresis. The siRNA-loaded nanoparticles were found to be stable in the presence of RNase A, serum and BALF respectively. Fine particle fraction (FPF) which was determined by a two-stage impinger (TSI) was $57.8\% \pm 2.6\%$. The particle size and zeta potential of the nanoparticles were 153.8 ± 12.54 nm and $+12.2 \pm 4.94$ mV. *In vitro* cell transfection studies were carried out with A549 cells. The cellular uptake was significantly increased. When the cells were incubated with siSurvivin-loaded nanoparticles, it could induce $26.83\% \pm 0.59\%$ apoptosis of A549 cells and the gene silencing level of survivin expression in A549 cells were $30.93\% \pm 2.27\%$. The results suggested that PBAE/GOCMCS nanoparticle was a very promising gene delivery carrier.

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1. Introduction

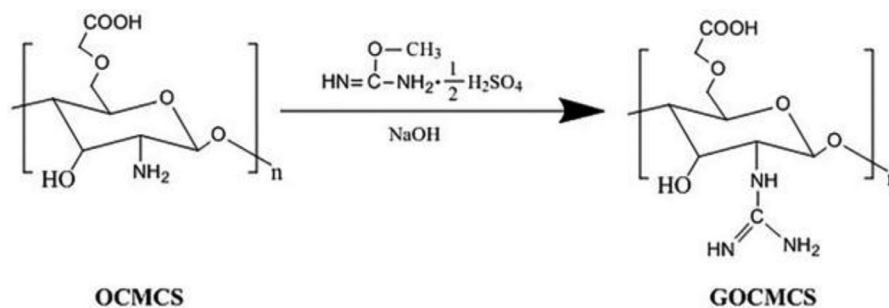
According to the latest statistics, lung cancer leads the highest fatality among all cancer diseases [1]. Lung cancer is divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC also has different types, such as adenocarcinoma, squamous cell carcinoma, large cell carcinoma and so on.

At present, the two common ways to treat this disease is surgery and chemo-radiotherapy [2]. But these approaches are expensive, painful and sometimes inefficient. Gene therapy can bring patients with high therapeutic effects and low side effects when treating cancer diseases [3]. It refers to correcting or replacing a defective gene by introducing normal gene or therapeutic gene fragment into target cells, and it has been extensively studied for treating primary

* Corresponding author. Department of Pharmacy, China Pharmaceutical University, Nanjing 211198, China. Tel.: +86 13851713608.

E-mail address: tangyue@cpu.edu.cn (Y. Tang).

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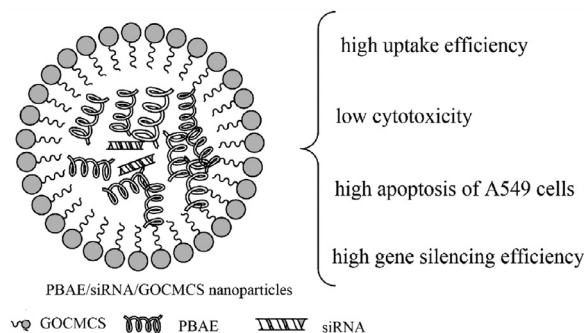
Scheme 1 – Synthetic route of GOCMCS.

immunodeficiency [4], hemophilia [5], heart failure [6]. RNA interference (RNAi) is a genetic surveillance mechanism that permits the sequence-specific post transcriptional manipulation of gene expression in an accurate manner [7,8]. siRNA-mediated cellular regulation by the RNAi-based pathway could offer a new paradigm in anti-cancer [9]. The clinical treatments show that delivery of gene to cancer cells recently face some obstacles for lack of suitable delivery vectors, proper therapeutic gene and feasible route of administration [10,11]. Hence, developing new delivery approaches is urging.

Delivery vectors used for gene therapy include viral vectors and non-viral vectors [12]. Compared with viral vectors, non-viral vectors show weaker immunogenicity and lower cytotoxicity, which have a wider application in gene therapy [13]. Poly(β -amino ester) (PBAE) is a brand-new type of cationic polymer material. PBAE is studied extensively for its biocompatibility and biodegradability [14]. To date, many research articles about PBAE focused on modifying polymers' structure or preparing ternary nanoparticles to improve the delivery efficiency of RNAi [15].

O-carboxymethyl chitosan (OCMCS) is a kind of amphiprotic ether derivative, which owns better physicochemical and biological properties [16]. It has emerged as a promising biomaterial in various fields, and it has been proved that OCMCS could significantly inhibit tumor cell migration *in vitro* [17]. However, there are still many challenges to address for its use in gene delivery system. To improve transfection efficiency, Park et al. modified OCMCS by grafting polyethylenimine [18]. Cell penetrating peptides (CPPs) can promote cell uptake of macromolecules because of the transmembrane transport capacity of guanidine groups. The guanidine group can be formed by the nucleophilic substitution reaction of primary amino groups with isobutamic compound. Luo et al. reported that guanidinylation of chitosan decreased cytotoxicity, facilitated cellular internalization and, at length enhanced the gene silencing efficiency [19]. In our previous study, guanidylated O-carboxymethyl chitosan (GOCMCS) was synthesised by converted guanidinos with aminos of OCMCS (Scheme 1), and the results had been proved that GOCMCS could improve the transfection efficiency compared with OCMCS [20].

Parenteral administration of siRNA has challenges due to nuclease degradation and renal clearance in the systemic circulation [21]. At current stage, most of non-viral gene therapy systems applied in clinical trials are through intravenous



Scheme 2 – The structure of PBAE/siRNA/GOCMCS nanoparticles and its biological properties.

and subcutaneous administration route. Only a few drug delivery systems are administered nasally [22]. Pulmonary administration takes the advantage of low drug dosage, quick absorption, bioavailability, stability and proves to be a patient-friendly delivery system [23,24]. Pulmonary administration includes aerosolized inhalation, metered-dose inhalation, and dry powder inhalation. The inhalation devices include nebulizers, pressurized metered dose inhalers (pMDIs) and dry powder inhalers (DPIs). Each type of inhalation device has its advantages and disadvantages [21].

The objective of this study was to develop nanoparticles with core-shell structure based on PBAE and GOCMCS for successful pulmonary delivery of siRNA. Scheme 2 shows the structure of the nanoparticles. The nanoparticles were prepared based on electrostatic self-assembly and the stability of the formulations were evaluated by gel retardation. The cytotoxicity, cell uptake and gene knockdown efficiency of these nanoparticles were tested in A549 cells. The aerodynamic behavior of nebulized nanoparticles were also assessed.

2. Materials and methods

2.1. Materials

The GOCMCS and amine-terminated PBAE were synthesized in previous studies [20]. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from D&B Biological Science and Technology Co.Ltd (shanghai, China).

Agarose was from Solarbio Biotech Co. Ltd. (Beijing, China). 1 × JJ Red was obtained from Biolite Biotech Co. Ltd. (Tianjing, China). Chlorpromazine, genestin and amiloride were purchased from Aldrich Chemical Co. (Shanghai, China). Filipin III were obtained from Cayman Chemical Co. (Ann Arbor, MI). Trypsin, penicillin, streptomycin, RPMI1640 cell culture medium, Annexin V-FITC and propidium iodide (PI) apoptosis assays kit, Hoechst 33,342 and Trizol total RNA extraction reagent were from KeyGen BioTech (Nanjing, China). The qRT-PCR quantitation kit, FAM labeled siRNA, negative control siRNA (siNC) and survivin siRNA (siSurvivin) were synthesized by GenePharm (Shanghai, China).

2.2. Cell lines and culture conditions

A549 (the human lung adenocarcinoma cells) were obtained from American Type Culture Collection (ATCC) and were cultured at 37 °C in 5% CO₂ atmosphere in complete RPMI1640 medium (containing 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin).

2.3. Preparation and characterization of nanoparticles

Appropriate amounts of PBAE (1 mg/ml) solution was mixed with siRNA (100 ng/µl) solution and vortexed for 10 s. Then PBAE/siRNA complexes were incubated at room temperature for 20 min to allow self-assembly. To prepare GOCMCS coated complexes, GOCMCS solution (1 mg/ml) was added to binary complexes at various weight ratio (50:1:20, PBAE:siRNA:GOCMCS,w/w/w), then, mixed well and incubated for another 20 min at room temperature [15]. The particles size and zeta-potential of PBAE/siRNA/GOCMCS complexes were determined with the dynamic light scattering technique (DLS) with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

PBAE/siRNA/GOCMCS complexes binding assay were confirmed by gel electrophoresis.

First, 1% agarose gel was prepared by dissolving agarose in 1 × TAE buffer (made of 40 mM Tris, 20 mM Acetate and 1 mM EDTA), the gel was heated to melt completely. and 1 × JJ Red was added in the gel. The previous nanoparticles were mixed with 6 × loading buffer (0.15% Bromophenol Blue, 0.15% xylene cyanol ff, 5 mmol/l EDTA and 50% Glycerol) and DEPC water was used to adjust the total volume. All the samples were loaded on each well (100 ng siRNA per well), and electrophoresed at a constant voltage of 100 V for 20 min. Finally, the gel was visualized by UV illumination and photoed by Gel imaging system.

The protection against RNase A degradation assay was detected by agarose gel electrophoresis [25]. Naked siRNA and siRNA-loaded nanoparticles was incubated with RNase A (25 mg/ml, 37 °C) for 24 h, respectively. At predetermined time interval (0 h, 0.5 h, 2 h, 4 h, 12 h, 24 h), 20 µl of the sample was removed and put into hot water at 80 °C to stop the reaction, and amounts of sodium heparin were added to release free siRNA.

To investigate the stability of PBAE/siRNA/GOCMCS nanoparticles in fetal bovine serum (FBS) and bronchoalveolar lavage fluid (BALF), the nanoparticles and naked siRNA

were incubated with 10% FBS or BALF at 37 °C at certain time interval (0 h, 0.5 h, 2 h, 4 h, 12 h, 18 h), naked siRNA was set as negative control. After all the samples were collected, heparin was added into each sample to detach siRNA from the nanoparticles. SiRNA integrity was investigated by the gel electrophoresis.

2.4. Nebulization of formulations and aerodynamic behavior

The siRNA-loaded nanoparticles were nebulized by using Omron NE-C801 (Kyoto, Japan) to deliver into lung. To assess the integrity of the siRNA-loaded nanoparticles after nebulization, gel electrophoresis was carried out. The naked siRNA was set as negative control. siRNA-loaded nanoparticles and naked siRNA were compared before and after nebulization respectively.

Aerosol performance was measured by using a two-stage impinger (TSI) according to Chinese Pharmacopoeia. First, the FITC-loaded GOCMCS was synthesized as previous studies [20]. Then PBAE/siRNA/FITC-GOCMCS nanoparticles (50:1:20,w/w/w) was prepared as mentioned above. Then, the prepared nanoparticles were move into the nebulizer reservoir which attached to TSI. Adjust the flow rate to 60 ± 5 l/min using vacuum pump. After TSI test, the aerosol formulations were collected completely. The fluorescence intensity of FITC-labeled nanoparticles in every stages was measured respectively at excitation wavelengths of 492 nm and emission wavelengths 518 nm by automatic microplate reader (POLARstar Omega, BMG, Germany). Fine particle fraction (FPF) was defined as the amount of FITC-loaded nanoparticles in Stage2 (mass median aerodynamic diameter < 6.4 µm) as percentage of the total nanoparticles released into the TSI.

2.5. In vitro cytotoxicity assay

FAM-labeled siRNA was used in order to be able to detect by flow cytometry (MACSQuant Analyzer 10, Miltenyi, Germany). PBAE/FAM-siRNA/GOCMCS nanoparticles was prepared as previous, briefly, FAM-labeled siRNA (50 nM) was added into PBAE solutions (1 mg/ml) at a weight ratio of 50:1, then vortexed 10 s and incubated at 37 °C for 30 min in the dark to prepare PBAE/FAM-siRNA binary nanoparticles. To prepare PBAE/FAM-siRNA/GOCMCS ternary nanoparticles, then, added the binary nanoparticles dropwise into GOCMCS (1 mg/ml) solution, and then incubated at room temperature for another 30 min in the dark.

A549 cells were seeded on 96-well plates at 2 × 10⁴ cells/ml, cultured in complete medium RPMI1640 for 24 h. The medium was replaced with free RPMI 1640 (200 µl) which contained PBAE/FAM-siRNA/GOCMCS nanoparticles at various concentration (10 µg/ml, 25 µg/ml, 50 µg/ml and 200 µg/ml). After incubation at 37 °C for 24 h, the medium was changed with MTT solution (180 µl free RPMI 1640 and 20 µl MTT (1 mg/ml)), continue incubated at 37 °C for 4 h. Then removed the medium, and added 200 µl DMSO, incubated for another 10 min. Using enzyme standard instrument to measure absorbance at 490 nm.

2.6. Cellular uptake and mechanisms

A549 cells were seeded in 6-well plates at a density of 2×10^5 cells/ml and incubated for 12 h. Cells were washed with PBS, and the medium was replaced with free RPMI 1640 which contained PBAE/FAM-siRNA nanoparticles (50:1, w/w), PBAE/FAM-siRNA/GOCMCS nanoparticles (50:1:20, w/w/w) respectively, and incubated for 6 h. Then the media was removed, the cells were washed twice with PBS, harvested with trypsin, centrifuged, re-suspended in PBS, and then analyzed by flow cytometry instrument to assay the cellular uptake ability. Furthermore, fluorescence microscope (Olympus IX53, Japan) was used to qualitatively observe the cells uptake. A549 cells were transfected with PBAE/FAM-siRNA/GOCMCS nanoparticles for 6 h, fixed with 4% paraformaldehyde, and stained with Hoechst 33342.

To obtain the best time when the mean fluorescence intensity for the cellular uptake was biggest, the cells were incubated with the PBAE/FAM-siRNA/GOCMCS nanoparticles for series time (2 h, 4 h, 6 h, 24 h), other operations are similar with cellular uptake assay.

To study the endocytosis pathway of PBAE/FAM-siRNA/GOCMCS nanoparticles, cells were pre-incubated at 4 °C or incubated with endocytic inhibitors at 37 °C for 30 min. The endocytic inhibitors we used were sodium azide (1 mg/ml), chlorpromazine (10 mg/ml), hypertonic sucrose (450 mM), genistein (200 μ M), filipin (5 μ g/ml), amiloride (13.3 μ g/ml) [20]. Then the cells were incubated with PBAE/FAM-siRNA/GOCMCS nanoparticles for another 4 h. Finally, the cells were subjected to flow cytometry analysis.

2.7. Apoptosis of A549 cells

A549 cells were seeded on 6-well plates and cultured overnight for attachment. Then the medium was replaced with free RPMI 1640 that contained PBAE/siSurvivin/GOCMCS nanoparticles, and incubated for 6 h, then replaced the solution with free RPMI 1640, and cultured for another 42 h. Finally, the cells were washed twice with PBS, detached by trypsin without EDTA, collected, centrifuged, and resuspend twice with PBS. Ultimately, the cells were suspended in 300 μ l binding buffer, added 5 μ l Annexin V-FITC for 15 min and further stained with PI before analyzed by flow cytometry instrument. All the operation need to be done in the dark. Cells incubated only with medium and cells incubated with naked siSurvivin were set as the control.

2.8. Gene silencing experiment

The gene silencing efficiency of PBAE/siSurvivin/GOCMCS nanoparticles were determined by quantitative real time reverse transcription polymerase chain reaction (qRT-PCR). A549 cells were seeded in 6-well plates. After the cells reaching 70%–80% confluence, the culture medium was changed with fresh complete medium respectively containing mocks, PBAE/siNC/GOCMCS nanoparticles, and amine-PBAE/siSurvivin/GOCMCS nanoparticles. After incubating for 6 h, the medium contained samples were replaced with fresh serum-free medium, and further cultured for another 42 h.

Then the cells were washed with PBS and lysed by Trizol reagent. The isolated total RNA was quantified by micro UV Spectrophotometer Nano 100 (All for life science, China) at 260 nm. RT-PCR was performed by using the SYBR Green I dye method. The data was analyzed by using the comparative $CT(2^{-\Delta\Delta CT})$ method.

3. Results and discussion

3.1. Formation and characterization of nanoparticles

The particle size and the zeta potential of various formulation with various weight ratio and concentration were determined by dynamic light scattering (DLS). The best particle size was 153.8 ± 12.54 nm, and the zeta potential was $+12.2 \pm 4.94$ mV when PBAE/siRNA/GOCMCS nanoparticles was 50:1:20(w/w/w, 1 mg/ml, 100 ng/ μ l, 1 mg/ml). A good gene delivery system must be able to bind to siRNA, and condense it into nanoparticles. To investigate the condensation ability of PBAE/siRNA/GOCMCS nanoparticles, agarose gel retardation electrophoresis assay was performed at various weight ratio(PBAE/siRNA). In our study, 50 nM naked siRNA was set as control, as the optimal transfection concentration of siRNA is in the range of 50–100 nM for *in vitro* experiments [26]. As shown in Fig. 1A, the complete retardation of siRNA mobility in the nanoparticles was achieved when the weight ratio was 50:1:20 (PBAE/siRNA/GOCMCS), at this point, the quantity of siRNA was 250 nM.

As shown in Fig. 1B, naked siRNA degraded rapidly and could not be observed within 0.5 h after it was incubated with RNase A. However, PBAE/siRNA/GOCMCS nanoparticles could be observed for a few hours. These results suggested that a certain amount of the siRNA was encapsulated into the nanoparticles, and the nanoparticles protected the siRNA from RNase A degradation.

In order to investigate the stability of the nanoparticles in lung and serum, naked siRNA and siRNA-loaded the nanoparticles were incubated with serum or BALF for 18 h. As Fig. 1C shown, naked siRNA and siRNA-loaded nanoparticles both could be observed after incubation in BALF for 18 h. Naked siRNA was hardly detected after incubation with serum for 12 h, while under the protection of the nanoparticles, the integrity of siRNA was maintained for 18 h (or longer). That is to say, the integrity of siRNA in serum was enhanced for the protection of nanoparticles compared with naked siRNA. Importantly, the results also suggested that siRNA was more stable in lung than in serum and indicated that it was more suitable for pulmonary delivery.

3.2. Nebulization of formulations and aerodynamic behavior

The nanoparticles were nebulized with Omron NE-C801 (Kyoto, Japan). The integrity of the siRNA after and before nebulization was evaluated by gel electrophoresis assays. As shown in Fig. 1D, the integrity of naked siRNA was decreased after nebulization (Fig. 1D, lane 2) due to the thermal energy generating from the process of nebulization [27]. The band of

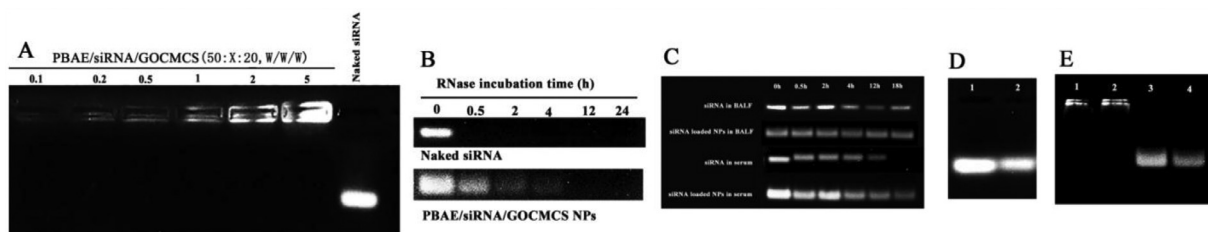


Fig. 1 – (A) Gel retardation analysis of siRNA binding with PBAE/siRNA/GOCMCS nanoparticles. (B) RNase A protection assay of naked siRNA and PBAE/siRNA/GOCMCS nanoparticles. (C) Gel electrophoresis assays the stability of siRNA in serum and BALF. (D) Gel electrophoresis of naked siRNA integrity before and after nebulization: Lane 1: pre-nebulization of naked siRNA; Lane 2: post nebulization of naked siRNA. (E) Gel electrophoresis of PBAE/siRNA/GOCMCS nanoparticles integrity before and after nebulization: Lane 1: pre-nebulization of nanoparticles; Lane 2: post nebulization of nanoparticles; Lane 3: nanoparticles dissociated with heparin; Lane 4: nebulized nanoparticles dissociated with heparin.

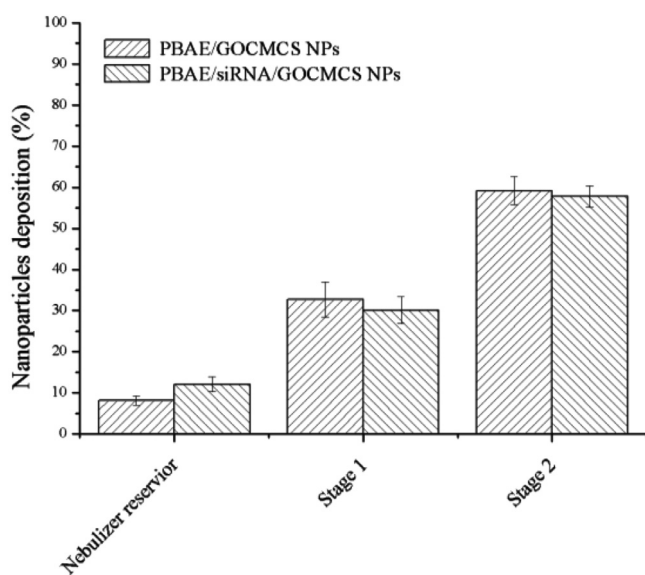


Fig. 2 – Nanoparticles deposition percentage of the nebulizer reservoir and each stage following nebulization (mean \pm SD, $n = 3$).

the siRNA-loaded nanoparticles was retarded in the well after nebulization (Fig. 1E, lane 2), and a bright and clear band of siRNA could also be observed after using heparin to dissociate nanoparticles (Fig. 1E, lane 4). The results suggested that siRNA can be successfully loaded into nanoparticles to overcome the shear force of aerosolization process and conserve its integrity.

FITC was used to quantify the concentration of the nanoparticles, FITC-labeled GOCMCS had been synthesized in previous study [20]. PBAE/siRNA/FITC-labeled GOCMCS nanoparticles were prepared as previous described. The portion of nanoparticles deposited in the stage 2 of impinger, representing the FPF of nanoparticles, was quantified by measuring the fluorescence intensity in every stages. As shown in Fig. 2., the FPF of PBAE/FITC-GOCMCS nanoparticles and PBAE/siRNA/FITC-GOCMCS nanoparticles were $59.2\% \pm 3.4\%$ and $57.8\% \pm 2.6\%$, respectively ($P > 0.05$), indicating that there

was no significant difference between the aerodynamic performance of PBAE/FITC-GOCMCS nanoparticles and that of PBAE/siRNA/FITC-GOCMCS nanoparticles. Hence, the loading of siRNA had no effect on the aerodynamic behavior of nanoparticles. The high FPF of PBAE/siRNA/FITC-GOCMCS nanoparticles suggested that this delivery system is suitable for deep lung deposition and could serve as potential candidate for siRNA pulmonary delivery.

3.3. Cytotoxicity assay

The basic cytotoxicity caused by the nanoparticles was evaluated in A549 cells by MTT assay, as presented in Fig. 3A, the PBAE/siNC nanoparticles showed low cytotoxicity. Although the same quantity of PBAE was utilized for gene complexation, PBAE/siNC /GOCMCS nanoparticles showed numerical slightly lower toxicity than PBAE/siNC nanoparticles. It was worth noting that cell viability was higher than 80% for the nanoparticles in contrast with PEI. Statistical analysis was performed using t-test and it had been proved that there was no significant difference between the cell viability of PBAE/siNC/GOCMCS nanoparticles and that of PBAE/siNC nanoparticles. It proved that PBAE can bind siRNA to form stable nanoparticles with low cytotoxicity, and it is a promising agent for non-viral gene delivery [28,29].

3.4. Cellular uptake and mechanisms

The cellular uptake of the siRNA was evaluated qualitatively and quantitatively via flow cytometric methods (Fig. 3B) and fluorescent microscopy (Fig. 4), respectively. As shown in Fig. 3B, the cellular uptake efficiency of PBAE/FAM-siRNA/GOCMCS nanoparticles was higher than PBAE/FAM-siRNA nanoparticles. The results proved that the group of guanidine has the similar effect of CPPs which promoted the uptake of the nanoparticles. On the other hand, GOCMCS balanced the positive charge of the nanoparticles, therefore, the nanoparticles was easily endocytosis into the cells [30]. Compared with PBAE/FAM-siRNA nanoparticles (Fig. 4A), the strongest green color in the merged image (Fig. 4B) represented that to some extents successful qualitative cellular uptake took place by the PBAE/FAM-siRNA/GOCMCS nanoparticle.

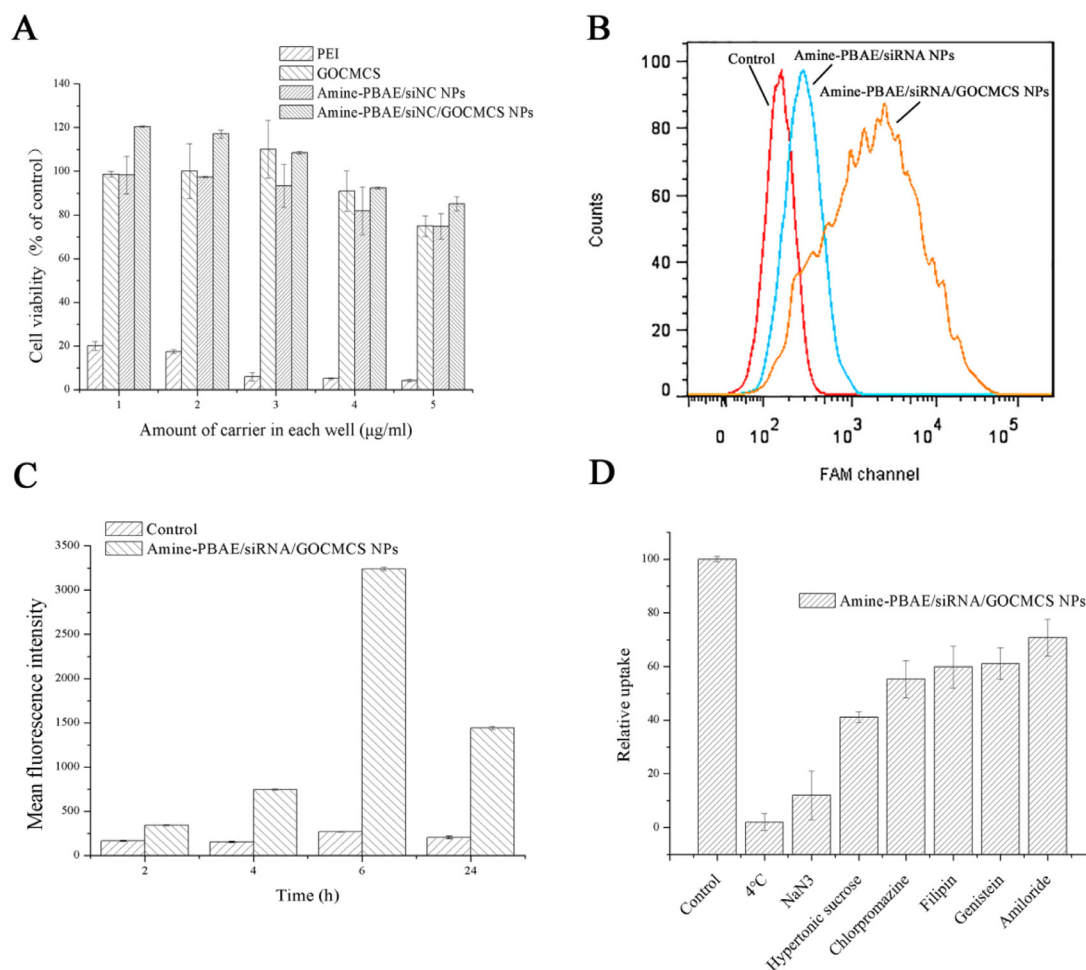


Fig. 3 – Cellular uptake and mechanisms of polymer/siRNA nanoparticles. (A) Cytotoxicity of siNC loaded polymers tested with A549 cells, (n = 6). (B) Internalization identification of nanoparticles in A549 cells with flow cytometric analysis. (C) Quantification of cell internalization at various time was shown by mean fluorescence intensity (MFI). (D) Effects of endocytosis inhibitors and temperature on the internalization of siNC loaded nanoparticles (n = 3), Relative uptake level(%) = $MFI(\text{treated group})/MFI(\text{control group}) \times 100\%$.

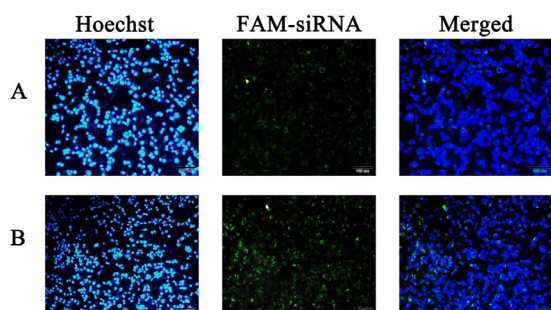


Fig. 4 – Fluorescence images of A549 about cellular uptake after incubated with FAM-siRNA loaded polymer nanoparticles for 6 h. (A) A549 cells were treated with PBAE/siRNA nanoparticles, (B) PBAE/siRNA/GOCMCS nanoparticles. Images showed fluorescent overlay of siRNA (green, FAM-labeled) and nuclear (blue, Hoechst33342-stained).

The nanoparticles exhibited time-dependent cellular uptake upon incubation. As shown in Fig. 3C, the highest cellular uptake efficiency was at 6 h, when the time prolonged to 24 h, the uptake efficiency had no obvious increase, this may be due to the cellular uptake of the nanoparticles had reached saturation, or FAM-siRNA was degraded and the fluorescence quenched gradually. Thus, the cellular uptake time was set at 6 h.

Endocytic inhibitors were applied to investigate the endocytosis pathways of the nanoparticles to delivery into the cell. Energy dependent processes endocytic pathways can be inhibited by low temperature and sodium azide which is an ATPase inhibitor. Clathrin dependent endocytosis can be inhibited by hypertonic sucrose and chlorpromazine. Filipin and genistein can be used as the inhibitors for caveolae dependent endocytosis. Amiloride can block macropinocytosis [31]. As shown in Fig. 3D, when the cell was incubated with sodium azide or at 4 °C, there revealed an significant inhibitory effects. This result indicated that the delivery of the nanoparticles was energy-dependent endocytosis. When the

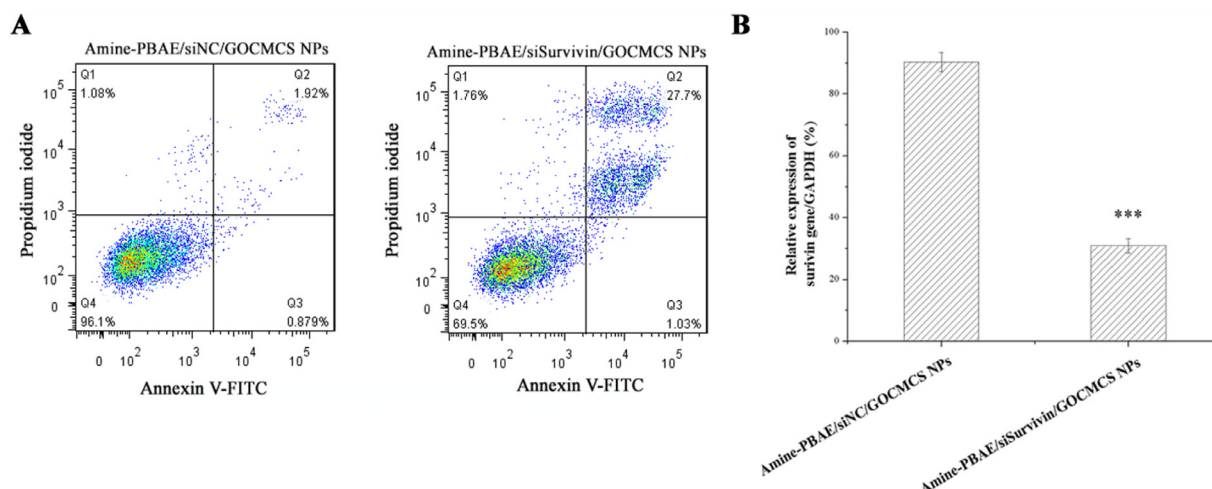


Fig. 5 – (A) Flow cytometry analysis of A549 cells for apoptosis after treatment with PBAE/siRNA/GOCMCS nanoparticles (n = 3). (B) siSurvivin-mediated silencing of A549 assessed by using qRT-PCR ($\Delta\Delta Cq$), GAPDH was set as endogenous reference gene (n = 3).

cells were incubated with chlorpromazine and hypertonic sucrose (clathrin-mediated endocytosis inhibitors) the cellular uptake level of the nanoparticles was relatively lower than filipin and genistein (caveolae-mediated endocytosis inhibitors). The uptake level of samples was lower than 70%. Therefore, clathrin-mediated and caveolae-dependent endocytosis both involved in mediating the cellular uptake of the nanoparticles, but more dependent on clathrin mediated endocytosis pathway.

3.5. Apoptosis of A549 cells

The apoptotic effect of the survivin siRNA carried by PBAE/GOCMCS nanoparticles was determined qualitatively using Annexin V/PI double staining assay [32]. The results were evaluated by flow cytometry (MACSQuant Analyzer 10, Miltenyi, Germany). The apoptotic cells are shown on Fig. 5A. Q1 indicated necrotic cells (UL), Q2 the late apoptotic cells (UR) Q3 early apoptotic cells (LR) and Q4 indicated the living cells (LL), each groups calculated cell number in the form percentage. The sum of Q2 and Q3 represented cell apoptosis rate, thus, the survivin siRNA introduced in the A549 cell by PBAE/GOCMCS nanoparticles caused $26.83\% \pm 0.59\%$ ($P < 0.01$) apoptosis cells compared with siNC-loaded nanoparticles treated group. The reason why the apoptosis increased might be attributed to the high cellular uptake efficiency into A549 cells.

3.6. The gene silencing

In this study, the gene silencing efficiency was evaluated by RT-PCR. Survivin siRNA was used as a model gene. siNC-loaded nanoparticles was set as a negative control, the results found that the carrier had no effect on the gene expression. The outcomes are shown in Fig. 5B as a bar chart. The results showed significant reduction

$30.93\% \pm 2.27\%$ ($P < 0.001$) in survivin mRNA level when treated with PBAE/siSurvivin/GOCMCS nanoparticles. These results suggested that PBAE/GOCMCS nanoparticles would be a promising gene delivery vector.

4. Conclusions

In summary, we have developed promising PBAE/siRNA/GOCMCS ternary nanoparticles based on electrostatical coatings as an efficient non-viral gene delivery system with excellent biocompatibility *in vitro* and *in vivo*. The addition of GOCMCS markedly performed high cellular uptake in A549, and the PBAE/siRNA/GOCMCS ternary complexes presented higher gene transfection efficiency *in vitro* and *in vivo* than the PBAE/siRNA binary complexes. It is concluded that PBAE/siRNA/GOCMCS may be a applicable gene delivery strategy of therapeutic genes to treat tumor *in vivo*.

Conflicts of interest

The authors declare no conflicts of interest.

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