Reversal of P-glycoprotein-mediated multidrug resistance by CD44 antibody-targeted nanocomplexes for short hairpin RNA-encoding plasmid DNA delivery

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ABSTRACT

Multidrug resistance (MDR) remains one of the major reasons for the reductions in efficacy of many chemotherapeutic agents in cancer therapy. As a classical MDR phenotype of human malignancies, the adenosine triphosphate binding cassette (ABC)-transporter P-glycoprotein (MDR1/P-gp) is an efflux protein with aberrant activity that has been linked to multidrug resistance in cancer. For the reversal of MDR by RNA interference (RNAi) technology, an U6-RNA gene promoter-driven expression vector encoding anti-MDR1/P-gp short hairpin RNA (shRNA) molecules was constructed (abbreviated pDNA-iMDR1-shRNA). This study explored the feasibility of using Pluronic P123-conjugated polypropyleneimine (PPI) dendrimer (P123-PPI) as a carrier for pDNA-iMDR1-shRNA to overcome tumor drug resistance in breast cancer cells. P123-PPI functionalized with anti-CD44 monoclonal antibody (CD44 receptor targeting ligand) (anti-CD44-P123-PPI) can efficiently condense pDNA into nanocomplexes to achieve efficient delivery of pDNA, tumor specificity and long circulation. The in vitro studies methodically evaluated the effect of P123-PPI and anti-CD44-P123-PPI on pDNA-iMDR1-shRNA delivery and P-gp downregulation. Our in vitro results indicated that the P123-PPI/pDNA and anti-CD44-P123-PPI/pDNA nanocomplexes with low cytotoxicity revealed higher transfection efficiency compared with the PPI/pDNA nanocomplexes and Lipofectamine™ 2000 in the presence of serum. The nanocomplexes loaded with pDNA-iMDR1-shRNA against P-gp could reverse MDR accompanied by the suppression of MDR1/P-gp expression at the mRNA and protein levels and improve the internalization and cytotoxicity of Adriamycin (ADR) in the MCF-7/ADR multidrug-resistant cell line. BALB/c nude mice bearing MCF-7/ADR tumor were utilized as a xenograft model to assess antitumor efficacy in vivo. The results demonstrated that the administration of anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes combined with ADR could inhibit tumor growth more efficiently than ADR alone. The enhanced therapeutic efficacy of ADR may be correlated with increased accumulation of ADR in drug-resistant tumor cells. Consequently, these results suggested that the use of pDNA-iMDR1-shRNA-loaded nanocomplexes may be a promising gene delivery strategy to reverse MDR and improve the effectiveness of chemotherapy.

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widely considered a promising approach for silencing target gene expression efficiently with high specificity at the transcriptional level and reducing the efflux of chemotherapeutic agents [5,11–14]. Small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) are ordinarily used by the gene silencing mechanism to inhibit gene expression, which has highlighted their potential use as therapeutic agents in cancer gene therapy. MDR1/P-gp-mediated MDR has been successfully reversed by the application of RNAi technology in different cell models derived from various tumors. shRNA is the precursor of siRNA in the intracellular microenvironment. Moreover, siRNAs can only achieve a transient therapeutic effect, but expression vectors encoding siRNA-like shRNAs have been developed to allow the long-term production of therapeutic RNAs in target cells [15]. For example, Ulrike and colleagues reported that the multidrug resistance phenotype could be reversed completely in vivo by the jet-injection of anti-MDR1 shRNA-encoding plasmid DNA. The results demonstrated that shRNA-expressing vectors effectively reverse MDR1/P-gp-mediated MDR in vivo and are therefore promising candidates for application to revert tumors with MDR1/P-gp-dependent MDR to a drug-sensitive state [16].

Regardless of the presence of siRNA, shRNA or plasmid DNA (pDNA) can ineffectively bind to cell–surface membranes by themselves, and their subsequent internalization is not possible. The identification of an efficient and safe gene delivery vector remains a major challenge to successful gene therapy. Nanoformulations based on cationic polymeric materials can assemble anionic nucleic acids into nanoparticles via ionic interactions and deliver nucleic acids to mammalian cells, leading to significantly increased transfection efficiency. A variety of cationic polymers have been designed as non-viral vectors and developed for gene delivery [17–23]. Among the different cationic polymers that have been reported, dendrimers have particular advantages [24–27]; (1) Dendrimers are highly branched with complete uniformity and dispersity, a regular structure and a high degree of symmetry; (2) There are a large number of cavities within the dendrimer molecules and a large number of functional groups on the surface of the dendrimer molecules; (3) The nano-size of dendrimers is suitable for cellular uptake and carrying foreign genes into the nucleus; and (4) The significant “proton sponge effect” of the dendrimers can promote the escape of pDNA from the lysosomes and thus avoid its degradation. The typical polycationic dendrimers are polyamidoamine (PAMAM) [28–30] and polypropyleneimine (PPI) [31–33], which have been widely used as carriers for gene delivery. Compared with PAMAM, PPI has not been widely studied. The transfections of G1-5 PPI have been performed in A431 cells, and the results have shown that PPI (G2 and G3) exhibits high transfection efficiency, equivalent to that of the cationic lipid carrier DOTAP, whereas PPI (G4 and G5) alone cannot be used for transfection because of its cytotoxicity [33]. There are two types of amino groups on the surface of G3 PPI, which can condense nucleic acids effectively with slight cytotoxicity and a significant “proton sponge effect”. Thus, G3 PPI was used as the core of the non-viral gene delivery vectors designed in this study.

A key issue of successful non-viral vectors for gene delivery in vivo is their stability in the blood circulation. Cationic biomaterials, such as PEI and PAMAM, show high transfection efficiency in vitro, but the particle size and dispersion of these polymer vector systems are very sensitive to serum and buffer when applied in vivo, resulting in low transfection efficiency, cytotoxicity, poor biodistribution and rapid clearance from the blood, which are mainly attributed to the strong positive charge of cationic polymers. Some hydrophilic polar molecules, such as poly(ethylene oxide) (PEO) [34] and poly(ethylene glycol) (PEG) [35–38], have often been introduced to modify a cationic polymer to inhibit non-specific protein adsorption by increasing particle stability and circulation time. PEGylated cationic polymers have been extensively studied as gene delivery vehicles. The modified cationic polymer may condense pDNA-forming core–shell nanostructures with a cationic polymer/pDNA core and a non-ionic hydrophilic shell and showed enhanced stability compared with non-PEGylated polyplexes. However, the use of the core–shell systems for gene delivery unavoidably compromised cellular internalization and transfection efficiency. This result is likely due to the hydrophilic shell reducing the interaction between the vector and the cell membrane and thereby resulting in poor cellular internalization [39]. Herein, Nguyen et al. proposed that cationic polymers can be modified with Pluronic rather than PEG [40]. This hypothesis was determined based on the following considerations: As non-ionic surfactants, the hydrophobic chain PO of Pluronic block copolymers has been shown to interact with the plasma membrane, increase the cell membrane fluidity and promote the cellular uptake of various small molecules and biomacromolecules. The EO chain of Pluronic molecules can make polyplexes stable in aqueous medium and reduce their interaction with plasma proteins, thereby protecting the pDNA from nuclease degradation, similarly to the effects obtained with Pluronic micelles [40]. In this manuscript, we describe the Pluronic P123, a triblock copolymer of ethylene oxide (EO) and propylene oxide (PO) subunits, as promising vehicles that not only exhibit important biological activities mentioned above but also acts as an inhibitor of P-glycoprotein (P-gp), which has been found to recuperate the sensitivity of multidrug-resistant (MDR) tumor cells to doxorubicin, paclitaxel and other anti-cancer agents [41].

To increase tumor-targeting drug delivery, many studies have focused on the molecular markers that are specifically overexpressed in cancerous cells and the tumor microenvironment. The cell surface membrane-bound protein CD44 is most widely overexpressed in various types of cancer cells [42–44] and is particularly interesting for the cancer-selective delivery of chemotherapeutic drugs, peptides and nucleic acid drugs. An antibody that binds to specific cell surface receptors has been well known to allow the insertion of a ligand into carriers to improve tumor targeting. Anti-CD44 antibody, which has been identified as a potent tumor-targeting ligand, can recognize CD44 receptors expressed exclusively within solid tumor. P-gp and CD44 are expressed in multidrug-resistant but not in parental, sensitive cell lines [45]. Breast cancer cells overexpressing CD44 receptors tend to be much more elastic than normal cells. The anti-CD44 antibody ligand—receptor system provides a new pathway for breast tumor-targeting diagnosis and therapy and prompts the current interest in the use of CD44 as a very important target for drug development [46–48].

The aim of this study was to re-sensitize doxorubicin-resistant breast cancer cells to the anticancer agent doxorubicin through the selective inhibition of P-gp. This study focused on developing an anti-CD44 antibody-conjugated Pluronic P123–PPI (anti-CD44-P123–PPI)-based nanocarrier to deliver pDNA–IMDR1-shRNA selectively into MCF-7/ADR cells to overcome drug resistance and administered ADR successively to achieve a combinatory effect of chemotherapy and gene therapy. To demonstrate the synergistic effects of gene therapy and chemotherapy, the nano-complexes loaded with pDNA–IMDR1-shRNA were evaluated based on their suppression of MDR1/P-gp expression at the mRNA and protein levels and the internalization and cytotoxicity of ADR in MCF-7/ADR cells in vitro. We also demonstrate the in vivo restoration of chemosensitivity to the MDR1/P-gp substrate ADR by delivering nano-complexes loaded with pDNA–IMDR1-shRNA intravenously into a xenograft tumor model (nude mice). Using a combination of gene therapy and chemotherapy, the therapeutic efficacy is reflected by the knock down of MDR1/P-gp in vivo, which
yields the same tumor volume as the chemosensitive tumor (MCF-7). The rational process of reversing multidrug resistance by the gene-silencing system is presented in Scheme 1.

2. Materials and methods

2.1. Materials

Pluronic P123 (MW 5750) was kindly provided by BASF (Germany). Polypropyleneimine (G3, MW 1684) was purchased from SyMO-Chem. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), N, N-carboxyldimidazole (CDI) and sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The anti-CD44 antibody was purchased from BD Bioscience (San Jose, CA, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Trypsin-EDTA (0.25%), penicillin-streptomycin and agarose were acquired from Gibco BRL (Gaithersburg, MD, USA). Lipofectamine™ 2000, YOYO-1, Lyso-Tracker Red and Hoechst 33342 were purchased from Invitrogen (Carlsbad, CA, USA). ADR was obtained from Beijing Huafeng United Technology Company (Beijing, China). Rabbit anti-P-gp monoclonal antibody, rabbit anti-i-actin monoclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody were obtained from Santa Cruz Biotechnology (USA).

The pDNA encoding enhanced green fluorescent protein (pEGFP-N2, 4.7 kb, CMV promoter) and the anti-MDR1 shRNA-encoding pDNA (abbreviated as pDNA-iMDR1-shRNA, U6 promoter) were amplified in Escherichia coli DH5a cells and purified using the Qiagen End-free™ Plasmid Mega Kit according to the provided manufacturer’s protocol. The purity was confirmed by UV spectrophotometry (A260/A280), and the concentration was determined from the absorbance at 260 nm. The targeted MDR1 mRNA sequence corresponded to the coding region at nucleotides 79-99 (5’-AAGGAAAAGAAACCAACTGTC-3’ relative to the start codon [49]. The negative control shRNA-expressing pDNA and pDNA-iMDR1-shRNA were obtained from GenePharm Co. Ltd. (Shanghai, China).

2.2. Cell lines and animals

Human umbilical vein endothelial cells (HUVEC), human breast adenocarcinoma cells (MCF-7) and human ADR-resistant breast adenocarcinoma cells (MCF-7/ADR) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in complete high glucose DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified 5% CO2 incubator. The MCF-7/ADR cells were maintained with free ADR at 0.5 mg/mL to continue their resistance phenotype.

Female BALB/c nude mice aged five weeks (18–22 g) were purchased from the laboratory animal center of Fudan University and maintained under a 12-h light/12-h dark cycle at the Animal Care Facility. The animals were administered fresh diet daily with free access to water and acclimatized for at least five days prior to the experiments. All of the animal experiments were performed according to the Guideline Principles evaluated and approved by the ethics committee of Fudan University.

2.3. Synthesis of P123-PPI and anti-CD44-P123-PPI

2.3.1. Synthesis of P123-PPI

The synthesis of P123-PPI was reported in detail in our previous publication [50]. P123 was directly activated by an equal molar quantity of CDI without terminal hydroxyl group protection and then reacted with PPI [40]. A brief schematic of the steps is shown in Fig. 1A. After dissolving 0.5 mmol of P123 in 25 mL of anhydrous acetonitrile and added dropwise to the PPI (210 mg) dissolved in 20 mL acetonitrile, 0.5 mmol of CDI was dissolved in 25 mL of anhydrous acetonitrile and added dropwise to the P123 solution in the presence of nitrogen gas. The mixture was stirred for 4 h at 40 °C, diluted with 75 mL of water and dialyzed six times against 20% ethanol. For P123-PPI conjugation, mono-imidazolylcarbonyl-activated P123 was added dropwise with constant stirring to PPI (210 mg) dissolved in 20 mL of water. The reaction mixture was incubated at 25 °C for 48 h. The products were dialyzed against water six times to remove unbound PPI. The resulting product, denoted P123-PPI, was collected, lyophilized, and stored at 4 °C for the preparation of P123-PPI/pDNA nanocomplexes without antibody conjugation.
2.3.2. Synthesis of anti-CD44-P123-PPI

Anti-CD44-P123-PPI was obtained as described previously using thiolated P123 (P123-SH) with some modifications [51–54]. The synthesis scheme is shown in Fig. 1B. Briefly, one terminal sulfhydryl group of Pluronic P123 was activated by CDI, and the mono-imidazolylcarbonyl-activated P123 was then conjugated to PPI. The synthesis process was the same as that described above in detail. In the next step, primary amines of anti-CD44 antibody were modified with the sulfo-SMCC cross-linker. Typically, 100 μL of 50 μM sulfo-SMCC cross-linker was first added to 400 μL of pure antibody solution in PBS-EDTA buffer (1 mg/mL) and shaken for 1 h at room temperature to introduce maleimide groups. To remove unconjugated sulfo-SMCC, the solution was washed three times using 10-kDa cutoff Amicon centrifugal filter units with PBS as an eluent. Finally, anti-CD44 antibody-conjugated SH-P123-PPI was obtained by the incubation of maleimide functionalized antibody with SH-P123-PPI under gentle shaking for 3 h at room temperature. The products were dialyzed to remove the free antibody and then lyophilized for further use.

2.4. Preparation and characterization of nanocomplexes

P123-PPI/pDNA and anti-CD44-P123-PPI/pDNA nanocomplexes were self-assembled by electrostatic interaction between the positively charged P123-PPI and the negatively charged pDNA at the appropriate N/P ratio. The optimal molar N/P ratio of nitrogen atoms in the copolymer to the phosphate groups on the plasmid was determined to be 20:1. To prepare the nanocomplexes, 24 μL of 2.5 mg/mL P123-PPI (or anti-CD44- P123-PPI) stock solution was diluted with 26 μL of deionized water and then added to pre-prepared 50 μL of 40 μg/mL pDNA solution. The samples were vortexed briefly and incubated at room temperature for 20 min to ensure complex formation.

The particle size, polydispersity index and zeta potential of the freshly prepared nanocomplexes were measured by dynamic light scattering (DLS) using a nano-Zetasizer (Malvern Instruments, UK) with a constant angle of 90° at 25 °C after the samples were appropriately diluted in distilled water. The measurements were performed in triplicate based on the refractive index and viscosity of water. The morphology of the nanocomplexes was observed and imaged by atomic force microscopy (AFM, BioScope, USA).

The condensation ability of the nanocomplexes was evaluated by agarose gel electrophoresis. After preparation of the nanocomplexes, 10 μL of the nanocomplexes (equivalent to 200 ng of pDNA) was added to 2 μL of 10 × loading buffer and then loaded into an 0.8% agarose gel with ethidium bromide (0.5 μg/mL). Gel electrophoresis was performed for 1.5 h at 90 V. The resulting pDNA migration pattern was visualized by UV illumination.

2.5. Cellular uptake and co-localization of nanocomplexes

To evaluate the intracellular uptake capacity and targeting efficacy of the anti-CD44 antibody-conjugated nanocomplexes in vitro, pEGFP-N2 was labeled with the fluorescent dye YOYO-1 (YOYO-1-pDNA) according to the manufacturer's...
protocol. HUVEC, MCF-7 and MCF-7/ADR cells were seeded into a six-well plate (Corning, NY, USA) at a density of 10^4 cells per well in DMEM containing 10% FBS and incubated for 24 h to obtain a cell confluence of 70–80%. Subsequently, the SYVOL®-pDNA-Loaded Nanocomplexes, P123-PPI/pDNA- and anti-CD44-P123-PPI/pDNA nanocomplexes were separately added to the cells, and the plate was then incubated for 4 h at 37°C. The concentration of pDNA was maintained at 4 μg/well/500 μL medium. At the end of the incubation period, the medium containing nanocomplexes was removed from the wells, and the cell monolayers were rinsed three times with cold PBS (0.1% w/v, pH 7.4), trypsinized and centrifuged at 1200 rpm for 10 min to harvest the cells. The harvested cells were then resuspended in PBS, and the fluorescence intensity was quantified by flow cytometry (BD FACS Calibur, USA).

Untreated cells were used as negative controls.

2.6.2. Transfection of therapeutic gene pDNA-iMDR1-shRNA and intracellular uptake of AD

To analyze the co-localization of the YOYO-1-pDNA-loaded nanocomplexes with lysosomes, MCF-7/ADR cells were seeded into 24-well-mm glass-based confocal dishes. After cellular uptake, Lysotracker Red (50 mL, Invitrogen) was added to the medium, and the cells were incubated for an additional 30 min at 37°C. Subsequently, 7.5 μL/mL Hoechst 33258 was added to the medium for 10 min to stain the nucleus. The cells were extensively washed with cold PBS and treated with Trypan blue (0.04%) to quench the extracellular fluorescence. The cells were then observed and pictured by confocal laser scanning microscopy (Leica TCS SPS, Germany).

2.6. Evaluation of transfection in vitro

2.6.1. Reporter gene transfection

The gene transfection ability of the nanocomplexes was evaluated using enhanced green fluorescent protein (EGFP) as the reporter protein. The transfection efficiency was assayed by the expression of EGFP in the mammalian cells after transfection. The MCF-7 and MCF-7/ADR cells were separately seeded in 24-well plates at a density of 5 × 10^4 cells/well and allowed to attach overnight. When the cells reached approximately 70–80% confluency, 450 mL of fresh DMEM and 50 mL of nanocomplexes (1 μg of pEGFP-N2) were added to each well. After incubation for 4 h, the medium containing nanocomplexes was replaced with 500 mL of fresh complete growth medium. After incubation for an additional 48 h, the EGFP-positive cells were further analyzed qualitatively by fluorescence microscopy (Leica, DMI 4000B, Germany). The cells were then trypsinized and quantified by flow cytometry analysis (FACSCalibur, Becton Dickinson, USA). The fluorescence parameters of untreated cells, the statistics of the cells that fluoresced above the control level were determined.

2.6.2. Transfection of therapeutic gene pDNA-iMDR1-shRNA and intracellular uptake of AD

To analyze the co-localization of the YOYO-1-pDNA-loaded nanocomplexes with lysosomes, MCF-7/ADR cells were seeded into 24-well plates at a density of 10^5 cells/well, and transfection was performed using the same protocol used for transfection of the reporter gene. After 48 h of incubation, the culture medium was removed, and the cells that were treated or untreated with the nanocomplexes were incubated with fresh culture medium containing 1 mg/mL ADR for 2 h. After incubation, the medium containing ADR was replaced with 500 mL of fresh culture medium, and the cells were incubated for an additional hour for efflux AD. The cells were then gently washed three times with cold PBS and photographed under a fluorescence microscope (Leica, DMI 4000B, Germany). The intracellular fluorescence intensity of ADR was quantified by flow cytometry analysis (FACS Calibur, Becton Dickinson, USA). For fluorescence microscopy observation, the cells were treated as described above. The cells were then washed three times with cold PBS and observed by fluorescence microscopy.

2.7. Reverse transcription-PCR

The level of MDRI mRNA was analyzed by reverse transcription-PCR (RT-PCR) [49]. MCF-7 and MCF-7/ADR cells at an initial density of 1 × 10^4 cells/well were seeded in six-well plates with 2 mL of growth medium and incubated for 24 h. Forty-eight hours after transfection, the cells were washed with cold PBS, and the total cells were harvested using a QIAamp RNAeasy® Kit and then reversely transcribed into complementary DNA (cDNA) using cDNA SuperMix Kit (Quanta Biosciences) according to the manufacturer’s recommendation. The MDRI primer sequences for amplification were the following: sense, 5'-ATATCCAGGCG- CACATCAT-3' and antisense, 5'-GGACGATGATCCTCG-3'. GAPDH served as a reference (sense, 5'-GCAAAAGGGTATCATC-3', and antisense, 5'-GTA- GACGAGGGATGTCACT-3') were used as an internal control. The amplification conditions were: 94°C for 1 min, 33 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, and final step at 72°C for 15 min. The PCR products were electrophoresed on a 1% agarose gel for 30 min at 100 V and visualized by staining with ethidium bromide under UV illumination.

2.8. Western blotting

For western blotting analysis, the cells were treated with lysis buffer, and the total protein after transfection, which was performed using the same protocol described above, was extracted. The total protein was quantified using the BCA protein assay kit (Promega, USA). Equal amounts of protein (50 μg) were separated by 10% SDS-PAGE, and the resultant proteins were transferred electrophoretically onto a nitrocellulose membrane. The membrane was then blocked with skimmed milk at room temperature for 2 h and incubated with rabbit anti-P-gp monoclonal antibody (1:500, v/v) and rabbit anti-β-actin monoclonal antibody and then with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:2000, v/v). β-actin served as an internal protein standard. The specific protein bands were visualized with an enhanced chemiluminescent system (ECL, Amersham) as described by the manufacturer. The band intensities were measured, and the protein signals were normalized to the β-actin levels.

2.9. In vitro cytotoxicity study

2.9.1. Cytotoxicity evaluation of polymers and nanocomplexes

The cytotoxicity of the polymers and nanocomplexes was evaluated by the MTT assay. MCF-7 and MCF-7/ADR cells were seeded at a density of 10^4 cells/well into 96-well plates in 100 mL of DMEM containing 10% FBS and incubated for 24 h. The medium was then changed, and the cell monolayers were incubated with 100 μL of fresh medium and added to the cells individually. Cells treated with fresh culture media only were used as negative control. After 4 or 24 h of incubation, the medium was changed and replaced with a mixture of 180 μL of growth medium and 20 μL of MTT (5 mg/mL in PBS). After the incubation period, all of the media was discarded carefully, and 150 μL of DMSO was added to each well. The plate was gently shaken for 10 min to dissolve the formazan crystals. The absorbance was measured at a wavelength of 570 nm using a 96-well plate reader (Synergy TM2, BIO-TEK Instruments Inc., USA). The results are expressed as the mean percentages of the cell viability relative to that of untreated cells. The viability of the non-treated cells was defined as 100%.

2.9.2. Cytotoxicity evaluation of ADR

To determine the cytotoxicity of ADR, MCF-7 and MCF-7/ADR cells were seeded at a density of 10^4 cells per well in 100 mL of growth medium in 96-well plates. After attachment, the medium was replaced with negative pDNA-loaded nanocomplexes or pDNA-imIDR1-shRNA-loaded nanocomplexes in fresh culture medium at a concentration of 0.2 μg of plasmid/well, and the cells were then incubated for 4 h at 37°C. Cells treated with fresh culture medium only were used as control. The nanocomplexes-containing medium was then replaced with fresh culture medium, and this step was followed by 48 h of incubation. The cells were then exposed to fresh culture medium containing different concentrations of ADR. After 48 h of incubation, the cell medium was replaced with fresh medium and subjected to MTT analysis.

2.10. Gene transfection and therapy in vivo

Female athymic BALB/c nude mice were xenografted subcutaneously in the right flank with 1 × 10^6 cells (MCF-7/ADR) and matrigel (BD Biosciences) suspended in 0.2 mL PBS. When the tumor sizes reached approximately 50–100 mm^3, the MCF-7/ADR tumor-bearing mice were randomly divided into eight groups (n = 8) such that each group exhibited a similar tumor size and subjected to in vivo transfection.

For luciferase expression in vivo, freshly prepared nanocomplexes were immediately injected intravenously into the tail vein at a dose of 50 μg pDNA per mouse. For quantification of luciferase expression, the heart, liver, spleen, lung, kidney, and tumor of mice were isolated at 48 h after the injection. The tissues were carefully washed twice with cold saline and homogenized with 1 mL lysis buffer (Promega, Madison, WI, USA) on ice. Homogenates were centrifuged at 15,000 rpm for 10 min and the supernatants were quantified for luciferase activity assays using a Luciferase Assay System. The tissue protein content was detected with BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China), respectively. Results about luciferase activity were expressed as fluorescence intensity associated with 1 mg protein (RLU/μg protein).

The mice were administered various formulations by i.v. injection as follows: (a) negative control (0.9% saline); (b) ADR solution; (c) P123-PPI/pDNA-iMDR1-shRNA nanocomplexes; (d) combination of P123-PPI/pDNA-iMDR1-shRNA nanocomplexes and ADR solution; (e) anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes; and (f) combination of anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes and ADR solution. The injection of nanocomplexes (2 mg pDNA/kg) was performed every three days for a period of 24 days. ADR at a dose of 10 mg/kg was administered 24 h after the injection of the nanocomplexes. On days 0, 4, 8, 12, 16, 20 and 24, the body weights of the mice and the tumor volumes (with calipers) were measured. The antitumor activity was assessed based on the individual tumor volumes (V), which was calculated using the formula: V = (L × W × W)/2, where the length (L) is the longest diameter and the width (W) is the shortest diameter perpendicular to the length. The tumor volume on the day of initial treatment (day 0) was normalized to 100% for all groups. The relative tumor volume (RTV) was calculated for each measurement time point (where RTV was equal to the tumor volume at a given time point divided by the tumor volume prior to initial treatment).
2.11. Statistical analysis

All of the data are presented as the means ± SD. The statistical significance of the differences was determined by unpaired Student's t-test. P < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Synthesis of P123-PPI and anti-CD44-P123-PPI

In this study, anti-CD44-P123-PPI was developed to improve the targeting properties of P123-PPI in gene delivery to MCF-7/ADR cells. The synthetic route of the targeting copolymers is presented in Fig. 1.

P123-PPI was synthesized as a gene carrier with low toxicity and high gene transfection in MDR cancer cells according to our previously reported procedure [50]. In preliminary experiments, P123-PPI was synthesized at varying P123/PPI molar ratios through two methods [40,55]: non-single-terminal protection graft reaction (Fig. 1A) and single-terminal protection graft reaction (Supplementary Fig. S1). As shown in Supplementary Table S1, the typical results of elemental microanalysis and the conjugation efficiency of P123-PPI were calculated based on the number of P123 molecules grafted to each PPI molecule. For non-single-terminal protection, equal molar amounts of P123 and CDI were fed, and the CDI solution was added dropwise to the P123 solution to control the single-terminal conjugation. In addition, the number of P123 molecules grafted to PPI increased with an increase in the P123/PPI molar ratio. Additionally, at a P123/PPI molar ratio greater than 4:1, the number of P123 molecules grafted to PPI no longer increased. However, in the single-terminal protection graft reaction, only 1.2 P123 molecules were grafted to each PPI molecule at a molar ratio of activated P123 to PPI of 4:1. The P123-PPI copolymer was also characterized and determined quantitatively by 1H-NMR (400 MHz, D2O). The 1H-NMR spectra of P123-PPI is shown in Supplementary Fig. S2. The signals at δ = 1.00 and 3.41 ppm correspond to the methyl protons and methenyl protons in the propylene oxide (PO) blocks of P123, respectively, and the peaks of the methylene protons in the ethylene oxide (EO) blocks of P123 were present at δ = 3.6 ppm. The presence of the PPI molecule could be confirmed by the signal at δ = 1.51–2.89 ppm corresponding to methylene protons. Most importantly, peaks appeared at δ = 4.09 ppm, and these were assigned to methylene protons close to the amide group, which indicated that P123 was conjugated successfully with the amino groups of PPI. According to the integral values obtained from all of the protons of P123 and PPI in the P123-PPI copolymer, the number of P123 conjugated to PPI with an increase in the P123/PPI molar ratio could be calculated. The results calculated from the 1H-NMR spectra were consistent with the elemental microanalysis results. Finally, non-single-terminal protection was selected for the synthesis of P123-PPI, which was used in the subsequent experiments with a P123/PPI molar ratio of 4:1, and approximately 2.5 P123 molecules were conjugated to one PPI molecule.

In this study, anti-CD44 antibody was conjugated to P123-PPI to further increase the transfection efficiency due to increased specific receptor binding. Because we selected thiolated P123, which contains sulfhydryl groups on the side chain, it is easy to conjugate maleimide functionalized antibody (Fig. 1) through a reaction between thiol groups and maleimide groups. This reaction between thiol groups and maleimide is rapid and proceeds close to completion. It occurs at nearly neutral pH, at ambient temperature, and even when rather low concentrations of the reactants are present.

3.2. Preparation and characterization of nanocomplexes

The P123-PPI/pDNA and anti-CD44-P123-PPI/pDNA nano-complexes were self-assembled by electrostatic interaction. The morphologies of free pDNA (Fig. 2A) and the representative nano-complexes (N/P ratio of 20) (Fig. 2B and C) were observed by AFM. The AFM images revealed that the prepared nano-complexes had a homogeneous and well-dispersed spherical structure with an average particle size of 155.9 ± 8.9 nm (without antibody conjugation) and 182.3 ± 5.4 nm (with antibody conjugation) in diameter. No obvious aggregation and free plasmid DNA (compared with the AFM image of free pDNA, Fig. 2A) was visible in Fig. 2B and C. These results demonstrated that P123-PPI and anti-CD44-P123-PPI could effectively condense pDNA into nanometer-size nano-complexes and that the P123 chains on the nano-complex surface could minimize the interactions among the particles to reduce the aggregation, even if the particles possessed neutral charges [40].

The particle size and surface charge of the polymer/pDNA complexes play an important role in determining the circulation time, biodistribution, intracellular internalization and transfection efficacy for in vivo applications [56,57]. The size distribution and zeta potential of the nano-complexes formed at various N/P ratios in an aqueous environment were investigated by the dynamic light scattering (DLS) technique (Fig. 2D). The results showed that all of the nano-complexes maintained a nanometer size (150–500 nm) within the tested N/P ratio range, which is suitable for endocytosis applications. The particle size decreased and then increased with an increase in the N/P ratio, and the following smallest sizes were observed at an N/P ratio of 20: 165.5 ± 12.1 nm for P123-PPI/pDNA nano-complexes and 198.0 ± 7.6 nm for anti-CD44-P123-PPI/pDNA nano-complexes. Because AFM measurements require the samples to air-dry before analysis, the particle sizes measured by AFM are always slightly smaller than those obtained using the DLS technique for samples suspended in solution. In general, the mean diameters measured through DLS and AFM analyses were consistent. The zeta potential increased from 1.9 ± 0.8 mV to 28 ± 2.7 mV for P123-PPI/pDNA nano-complexes and from 1.1 ± 0.5 mV to 25.8 ± 2.8 mV for anti-CD44-P123-PPI/pDNA nano-complexes as the N/P ratios increased from 1:1 to 40:1. These results implied that all negative charges of the pDNA molecules were neutralized by the positive charges of the polymer molecules, which contributed to the excess positively charged PPI chains in the nano-complexes. Moreover, the introduction of P123 into PPI molecules did not hamper efficient charge interaction between cationic P123-PPI and pDNA. The conjugation of antibody had little influence on the zeta potential of the nano-complexes.

The pDNA condensation abilities of P123-PPI and anti-CD44-P123-PPI at different N/P ratios were studied through a gel electrophoresis assay. As shown in Fig. 2E, the condensation abilities of the polymers enhanced with an increase in the N/P ratio, which was consistent with the results of the particle size analysis. The pDNA migrations in the agarose gel were completely retarded at N/P ratios of at least 5:1, indicating that all of the polymers could electrostatically neutralize the pDNA and deter electrophoretic mobility. However, the polymers could not completely prevent the electrophoretic behavior of pDNA at an N/P ratio of 1:1 (Fig. 2E), even though the zeta potentials were 1.9 ± 0.8 mV and 1.1 ± 0.5 mV (Fig. 2D), respectively. This result demonstrates that the polymers bound to the pDNA and neutralized its charge at an N/P ratio of 1:1, but the formed nano-complexes were thought to be loose and unstable. Additionally, the pDNA condensation abilities of P123-PPI did not show any significant difference after antibody conjugation.
3.3 In vitro transfection efficiency of reporter gene

The transfection activity of cationic polymers has often been shown to be cell line-dependent [58,59]. Moreover, P-gp and CD44 receptor are expressed in multidrug-resistant cell lines (MCF-7/ADR cells) but not in sensitive cell lines (MCF-7 cells) [45]. To investigate whether P123 and anti-CD44 play a role in gene transfection, transfection experiments were performed in MCF-7 and MCF-7/ADR cells treated with PPI/pEGFP, P123-PPI/pEGFP, scrambled antibody-P123-PPI/pEGFP, anti-CD44-P123-PPI/pEGFP nanocomplexes and Lipofectamine™ 2000/pEGFP (positive control), respectively. Fig. 3 shows the EGFP expression and transfection efficiency of the nanocomplexes in the two cell lines, which were visualized by the observation of EGFP-positive cells using a fluorescence microscope and quantified by flow cytometry.

Interestingly, the P123-PPI/pEGFP nanocomplexes achieved markedly higher transfection efficiency than the PPI/pEGFP nanocomplexes in serum-containing medium. Quantitatively, the P123-PPI/pEGFP nanocomplexes mediated a transfection efficiency in MCF-7 cells that was 1.6-fold greater (Fig. 3B) than that obtained with the PPI/pEGFP nanocomplexes. A similar tendency was also observed in MCF-7/ADR cells. An approximately 2.2-fold higher...
transfection efficiency (Fig. 3B) was observed in MCF-7/ADR cells treated with the P123-PPI/pEGFP nanocomplexes compared with that obtained with the PPI/pEGFP nanocomplexes. These results were consistent with those of previous studies, which have shown that Pluronic in different polyplex formulations can enhance transfection [39,60]. Kabanov and his group have discussed possible reasons for the increased transfection activity of Pluronic-modified polycation/pDNA complexes in a previous publication [60,61]. It has been hypothesized that polycations can condense pDNA to form polycation/pDNA complexes that bind to the cell membrane to facilitate endocytosis due to the positive charge of the surface. The hydrophobic PO segments of Pluronic in the polyplexes can interact with the plasma membrane and thereby increasing the fluidity of the membrane phospholipid bilayer and lowering the microviscosity. Therefore, Pluronic copolymer may intensify the endocytic processes in eukaryotic cells and may thus promote cellular uptake and enhance the transfection of the complexes. Furthermore, the transfection inhibition in the presence of serum has been a common problem for cationic non-viral gene delivery systems, and this inhibition has been explained by the fact that serum destroys the stability of polycation/pDNA complexes due to the non-specific interaction between the complexes and the complicated negative ingredients within the serum. Compared with PPI/pEGFP nanocomplexes and Lipofectamine™ 2000/pEGFP, a marked increase was observed in the cells treated with P123-PPI/pEGFP nanocomplexes, which indicated that the Pluronic copolymer may act as a shield for stopping the interaction between cationic complexes and serum.

In MCF-7/ADR cells, the strongest fluorescent spot density (Fig. 3A) and the highest transfection efficiency (Fig. 3B) were observed after transfection with the anti-CD44-P123-PPI/pEGFP nanocomplexes. However, no obvious difference was discovered in the transfection efficiency of P123-PPI/pEGFP and anti-CD44-P123-PPI/pEGFP nanocomplexes in CD44-negative MCF-7 cells (P > 0.05). Based on these results, it could be concluded that the anti-CD44-P123-PPI/pEGFP nanocomplexes were internalized into cells by CD44 receptor-mediated endocytosis.

3.4. Cellular uptake study and specific targeting

3.4.1. Effect of P123 on cellular uptake

The mechanism underlying the improvement of transfection efficiency achieved by the P123-PPI/pDNA nanocomplexes was further confirmed by analyses of the cellular uptake of nanocomplexes in MCF-7 and MCF-7/ADR cells. After 4 h of incubation, the mean fluorescence intensity of YOYO-1-positive cells was quantified by flow cytometry. As shown in Fig. 4, the P123-PPI/pDNA nanocomplexes showed markedly more efficient cellular internalization, which was 2.1- and 3.7-fold higher than that obtained with the PPI/pDNA nanocomplexes in MCF-7 and MCF-7/ADR cells, respectively. Polycation/pDNA complexes interact with the cell membrane depending on the positive charge. By introducing P123 into a polycation, the positive charge of the complex surface would be partly shielded, which would theoretically result in decreased cellular uptake. However, compared with those treated with PPI/pDNA nanocomplexes, the mean fluorescence intensity of YOYO-1-positive cells in the group treated with P123-PPI/pDNA nanocomplexes was significantly improved, which is an indication of greater cellular uptake. The overall trend of cellular uptake of nanocomplexes was the same as that obtained with the transfection experiments. In addition to the interaction between Pluronic and the plasma membrane, the difference in cellular uptake could result from the difference in their interaction with the serum because the positive potential of the P123-PPI/pDNA nanocomplexes was significantly lower than that of the PPI/pDNA.
complexes. It has been reported that the higher potential would result in increased non-specific interaction between the polyplex and serum. P123 may protect the nanocomplexes and consequently shield the interaction between serum proteins and the nanocomplexes, thereby reducing ionic interaction and adsorption to promote cellular uptake. It was also observed that the cellular uptake of the P123-PPI/pDNA nanocomplexes in MCF-7 cells was less than that in MCF-7/ADR cells. This result is likely due to the stronger selective effects of Pluronics to the drug-resistant cells [62]. This phenomenon was also found in the paclitaxel-resistant A549/T cell line [57].

3.4.2. In vitro quantitative evaluation of specific targeting to the CD44 receptor

To achieve efficient tumor targeting, anti-CD44 antibody was introduced into the nanocomplexes, which could bind to the CD44 receptor overexpressed in neovasculature endothelial cells and tumor cells. To confirm the target specificity, MCF-7, MCF-7/ADR and HUVEC cells were incubated with YOYO-1-pDNA-loaded nanocomplexes and then evaluated by flow cytometry. As shown in Fig. 4, the mean fluorescence intensity in MCF-7/ADR cells treated with anti-CD44-P123-PPI/pDNA nanocomplexes displayed stronger green fluorescent signals, which were increased 1.7-fold compared with that obtained after treatment with the P123-PPI/pDNA nanocomplexes, suggesting that conjugation with anti-CD44 antibody selectively increased the special targeting of nanocomplexes to the breast adenocarcinoma cells overexpressing the CD44 receptor. However, compared with the P123-PPI/pDNA nanocomplexes, the anti-CD44-P123-PPI/pDNA nanocomplexes did not show any efficient enhancement in fluorescence in the MCF-7 cells, and their level of internalization was similar to that obtained with the P123-PPI/pDNA nanocomplexes, indicating that no CD44

![Graphical data](image-url)
receptor is expressed on the surface of MCF-7 cells that could be recognized by anti-CD44 antibody. These results are consistent with the findings of the transfection study, as shown in Fig. 3. Interestingly, increased uptake could also be observed in HUVECs, which highly suggests that anti-CD44-P123-PPI/pDNA nanocomplexes may target HUVECs. This finding is due to the high expression of CD44 receptors in HUVECs [63]. This experiment demonstrated that the anti-CD44-P123-PPI/pDNA nanocomplexes can target both ADR-resistant breast adenocarcinoma cells (MCF-7/ADR) and neovascularure cells (HUVECs).

3.5. Intracellular distribution and co-localization

It is well known that nanoparticles enter cells through the endocytosis pathway. However, successful escape from the endosome is very crucial for gene carriers to prevent pDNA degradation in the lysosome and improve gene transfection efficiency [64]. To gain additional insights into the intracellular distribution and localization of pDNA after cellular uptake, confocal laser scanning microscopy was employed to observe and image the cells incubated with anti-CD44-P123-PPI/pDNA nanocomplexes for different durations. The cell nucleus was stained with Hoest33258 (blue), and pDNA was labeled with YOYO-1 (green). Late endosomes/lysosomes were stained with LysoTracker Red, as shown in red in the confocal images. As shown in Fig. 5, representative co-localization of pDNA and late endosomes/lysosomes produced yellow pixels due to the overlapping of the red and green fluorescences, and only a little amount of pDNA entered the nucleus after incubation for 4 h, suggesting that some of the nanocomplexes still resided in the late endosomes/lysosomes as well as the partial escape of the nanocomplexes from late endosomes/lysosomes at this time point. Moreover, co-localization was still obvious, and uptake was sustained for 4 h, which suggested that the internalization of nanocomplexes by the cells was a slow process. Additionally, it was obviously shown that the incubation of cells with nanocomplexes for 24 h resulted in separate localization of green and red fluorescences: as shown in the merged images, all of the green fluorescence completely co-localized to the nucleus as all of the nanocomplexes successfully escaped from the acidic vesicles. In the case of Pluronic-modified polycation/pDNA complexes, the results suggested that the nanocomplexes with different compositions followed the same trend (proton sponge effect) as the polyplexes in the intracellular trafficking pathway and fate.

3.6. Intracellular accumulation of ADR in MCF-7 and MCF-7/ADR cells

The finding that the overexpression of P-gp leads to drug efflux in MDR has attracted considerable attention, and increasing efforts have been devoted to the inhibition of P-gp to increase intracellular drug accumulation [9]. In this study, the intracellular accumulation of ADR was observed under a fluorescence microscope after the MCF-7 and MCF-7/ADR cells were treated with ADR solution only and anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes combined with ADR, respectively. As shown in Fig. 6A, MCF-7/ADR cells treated with free ADR only exhibited weak red fluorescence, which illustrated that the markedly lower accumulation of ADR in MCF-7/ADR cells compared with MCF-7 cells resulted partly from P-gp-mediated drug efflux. However, exposure of the MCF-7/ADR cells to ADR after transfection with anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes led to significant increases in intracellular ADR accumulation, which was related to the downregulation of P-gp and the weakened drug efflux. As expected, no changes in cellular uptake of ADR were observed in the case of MCF-7 cells pretreated or not treated with the anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes. The quantitative measurements of ADR accumulation in MCF-7 and MCF-7/ADR cells were performed by flow cytometry (Fig. 6B), and the results were in accordance with those presented in Fig. 6A. After transfection, the ADR accumulation in MCF-7/ADR cells increased approximately 3.3-fold ($P < 0.01$) when combined with MDR1 gene silencing. In contrast, there was no significant difference in the mean fluorescent intensity of ADR between MCF-7 cells treated with ADR solution only and those treated with anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes combined with ADR.

3.7. Inhibition of P-gp expression in MCF-7/ADR cells

RNAi technology is well known as an efficient and specific way to knock down the expression of the MDR1 gene. To validate whether the pDNA-iMDR1-shRNA targeting the MDR1 gene can effectively downregulate gene expression, the expression of MDR1 mRNA and P-gp in both MCF-7 and MCF-7/ADR cells after transfection was determined by RT-PCR and western blotting, respectively (Fig. 7). As shown in Fig. 7A and B, the multidrug-resistant MCF-7/ADR cells exhibited more overexpression of MDR1 mRNA compared with the drug-sensitive cell line MCF-7. However, the
level of MDR1 mRNA expression in MCF-7/ADR cells was significantly decreased after transfection with anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes compared with the untreated control cells. The MDR1 mRNA was only 25% of the initial amount. However, no significant difference was observed in MCF-7 cells before and after transfection. The GAPDH-encoding mRNA was not affected by these treatments. Similar to the mRNA results, the expression of P-gp in MCF-7/ADR cells was significantly decreased after transfection with pDNA-iMDR1-shRNA-loaded nanocomplexes. However, MCF-7 cells also did not show any changes. As shown in Fig. 7C and D, the results were consistent with the RT-PCR findings. These results indicated that the anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes can specifically and effectively silence MDR1 to inhibit P-gp expression in drug-resistant MCF-7/ADR cells. Therefore, the inhibition of the function of P-gp may be one of the effective ways to re-sensitize tumor cells to anticancer drugs during breast cancer chemotherapy.

3.8 Cytotoxicity in vitro

The cytotoxicity of PPI/pDNA, P123-PPI/pDNA and anti-CD44-P123-PPI/pDNA nanocomplexes against MCF-7 (Fig. 8A) and MCF-7/ADR (Fig. 8B) cells at different polymer concentrations was investigated by MTT assays to determine the safety of the carriers. The in vitro results demonstrated that the cytotoxicity increased obviously with an increase in the polymer concentrations, and all of the nanocomplexes exhibited a similar cytotoxicity tendency in MCF-7 and MCF-7/ADR cells. Notably, the nanocomplexes exhibited low cytotoxicity to MCF-7 and MCF-7/ADR cells (cell viability > 90%) at a polymer concentration of 50 μg/mL or lower for 4 h of incubation. In contrast, PPI/pDNA, P123-PPI/pDNA and anti-CD44-P123-PPI/pDNA nanocomplexes showed some cytotoxicity at various polymer concentrations after 24 h of incubation. No difference in cytotoxicity was observed between P123-PPI/pDNA and anti-CD44-P123-PPI/pDNA nanocomplexes (P > 0.05), which indicates that antibody conjugation did not have a significant effect on the cytotoxicity of the copolymer. However, lower cytotoxicity was observed in the cells treated with P123-PPI/pDNA or anti-CD44-P123-PPI/pDNA nanocomplexes compared with those treated with PPI/pDNA nanocomplexes. For instance, the cell viability of P123-PPI/pDNA was much higher than that of PPI/pDNA at the highest polymer concentration tested (P < 0.01), which were (45 ± 7.9)% and (9 ± 3.7)% against MCF-7/ADR cells, respectively. The lower cytotoxicity of P123-PPI/pDNA and anti-CD44-P123-PPI/pDNA nanocomplexes may be attributed to the lower positive surface potential of the nanoparticles because of the shielding effect of P123. Moreover, at the polymer concentration used in the transfection experiments with 4 h of incubation, the cytotoxicity of P123-PPI/pDNA nanocomplexes was accordingly negligible in MCF-7/ADR cells.

The cell viability-drug concentration curves, which indicated the anti-proliferation activity of ADR against MCF-7 and MCF-7/ADR cells before and after treatment with anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes, are shown in Fig. 8C and D. The sensitivity of MCF-7/ADR to ADR was first compared with that obtained with drug-sensitive MCF-7 cells. The IC50 values of free ADR against MCF-7/ADR cells was 18.2-fold greater than that in MCF-7 cells, which suggested that MCF-7/ADR cells exhibited high drug resistance to ADR. As a negative control, anti-CD44-P123-PPI/negative-pDNA nanocomplexes did not influence the IC50 of ADR in MCF-7 and MCF-7/ADR cells because it exhibited similar cytotoxicity compared with free ADR. Moreover, after treatment with anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes, the IC50 values of ADR in MCF-7 cells almost did not change, which indicated that anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes had little effect on the drug-sensitive cells. However, the IC50 value of ADR for MCF-7/ADR cells was decreased by 12.4-fold after anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes treatment, which displayed increased cytotoxicity compared with free ADR.

![Image](Fig. 7. Inhibition of MDR1 mRNA and P-gp expression in MCF-7 and MCF-7/ADR cells. (A–B) The suppression of MDR1 mRNA was quantified by RT-PCR. The PCR fragments were visualized through ethidium bromide staining using GAPDH as an internal control. (C–D) The expression of P-gp was evaluated by western blotting using β-actin as an internal control. Lanes 1 and 2 represent the untreated control group and the anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes-treated group of MCF-7/ADR cells; Lanes 3 and 4 represent the untreated control group and the anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes-treated group of MCF-7 cells. The results are expressed as the means ± SD from three independent experiments. *P < 0.05 and **P < 0.01.)
ADR is one of the substrates of P-gp. The overexpression of P-gp, which is one of the reasons for the resistance of MCF-7/ADR to ADR, mediated the efflux of ADR and the reduction in ADR accumulation, which resulted in the weakened sensitivity of MCF-7/ADR to ADR. To verify whether the stable expression of MDR1 shRNA could inhibit the function of P-gp and enhance the chemosensitivity of MCF-7/ADR to ADR, the results of ADR cellular uptake, P-gp expression and cytotoxicity of ADR in vitro were summarized and compared. The results showed that the cellular uptake of ADR in MCF-7 cells was 2.5-fold greater than that in MCF-7/ADR cells under the same free ADR concentration. After transfection of anti-CD44-P123-PPI/pDNA-iMDR1-shRNA, the cellular uptake of ADR significantly increased, as depicted by an increase in the mean fluorescent intensity from 15.2 ± 3.7 to 49.1 ± 6.9 (P < 0.01), as shown in Fig. 6. The increased cellular uptake of ADR could significantly increase the cytotoxicity of ADR. Moreover, ADR revealed dose-dependent cytotoxicity against MCF-7/ADR cells (Fig. 8C and D). The combination of anti-CD44-P123-PPI/negative-pDNA nanocomplexes and ADR showed almost the same toxicity as ADR alone, but the combination of anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes and ADR exhibited significantly increased ADR cytotoxicity, which indicated that increased ADR cytotoxicity could result from the expression of MDR1 shRNA and thereby the functional inhibition of P-gp. These results demonstrated that anti-CD44-P123-PPI/pDNA-imDR1-shRNA nanocomplexes could downregulate P-gp expression, which indicated that the weakened drug efflux led to increased ADR accumulation and enhanced chemosensitivity of MCF-7/ADR to ADR. These results may explain why the effective gene transfection caused an increase in drug uptake and thus mediated more effective anti-tumor activity.

3.9. In vivo distribution and tumor suppression study

As shown in Fig. 9A, luciferase expression level in vivo was determined to characterize the tissue distribution of nanocomplexes administered by intravenous injection. Deducing background signal and instrument noise, naked pDNA showed no any luciferase positive signal which proved that naked pDNA was rapidly eliminated from the circulation. The difference of P123-PPI/pDNA and anti-CD44-P123-PPI/pDNA nanocomplexes in tumor gene expression was remarkable. Luciferase expression for
Fig. 9. Transfection in vivo. (A) Tissue distribution of nanocomplexes administered by intravenous injection. (B) Body weights of the mice over the experimental period. (C) Antitumor effects (relative volume of tumor) of various treatments on nude mice bearing MCF-7/ADR breast tumors. The data are provided as the means ± SD (n = 8).
anti-CD44-P123-PPI/pDNA nanocomplexes was 2.5-fold higher than that of P123-PPI/pDNA nanocomplexes, indicating that anti-CD44 dramatically improved the gene transfection in tumor in vivo.

The reversion effects of pDNA-iMDR1-shRNA on MDR and the combination therapy (copolymer/pDNA-iMDR1-shRNA nanocomplexes and ADR) were evaluated in vivo on a MCF-7/ADR breast tumor xenograft model. First, the in vivo studies confirmed that no lethal toxicity was observed for P123-PPI/pDNA-iMDR1-shRNA and anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes at an N/P ratio of 20 and a dose of 2 mg pDNA/kg. Second, P-gp was still over-expressed in ex vivo cells extracted from the MCF-7/ADR tumor compared with MCF-7 cells, and the resistance to ADR was maintained in the mice tumor xenograft model, although the expression level was lower than that observed in MCF-7/ADR cells cultured in vitro. Furthermore, a dose of 10 mg/kg ADR, which is well-accepted in mice, was administered, and a schedule of multiple dosing was performed to enhance the efficacy of the in vivo therapy according to previously reported results. The mice were administered pDNA-iMDR1-shRNA-loaded nanocomplexes on days 0, 3, 6, 9, 12, 15, 18 and 21. Because the suppression of P-gp in an MDR tumor requires a period for gene expression, ADR was administered at 24 h after the nanocomplexes were injected, i.e., on days 1, 4, 7, 10, 13, 16, 19 and 22. The average tumor size was measured to monitor the antitumor efficacy from the first injection (Day 0) on days 0, 4, 8, 12, 16, 20 and 24.

The body weights of mice (Fig. 5B) with any treatments had no serious loss, which suggested that no significant toxicity was induced by nanocomplexes and ADR. As shown in Fig. 9C, compared with the saline control group, the groups treated with the nanocomplexes alone (P123-PPI/pDNA-iMDR1-shRNA and anti-CD44-P123-PPI/pDNA-iMDR1-shRNA) did not show any inhibitory effect on the growth of the MCF-7/ADR tumor, which indicated that only the inhibition of P-gp expression occurred. Tumor growth was inhibited in the groups treated with ADR alone and in combination with the copolymer/pDNA-iMDR1-shRNA nanocomplexes, which reflected the continuously inhibiting tendency of the RTV after the second administration. Up to eight days after in vivo therapy, the groups treated with ADR alone, with the combination of P123-PPI/pDNA-iMDR1-shRNA nanocomplexes and ADR, and with the anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes and ADR showed weak tumor-inhibitory effects but continued to show a moderate increase with RTV (410.7 ± 150.0%), i.e., (301.9 ± 100.6%) and (262.1 ± 80.6%) of the initial tumor volume, respectively (P < 0.05). As the treatment progressed, the antitumor efficacy was significantly enhanced in the three groups. The combination of anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes and ADR showed the strongest inhibition activity, as reflected by the RTV values. The RTV of the combination therapy group (anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes and ADR) was only (290.6 ± 57.4%) of the initial tumor volume, which indicated that the volume of the tumors treated with the combination of anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes and ADR was approximately one-quarter of those treated with ADR after only two weeks of treatment, whereas the tumors in the control group grew to 18-fold of the initial RTV (P < 0.01). At the end of the 24-day experimental period, the RTV obtained for the combination of anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes and ADR was significantly decreased to (150.3 ± 180%) of the initial RTV, which was 10-fold and 6-fold lower than those obtained with free ADR and the combination of P123-PPI/pDNA-iMDR1-shRNA nanocomplexes and ADR, respectively.

These results demonstrated that the sensitivity of MCF-7/ADR cells to doxorubicin was enhanced significantly after transfection with pDNA-iMDR1-shRNA. The combination of gene therapy and chemotherapy had a synergistic effect on a doxorubicin-resistant breast cancer cell line (MCF-7/ADR). The P123-PPI/pDNA-iMDR1-shRNA nanocomplexes, particularly in the absence of anti-CD44 antibody, could effectively transfect pDNA-iMDR1-shRNA to express MDR1 shRNA, suppress the expression of P-gp, and decrease the drug efflux in tumor cells, which led to enhancements in the antitumor effects of ADR. The antitumor efficacy in vivo was consistent with the results from the in vitro experiments. No side effects, such as infection and diarrhea, were observed in any of the groups, indicating that the nanocomplexes were well tolerated in vivo.

4. Conclusions

In the present study, P123-PPI and anti-CD44-P123-PPI copolymers were synthesized and evaluated functional materials for improving transfection efficiency and overcoming the multidrug resistance of cancer cells (MCF-7/ADR) in vitro and in vivo. The results of a quantitative transfection assay showed that P123-PPI and anti-CD44-P123-PPI displayed higher transfection efficiencies than P123-PPI in the tested cells, and anti-CD44-P123-PPI exhibited higher transfection efficiency than P123-PPI in MCF-7/ADR cells but presented no difference in CD44-negative MCF-7 cells. The developed anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes could enhance ADR uptake by reversing P-gp-mediated MDR in MCF-7/ADR cells in vitro and improve therapeutic efficacy combined with ADR in vivo. We believe that this study will provide a basis for future preclinical and clinical development of combination therapy treatment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiomater.2014.12.030.

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