PSA-responsive and PSMA-mediated multifunctional liposomes for targeted therapy of prostate cancer

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\textbf{Abstract}

In the hormone-refractory stage of prostate cancer (PC), the expression of prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) often remains highly active. Accumulating studies have demonstrated that these two proteins are attractive targets for specific delivery of functional molecules to advanced PC, not merely as potential sensitive markers for PC detection. In this study, we constructed a dual-modified liposome that incorporated PSA-responsive and PSMA-mediated liposomes and potentially offers double selectivity for PC. The folate moiety binds quickly to PSMA-positive tumors, and the PSA-responsive moiety is cleaved by PSA that was enriched in tumor tissues. The activated liposomes (folate and cell-penetrating peptides dual-modifications) are subsequently taken up by the tumor cells via polyarginine's penetrating effects and receptor-mediated endocytosis. To corroborate these assumptions, a series of experiments were conducted, including PSA-responsive peptide hydrolysis kinetics, cellular uptake, internalization mechanism and escape from endosomes in PC-3 and/or 22Rv1 cells, biodistribution and antitumor activity of siRNA-loaded liposomes after systemic administration, gene silencing and cell apoptosis \textit{in vitro} and \textit{in vivo}. The results reveal that multivalent interactions play a key role in enhancing PC cell recognition and uptake while reducing nonspecific uptake. The dual-modified liposomes carrying small interfering RNA (siRNA) have significant advantages over the control liposomes, including single-modified (folate, CPP, PSA-responsive only) and non-modified liposomes. The dual-modified liposomes elevated cellular uptake, downregulated expression of polo-like kinase 1 (PLK-1) and augmented cell apoptosis \textit{in vitro} and \textit{in vivo}. The entry of the dual-modified liposomes into 22Rv1 cells occurred via multiple endocytic pathways, including clathrin-mediated endocytosis and macropinocytosis, followed by an effective endosomal escape of the entrapped siRNA into the cytoplasm. \textit{In vivo} studies conducted on a 22Rv1 xenograft murine model demonstrated that the dual-modified liposomes demonstrated the maximized accumulation, retention and knockdown of PLK-1 in tumor cells, as well as the strongest inhibition of tumor growth and induction of tumor cell apoptosis. In terms of targeting capacity and therapeutic potency, the combination of a PSA-responsive and PSMA-mediated liposome presents a promising platform for therapy and diagnosis of PSMA/PSA-positive PC.

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\textbf{1. Introduction}

According to a report on cancer statistics in the United States [1], prostate cancer (PC) is the top newly diagnosed cancer in men, accounting for 29% of cancer incidence in 2012. PC continues to be the second-leading cause of cancer mortality in males. Despite curable in its early stages by surgical or radiation ablation, the consequences of advanced or recurrent disease are less positive, and certain PCs ultimately evolve into an aggressive and castration-resistant pattern [2]. Currently, treatment of advanced PC relies mainly on nonspecific therapies (i.e. chemotherapies), and there have been more PC drugs approved by the US Food and Drug Administration (FDA) in the past 3 years than in the prior 3 decades combined [3]. However, the accumulating clinical data have suggested that chemotherapy may lengthen survival in only a subset of men and that the benefit of conventional chemotherapy is limited...
due to its systemic toxicity. In contrast, RNA interference (RNAi) may offer several advantages, including higher specificity and the possibility of modulating the expression of any gene involved in tumor initiation, growth and metastasis formation [4]. As a potentially new, specific and more tolerated therapeutic strategy, gene-specific mRNA knockdown with synthetic small interfering RNA (siRNA) has been utilized to silence critical genes involved in PC growth [5–7].

However, the clinical application of siRNAs remains a considerable challenge, owing to their limited cellular uptake, low biological stability, and unfavorable pharmacokinetics. To ameliorate these challenges, several different types of nanocarriers have been developed for the delivery of siRNA, with cationic liposomes being by far the most extensively used and studied [8]. The additional incorporation of poly(ethylene glycol) (PEG) into the liposome surface reduces the probable complement activation and inflammation, resulting in prolonged blood circulation time and providing nanocarriers passive targeting to tumors via the enhanced permeability and retention (EPR) effect. However, the EPR effect may not be sufficient to direct the tissue distribution of siRNA due to nonspecific behavior [9]. There is a requirement of a desirable delivery platform to have reinforced specificity toward the tumor; efficient cellular uptake and endosomal escape of the nucleic acid.

Cell-penetrating peptides (CPPs, also known as protein transduction domains, PTDs), such as TAT and polyarginine, have been shown to promote intracellular delivery of bioactive molecules with low membrane permeabilities. However, the majority of known CPPs are not cell- or tissue-specific; they come into contact with and are internalized by cells via heparan sulfates and other glycosaminoglycans in nearly all cell types in vitro and in vivo [10,11]. With the focus of solving this dilemma, tumor microenvironment stimulus-responsive nanostructures have been introduced to build ‘off-on’ switches to CPP activity based on sensitivity to local environmental conditions (e.g., overexpressed protease [12–14], lower pH [15,16]) and external triggers [17] (e.g., heat and light irradiation).

A local tumor environment, which is notably enriched with a broad spectrum of proteases, differs greatly from normal tissues. The protease-responsive strategy is explorative and utilizes the stimulation of the unique proteases in the tumor environment based on the principle of proteolysis, a simple hydrolytic process that separates two adjacent amino acid residues at the amide bond. Prostate-specific antigen (PSA), a serine protease, is highly upregulated by PC and has a lower secretion in normal prostatic glandular cells [18]. The differential enzymatic activity of PSA has made it a logical target for PC therapies and imaging. Recently, several promising prodrugs, imaging techniques and microgel particles have been developed [18–22] in which either the transformation into theranostic agents or the efflux of the loaded cargo was accomplished on the basis of an internal linker subjected to proteolysis by PSA. Franc and co-workers [23] built an activatable protein transduction domain (APTD or ACPP)-based molecule for selective uptake into cells only after proteolytic cleavage of a tumor microenvironment stimulus-responsive sequence by the PSA, which may provide a favorable approach to the targeted delivery of therapeutics or imaging agents to PSA-positive PCs.

The introduction of various biological ligands or antibodies into drug delivery systems has provided an opportunity for the selective delivery of drugs to tumor cells. Such ligands are recognized by specific receptors on certain types of cancer cell surfaces, which later induce the cellular uptake of the ligand-decorated carriers via receptor-mediated endocytosis [24]. Prostate-specific membrane antigen (PSMA) is a 100-kDa membrane-bound glycoprotein that is upregulated in correlation with PC, especially in the advanced stages [25]. PSMA was identified as a folate transporter, significantly improving folate entry into PSMA expressing cells undergoing folate-deficient culture [26]. Several studies on gene targeting therapy have demonstrated the strength of folate-modified vehicles, such as dendrimers [27], lipid-based nanoparticles [28] and nanoplexes [29], in treating PSMA-positive PC cells and xenografts. The high transfection efficiency and enhanced therapeutic effect indicate that folate-decorated nanocarriers are potentially targeted vectors to PSMA-positive PC cells for gene delivery.

In this study, a rational strategy was employed to take advantage of the combination of both PSA-sensitive peptide and folate-modification for PSMA targeting to create a more selective and efficient drug delivery system to PC cells (Scheme 1). The PSA-responsive peptide includes three units: the cell-penetrating domain (polyarginine), the PSA-sensitive cleavable peptide (HSSKYQ) and the polyanionic inhibitory peptide (DGGDGGDGGDGG). We hypothesized that the multivalent interactions play a key role in enhanced cancer cell recognition and uptake and in the reduction of nonspecific uptake. The folate moiety binds quickly to PSMA-positive tumors, and the PSA-responsive moiety is cleaved by PSA, which is enriched in tumor tissues. The activated liposomes (folate and CPP dual-modifications) are later taken up by the PC cells via the CPP penetrating effect and receptor-mediated endocytosis, whereas in circulation, the penetration effect of CPP is shielded. The dual-modification is intended to improve the selective delivery to PC cells and to reduce the intrinsic toxicity to healthy cells beyond the reliance upon the EPR effect and monograft modification. Simultaneously, CPP penetration, which becomes active after cleavage in the tumor microenvironment with abundant proteases, can overcome the elevated interstitial pressure in tumors.

2. Materials and methods

2.1. Materials

1.2-distearyl-sn-glycerol-3-phosphoethanolamine-N-methoxy(polyethylene glycol) (aminum salt) (DSPE-mPEG2000). 1,2-distearyl-sn-glycerol-3-phosphoethanolamine-N-maleimide(polyethylene glycol) (DSPE-PEG2000-MAL), and 1,2-distearyl-sn-glycerol-3-phosphoethanolamine-N-amino(polyethylene glycol) (DSPE-PEG2000-NH2) were purchased from NOF (Tokyo, Japan). ACPP (Asp-Gly-Lys-Ser-Ser-Leu-Tyr-Gln-Gly-D-(Arg)8-Gly-Cys, MW 3132.33) and CPP (D-(Arg)8-Gly-Cys, MW 1484.75) were custom synthesized by GL Biochem (Shanghai, China). Folate and dicyclohexylcarbodiimide (DCC) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). N-Hydroxysuccinimide (NHS) was purchased from Advanced Technology and Industrial Co., Ltd. (Shenzhen, China). 5-(6)-carboxyfluorescein diacetaate (CFDA) and LysoTracker Red were obtained from Invitrogen/Molecular Probes (Eugene, OR, USA). Soybean phosphatidylcholine (SPC) was from Lipoid (Ludwigshafen, Germany). Cholesterol was purchased from Wako (Tokyo, Japan), [3H]-N,N′-dimethylaminoethane)-carbamoyl cholesterol (DC-Chol) was synthesized by our laboratory [30]. Negative control siRNA (siNC), FAM-labeled negative control siRNA (FAM-siRNA) (antisense strand, 5′-AGCUAGACCGUGCGAGCTTT-3′) and siRNA targeting PLK-1 mRNA (siPLK-1, antisense strand, 5′-GUGAAUUCCCUUCUCAAGGdTdT-3′) were synthesized by GenePharma (Shanghai, China). Silencer Cy5-labeled Negative siRNA (Cy5-siRNA) was purchased from Ribobo (Guangzhou, China). All primers were synthesized by AuGCT Biotechnology (Beijing, China). Enzymatically active prostate-specific antigen (PSA) was purchased from EMD Biosciences (Gibbstown, NJ, USA). s1-Antichymotrypsin (ACT) was obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Folate-deficient RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen/Gibco (Grand Island, NY). Ham’s F12–K medium, poly-L-lysine, Hoechst 33258 and 4′,6-diamidino-2-phenylindole were purchased from Marge (Beijing, China). Triton X-100 was purchased from Amresco (Solon, OH, USA).

2.2. PSA-responsive peptide hydrolysis by PSA

To verify the PSA-mediated hydrolysis of PSA-responsive peptide, this peptide was incubated with PSA in pH 7.4 Tris-buffered saline (50 mM Tris, 100 mM NaCl, TBS) in a shaking incubator, and a final concentration of PSA in the reaction system of 5 μg/mL. During the incubation at 37 °C, aliquots of the mixture were analyzed by high performance liquid chromatography (HPLC) after being quenched with an equal volume of 0.1% TFA in methanol at the discrete time points (0, 6, 12, 24 and 36 h). Buffer without PSA enzyme was loaded as a control. ACPP contained sample (100 μg/mL) was also chromatographically analyzed.

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2.3. Synthesis of functional conjugates

HPLC was performed with a Diamonsil ODS C18 column (250 mm × 4.6 mm, 5 μm) on an LC-20A HPLC system (SHIMADZU), and the chromatograms were recorded at 220 nm using gradient solvent conditions: solvent A, 0.05% TFA in acetonitrile (ACN); solvent B, 0.05% TFA in water; 0–25 min, 10–30% ACN; 25–30 min, 30–100% ACN, with a flow rate of 1.0 mL/min at room temperature.

2.3. Synthesis of functional conjugates

The DSPE-PEG2000-ACPP and DSPE-PEG2000-CPP were synthesized as described by Zhu et al. [12], with minor modifications. Briefly, the designated peptide (20 mg of cysteine-modified ACPP or 10 mg of cysteine-modified CPP) was dissolved in 4 mL of HEPES buffer (20 mM HEPES, 10 mM EDTA-2Na, pH 6.5). Dried lipid film containing 20 mg DSPE-PEG2000-MAL was hydrated in the HEPES buffer and added immediately to the ACP or CPP peptide solutions with gentle agitation at room temperature. After 48 h of stirring under nitrogen protection, the resulting solution was incubated with L-cysteine (10 times the molar ratio to maleimide residues) for 4 h for capping unreacted maleimide group. To remove the excess peptides and quencher, the reaction mixture was centrifuged to remove trace insolubles, and the solution was dialysed against distilled water for 48 h. The puriﬁcation of the derivatized lipid was accomplished using a 5% glucose solution (pretreated with DEPC) for the formation of hydrated micelles and incubated for 15 min at 60 °C. The lipid was hydrated using a 10% glucose solution (pretreated with DEPC) for the formation of hydrated micelles and incubated for 15 min at 60 °C. The purified dialysate was lyophilized and stored at −20 °C.

The synthesis of DSPE-PEG2000-Folate followed the procedure previously reported by Gao et al. [14] with minor modiﬁcations, which consists of two reaction steps. First, 100 mg folate dissolved in 4 mL of anhydrous DMSO was reacted with 93.5 mg of DCC and 21 mg of NHS. After stirring for 6 h, the white precipitate, dicyclohexylurea, was ﬁltered by centrifugation, which consists of two reaction steps. First, 100 mg folate dissolved in 4 mL of anhydrous DMSO was reacted with 93.5 mg of DCC and 21 mg of NHS. After stirring for 6 h, the white precipitate, dicyclohexylurea, was ﬁltered by centrifugation, which consists of two reaction steps. First, 100 mg folate dissolved in 4 mL of anhydrous DMSO was reacted with 93.5 mg of DCC and 21 mg of NHS. After stirring for 6 h, the white precipitate, dicyclohexylurea, was ﬁltered by centrifugation, which consists of two reaction steps. First, 100 mg folate dissolved in 4 mL of anhydrous DMSO was reacted with 93.5 mg of DCC and 21 mg of NHS. After stirring for 6 h, the white precipitate, dicyclohexylurea, was ﬁltered by centrifugation. After 48 h of stirring under nitrogen protection, the resulting solution was incubated with L-cysteine (10 times the molar ratio to maleimide residues) for 4 h for capping unreacted maleimide group. To remove the excess peptides and quencher, the reaction mixture was centrifuged to remove trace insolubles, and the solution was dialysed against distilled water for 48 h. The puriﬁcation of the derivatized lipid was accomplished using a 5% glucose solution (pretreated with DEPC) for the formation of hydrated micelles and incubated for 15 min at 60 °C. The lipid was hydrated using a 10% glucose solution (pretreated with DEPC) for the formation of hydrated micelles and incubated for 15 min at 60 °C. The purified dialysate was lyophilized and stored at −20 °C.

The MW distribution of DSPE-PEG2000-ACPP, DSPE-PEG2000-CPP and DSPE-PEG5000-Folate was determined on a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Germany) using 2,5-dihydroxybenzoic acid or α-cyano-hydroxycinnamic acid as the matrix for mass spectrometric analysis.

2.4. Preparation and characterization of liposomes

To prepare the normal liposomes (N-L) and folate-modified liposomes (F-L), a lipid composition (molar ratio) of SPC (48%), cholesterol (8%), DC-cholesterol (40%), DSPE-mPEG2000 (3%) and DSPE-PEG5000-Folate (1%, only for F-L) was used. A thin lipid film was formed in a round bottom flask by dissolving the lipids in chloroform-methanol (3:1 v/v) and removing the solvent in a rotary evaporator for 15 min at 40 °C. After continued vacuum drying for 1 h at room temperature, the lipid film was hydrated using a 10 μM siRNA (siC14, siPLK-1, FAM-siRNA, or Cy5-siRNA) solution in 5% dextrose (w/v) pretreated with DEPC. To control the size, the lipid dispersion was extruded sequentially through polycarbonate membranes with 0.4-μm (5 times) and 0.2-μm (5 times) pore sizes using a hand-extruder (Avanti, Canada).

ACPP-modiﬁed liposomes (A-L), CPP-modiﬁed liposomes (C-L) and ACPP/Folate-commodiﬁed liposomes (AF-L) were formed by the postinsertion method. Briefly, a lipid film of DSPE-PEG2000-ACPP or DSPE-PEG2000-CPP was prepared by rotary evaporation and further dried under vacuum for 1 h. The dried lipid ﬁlm was later hydrated with a 5% glucose solution (pretreated with DEPC) for the formation of micelles and incubated for 15 min at 60 °C. For the A-L or C-L preparations, 0.25 mL of micelle solution (DSPE-PEG2000-ACPP or DSPE-PEG2000-CPP micelles) was added into 1 mL of preformed N-L at the required molar ratio (3% DSPE-PEG-peptides of total lipid) and incubated for 4 h at 37 °C in a water bath. For the AF-L preparation, micelles of DSPE-PEG2000-ACPP were incubated with preformed F-L at the above molar ratio and conditions. All of the resulting liposomes were permitted to cool to room temperature before use.

The size distribution and zeta-potential of each formulation were determined in three serial measurements with dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS, Malvern, UK).

2.5. Cell culture

A human prostate tumor cell line (PC-3 cells) from the Cell Culture Centre of Peking Union Medical College (Beijing, China) was maintained in Ham’s F12-K medium supplemented with 10% FBS (GIBCO), 100 IU/mL penicillin, and 100 μg/mL streptomycin. The 22Rv1 xenograft prostate tumor cell line obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) was grown in folate-deﬁcient RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 1.0 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% FBS (GIBCO), 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained in a 37 °C humididified incubator with a 5% CO2 atmosphere.

2.6. Cellular uptake and flow cytometric analysis

For the flow cytometric analysis, PC-3 cells were seeded onto 6-well plates at 2 × 104 cells per well in 2 mL of complete Ham’s F12-K medium and cultured at 37 °C in a 5% CO2 humidified atmosphere for 24 h. After the attachment period, the cells were rinsed with PBS and incubated with different liposomal formulations containing FAM-siRNA with or without pretreatment with a 5 μg/mL PSA solution for 16 h in serum-free media. The concentration of FAM-siRNA was 75 nM. Following the treatment for 2 h at 37 °C, the cells were trypsinized and washed with cold PBS containing heparin (125 U/mL). After two further washes with cold PBS, the cells...
were filtered and subjected to flow cytometric analysis utilizing a BD FACSCalibur flow cytometer.

The 22Rv1 cells were seeded onto 6-well plates at a density of 3 × 10⁵ cells per well in 2 mL of complete folate-deficient RPMI 1640 medium and cultured at 37°C in a 5% CO₂ humidified atmosphere for 24 h. After rinsing with PBS, the cells were incubated for 2 h with medium containing free FAM-siRNA or various liposomes containing FAM-siRNA, with or without pretreatment with a PSA solution for 16 h. The concentration of FAM-siRNA was 75 ns. The following process involved the same treatment as above. For ACT inhibition, the preincubation of liposomal samples with PSA (as described in the previous paragraph) was introduced. The final concentration of FAM-siRNA in the culture medium was 225 ns. Subsequently, the cells were incubated for 3 h at 37°C and washed twice with cold PBS containing heparin (125 U/mL). Fixation with 4% formaldehyde was then performed for 15 min at 4°C. After three 5-min rinses with cold PBS, the fixed cells were stained with Hoechst 33342 for an additional 25 min at 37°C and were imaged via a confocal laser scanning microscope (Leica, Heidelberg, Germany). FAM-siRNA and Hoechst were excited using 488 nm and 345 nm lasers, respectively.

To track the internalization and endosomal release of liposomal FAM-siRNA, following a 3 h incubation with PSA-pretreated AF-L as mentioned above, 22Rv1 cells were washed three times with cold PBS and cultured in complete medium for an additional 0.5 h or 2.5 h. Endosomal/lysosomal labeling was performed for 0.5 h by Lysotracker Red (Invitrogen/Molecular Probes, CA, USA) at a concentration of 500 ns. Subsequently, the cells were rinsed with cold PBS containing heparin (125 U/mL) and were then treated as described above. The Lysotracker Red was excited by a 561-nm laser.

2.7. In vitro confocal image

To assess the cellular uptake, 22Rv1 cells (2 × 10⁵ cells) were seeded onto a sterile glass-bottom dish (35 × 10 mm) coated with 0.1% poly-l-lysine (150–300 kDa) and incubated for 48 h in complete folate-deficient RPMI 1640 medium. Following two washes with PBS, the serum-free medium containing free or liposome-entrapped siRNA (with or without pretreatment with PSA as described in the previous paragraph) was introduced. The total concentration of FAM-siRNA in the culture medium was 225 ns. Subsequently, the cells were incubated for 3 h at 37°C and washed twice with cold PBS containing heparin (125 U/mL). Fixation with 4% formaldehyde was then performed for 15 min at 4°C. After three 5-min rinses with cold PBS, the fixed cells were stained with Hoechst 33342 for an additional 25 min at 37°C and were imaged via a confocal laser scanning microscope (Leica, Heidelberg, Germany). FAM-siRNA and Hoechst were excited using 488 nm and 345 nm lasers, respectively.

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2.8. In vitro transfection and analysis of gene expression

22Rv1 cells were seeded into 25 cm² tissue culture flasks at a density 1.5 × 10⁶ cells/flask in 5 mL of complete folate-deficient RPMI 1640 medium. After 24 h of culture in a humidified atmosphere of 5% CO₂ at 37°C, the medium was exchanged with fresh serum-free medium containing siRNA-loaded samples (with or without pretreatment with PSA as mentioned above). The final concentration of siRNA (siPLK-1 or siN.C.) utilized in the experiment was 100 nM. Following 5 h of incubation, the medium was replaced with complete medium for an additional 48 h for mRNA assay or 72 h for protein quantification of culture at 37°C. Subsequently, PLK-1 mRNA and protein were evaluated using qRT-PCR (quantitative real-time polymerase chain reaction) and western blot analysis, respectively.

For the qRT-PCR assessment, cells were collected, and the total RNA from transfected cells was isolated using TRNZol A (Tiangen, China). PLK-1 mRNA and protein were evaluated using qRT-PCR (quantitative real-time polymerase chain reaction) or 72 h (for protein quantification) of PSA mRNA assay or 72 h (for protein quantification) of PSA in serum-free medium at 37°C for an additional 2 h. After the procedure as described before, the cells were subjected to flow cytometry for analysis. All experiments were performed in triplicate.

2.9. Cell apoptosis assay

22Rv1 cells were plated on 25 cm² tissue culture flasks at 1.5 × 10⁵ cells per flask in 5 mL of complete folate-deficient RPMI 1640 medium. After a 24 h culture at 37°C in 5% CO₂ humidified atmosphere, the cells were washed with PBS and exposed to fresh serum-free medium containing siRNA-loaded samples (with or without pretreatment with PSA as mentioned above). The final concentration of siRNA (siPLK-1 or siN.C.) used in the experiment was 100 nM. Following 5 h of incubation, the medium was replaced with complete medium for an additional culture of 72 h at 37°C. Subsequently, the cells were collected and stained with the Annexin V-FITC apoptosis detection kit (KeyGEN, Nanjing, China) according to the manufacturer's instructions, and were immediately analyzed via the FACSscan flow cytometer with 10,000 events collected (Ex: 488 nm; Em: 530 nm).

2.10. In vivo imaging

All procedures involving animal housing and treatment were approved by the Institutional Authority for Laboratory Animal Care of Peking University. First, 22Rv1 cells were suspended in a 50% mixture of Matrigel (BD Biosciences, San Jose, CA) and RPMI 1640 medium and were then treated as described above. The LysoTracker Red was excited by a 561-nm laser.

For the detection of PLK-1 expression in tumor tissues

To assess the antitumor efficacy, the xenograft tumor model was built by subcutaneous injection of 22Rv1 cells (5.0 × 10⁶ cells) as described above. After the tumors had developed to approximately 100 mm³, the mice were randomly divided into eight groups (n = 6–7) and treated with 5% glucose (control), free siPLK-1, various liposomal formulations (N-L, F-L, C-L, A-L or AF-L) carrying siPLK-1 or AF-L carrying siN.C. by intravenous injection (at 1.5 mg/kg; 200 μL) every other day for a total of 10 d. The body weight and tumor size were measured at least once every two days throughout the post-exposure period. The tumor volume was calculated using the formula V = 1/2 × (larger diameter) × (smaller diameter)².
2.13. Immunohistochemical analysis

To evaluate the apoptosis of tumor tissue, 24 h after the last injection, mice were sacrificed and tumor tissues were excised. The tumor tissues were frozenly sectioned into 4-μm slices for immunohistochemical analysis. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining for apoptosis was conducted on the specimens using the TRITC staining in situ Apoptosis Detection Kit (KeyGEN, Nanjing, China) according to the package instructions. Briefly, frozen sections were fixed in 4% formaldehyde for 30 min at room temperature and rinsed three times in PBS. After incubation in permeabilization solution (freshly prepared 1% Triton X-100 in PBS pH 7.4) for 5 min, the sections were washed three times in PBS followed by blockage of endogenous peroxidase activity with 3% hydrogen peroxide diluted in methanol for 10 min at ambient temperature. Each sample was treated with 50 μL of labeling reaction mixture (45 μL equilibration buffer, 1.0 μL TRITC-5-dUTP and 4.0 μL TdT enzyme) for 60 min at 37 °C in a dark and humidified atmosphere. For a positive control, the sample was added 100 μL of DNasel reaction solution and treated for 30 min at 37 °C before introduction of the TdT enzyme. For a negative control, the TdT enzyme was excluded from the labeling reaction mixture. Nuclei were stained with Hoechst 33258 for 25 min at 37 °C, and the slides were examined under a confocal laser scanning microscope (Leica, Heidelberg, Germany).

2.14. Statistical analysis

Data were presented as the mean ± standard deviation (SD). The difference between any two groups was determined by ANOVA. P < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Design and enzymatic hydrolysis of PSA-responsive peptide

The PSA-responsive peptide sequence NH2-DGDGDGDGG DGHHSSKYQ-Grrrrrrr-GC-COOH includes three units: polyarginine (r8), the PSA-selective substrate (HSSKYQ) and the polyglycine and the adjacent linked glutamine and produced two secondary structures of the PSA-responsive peptide (Fig. 2A). The two major multi-peaks centered at 6024.95 (Fig. 2C) and 4379 (Fig. 2D) mass-charge ratios verified that the mean MWs of DSPE-PEG5000-ACP and DSPE-PEG5000-CPP were 6024 Da and 4378 Da, respectively, which was in agreement with the calculated mean MW of the corresponding conjugate (6024 and 4378, respectively).

Based on the high affinity between folate and the extracellular membrane fraction of PSMA [26,29], DSPE-PEG5000-Folate was introduced to promote targeted delivery to PSMA-positive prostate tumor cells. To obtain the folate-labeled conjugates, our approach involved carbodiimide-mediated coupling of folate to readily attainable DSPE-PEG5000-NH2 (Fig. 2B). As shown in Fig. 2E, the major multi-peaks centered at a 5898 mass-charge ratio indicated that the mean MW of DSPE-PEG5000-Folate was consistent with the theoretical data (5897). The MW increment of DSPE-PEG5000-Folate compared to DSPE-PEG5000-NH2 is 424, which was similar to the MW difference (423) between folate and the leaving group, H2O. That indicated that the reaction successfully connected folate with DSPE-PEG5000-NH2.

3.2. Synthesis and identification of functional conjugates

We synthesized DSPE-PEG2000-ACP and DSPE-PEG2000-CPP via the thiol-ene “click” reaction of the maleimide group with the sulphydryl group of PSA-responsive peptide or CPP peptides (Fig. 2A). The two major multi-peaks centered at 6024.95 (Fig. 2C) and 4379 (Fig. 2D) mass-charge ratios verified that the mean MWs of DSPE-PEG2000-ACP and DSPE-PEG2000-CPP were 6024 Da and 4378 Da, respectively, which was in agreement with the calculated mean MW of the corresponding conjugate (6024 and 4378, respectively).

Five types of liposomes were formed, including N-L, F-L, C-L, A-L and AF-L (Table 1). To form A-L and AF-L, the N-L and F-L were individually surface-modified with ACPP via the postinsertion technique. The resultant insertion percentages (%) of the A-L and AF-L were 71.62 ± 0.74 and 67.03 ± 1.11, respectively (see Supplementary information).

The threshold vesicle size for the extravasation into a tumor’s extracellular space was approximately 400 nm [34], and the drug delivery system smaller than 200 nm was recommended [35]. All of the types of liposomes had a size range of 180–210 nm and a good uniformity with the PDI range of 0.080–0.250 (Table 1), which endowed the liposomal vectors with potential beneficial behavior in vivo. In addition, the zeta-potentials of the liposomal formulations corresponded with the liposomes components, indicating that the modifications of the folate and the peptides were shielded on the liposomal surface. The positive potential of N-L (9.34 mV) was provided by DC-Chol, which has one positive charge head group. The zeta-potential of F-L (0.20 mV) and AF-L (2.79 mV) were smaller than that of N-L owing to the folate’s acidic charge on the liposomal surface, and C-L (16.07 mV) was more positive due to the...
cationic charge of the CPP. Considering the comparison between the ACPP and CPP inserting liposomes, the zeta-potential of A-L (10.51 ± 1.43 mV) was less than C-L (16.07 mV) but similar to N-L (9.34 ± 0.91 mV), implying that the positive charges of the polycationic cell-penetrating domain were effectively shielded by the polyanionic shielding domain. The ligands incorporated into lipid membranes by the postinsertion technique were fitted exclusively on the outer leaflet of the liposomal bilayers with the hydrophilic segment exposed, which exhibits efficient utilization of ligand-PEG-lipid conjugates.

3.4. Cellular internalization and competition studies

According to the design idea, the cleavage of the PSA-responsive peptide anchored on the surface of the liposome could activate CPP and promote the cellular uptake of the siRNA-loaded liposome via
mediation of the polyarginine. To verify this hypothesis, a PSA-negative PC-3 cell line was used to investigate the effect of the absence and presence of exogenous PSA at a concentration level of 5 μg/mL, which is the same as for the hydrolysis of ACPP. The PC-3 cells were incubated with the liposomes carrying FAM-siRNA at a final concentration of 75 nM. As shown in Fig. 3A, in the presence of PSA, PC-3 cells exhibited stronger intracellular fluorescence than in the absence of PSA, which revealed the contribution of PSA-triggered cleavage on the PSA-responsive peptide in the A-L liposomes to cellular uptake. On the contrary, the internalization of N-L on PC-3 cells was not significantly influenced by the presence of PSA, where very similar fluorescence intensities were exhibited. This suggests that the exogenous PSA can cleave PSA-responsive peptide effectively, and the liposomes then commit an advanced translocation via the exposed CPP.

To investigate systematically what effects various liposomal formulations might have on the cell internalization of siRNA, we individually incubated various liposomes (N-L, F-L, C-L, A-L and AF-L with or without pretreatment with PSA) on 22Rv1 cells, a PSMA-positive [36,37] and PSA moderately positive cell line [33,38,39]. Without the stimulus of PSA, the highest uptake of FAM-siRNA (Fig. 3B) occurred with the C-L treatment, indicating a CPP-assisted translocation into the cell. Following the pretreatment with exogenous PSA due to the low level of PSA existing in the 22Rv1 cells’ culture media [32], only two groups, A-L and AF-L, presented a significant promotion in cellular uptake, suggesting the intended activation of PSA-responsive peptide. However, the C-L and N-L groups were not affected by pretreatment with PSA. PSMA, a folate binding protein and transporter [26], promotes the translocation of folate-modified nanocarriers into cells [28,29]. In this study, a significantly increased (1.7-fold) mean fluorescence intensity was observed in cells treated with F-L compared with N-L, suggesting the mediation of PSMA. Additionally, among all of the liposomal formulations with PSA pretreatment, AF-L expressed the greatest capacity toward improving the uptake of FAM-siRNA, proving the strength of CPP in combination with folate. However, the internalization of A-L by 22Rv1 cells failed to reach the degree of the cells treated with C-L, which could be explained by the incomplete cleavage of the PSA-responsive peptide and/or the insufficient separation of the shielding domain with the CPP segment after hydrolysis.

The proteolytic activity of PSA is substantially reduced by interaction with the major serum protease inhibitors ACT [40] to form a coherent stable PSA-Act complex at a 1:1 molar ratio. An excessive amount of ACT was added and followed by pretreatment of the liposomes (A-L and AF-L) with PSA to eliminate the PSA activity. The results (Fig. 3C) indicated that both liposomes treated with PSA combined with ACT, expressed a significantly decreased cellular uptake compared to the corresponding formulation incubated with only PSA. This observation suggested that the PSA-responsive peptide was not cleaved after formation of the PSA-Act complex, which indirectly supported the hypothesis that it was the introduction of PSA that enhanced cellular uptake of PSA-responsive peptide-modified liposomes.

The cellular uptake was also evaluated by confocal laser scanning microscopy (CLSM). As shown in Fig. 3D, no translocation of free siRNA into the 22Rv1 cells was observed, owing to the large MW (~13 kDa) and the hydrophilic nature prevents entrance into cells by passive diffusion mechanisms [9]. In groups without PSA pretreatment, some of the liposomes (F-L, C-L, and AF-L) exhibited significantly increased intracellular distribution, which could stem from the effects of their individual or cooperative ligands, as mentioned in the above paragraph. For A-L and AF-L, the PSA pretreatment significantly promoted the absorbance of FAM-siRNA by cultured cells. In addition, among all of the tested formulations, the AF-L with PSA preincubation exhibited the maximum intracellular fluorescence intensity. The result could be a consequence of the synergism between PSMA mediation and PSA activation and is consistent with the data from the flow cytometry analysis.

### Table 1

<table>
<thead>
<tr>
<th>Liposomes components (mol ratio of total lipid)</th>
<th>Diameter (nm)</th>
<th>PDI</th>
<th>Zeta-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-L SPC/Chol/DC-chol/DSPE-PEG2000 (48.5:8.1:40:4:3.0)</td>
<td>183.1 ± 6.1</td>
<td>0.088 ± 0.013</td>
<td>9.34 ± 0.91</td>
</tr>
<tr>
<td>F-L SPC/Chol/DC-chol/DSPE-PEG/DSPE-PEG2000-Folate (48.0:8.0:40.0:3.0:1.0)</td>
<td>186.8 ± 8.8</td>
<td>0.081 ± 0.016</td>
<td>0.20 ± 0.22</td>
</tr>
<tr>
<td>C-L SPC/Chol/DC-chol/DSPE-PEG/DSPE-PEG2000-CPP (47.1:7.8:39.2:2.9:2.9)</td>
<td>193.0 ± 10.0</td>
<td>0.183 ± 0.032</td>
<td>16.07 ± 1.50</td>
</tr>
<tr>
<td>A-L SPC/Chol/DC-chol/DSPE-PEG/DSPE-PEG2000-ACPP (47.1:7.8:39.2:2.9:2.9)</td>
<td>201.2 ± 10.5</td>
<td>0.246 ± 0.049</td>
<td>10.51 ± 1.43</td>
</tr>
<tr>
<td>AF-L SPC/Chol/DC-chol/DSPE-PEG/DSPE-PEG2000-Folate/DSPE-PEG2000-ACPP (46.6:7.8:38.2:9.0:2.9:2.9)</td>
<td>208.4 ± 10.7</td>
<td>0.228 ± 0.034</td>
<td>2.79 ± 1.24</td>
</tr>
</tbody>
</table>

The data are expressed as the mean ± SD for three different preparations (n = 3).

3.5. Endocytosis mechanism of dual-modified liposomes

In most cases, endocytosis is an energy-dependent pathway and is usually suppressed at low temperatures [41]. Lowering the incubation temperature from 37 °C to 4 °C induced a significant drop in mean fluorescence intensity (Fig. 4A), which supported that the entry pathway of AF-L into 22Rv1 cells is mostly by ATP-dependent internalization. A similar result was observed in the cell uptake of CPP-avidin as described in our previous publication [11].

We extended our studies by using several endocytotic inhibitors to investigate the entry mechanism. As shown in Fig. 4A, the uptake of liposomal FAM-siRNA-loaded in PSA-pretreated AF-L was partially inhibited by chlorpromazine and chloroquine, indicating that PSA-pretreated AF-L entered cells via clathrin-mediated endocytosis and required endosome acidification. Additionally, macropinocytosis was involved in the transduction of PSA-pretreated AF-L into 22Rv1 cells due to partial inhibition by EIPA.

Among the CPPs reported, the most well-studied mechanisms of internalization may be that of arginine-rich CPPs. The fate of the CPPs after uptake varies with the physicochemical properties of the CPPs and attached cargo molecules [42]. Furuhata et al. [43] evaluated the effect of oligoarginine length on the intracellular delivery of olioarginine-modified liposomes. As a result, the uptake of R10-lipoplexes occurred mainly through macrophagocytosis, whereas clathrin-mediated endocytosis dominates the internalization of R4-lipoplexes. Immunoelectron microscopy proved that endocytosis of the PSMA antibody complex occurred via clathrin-coated pits [44]. The internalization of iron oxide nanoparticles with incorporated anti-PSMA antibody was also proved to involve the formation of clathrin-coated vesicles [45]. Although several findings on the folate-modified vehicle have exhibited enhanced targeting and promoted therapy to PSMA-positive PC cells and/or their xenografts [27–29], the probable pathway by which PSMA mediates the internalization of folate-coupled carriers remains unclear. Folic acid inhibited the hydrolyzing activity of PSMA and suggested a direct binding between PSMA and folic acid [26]. Additionally, folic acid binds to the membrane fraction and cross-reacts with anti-PSMA.
Fig. 3. Assessments of cell uptake by flow cytometry and confocal laser scanning microscopy (CLSM). Flow cytometric measurement of liposomal FAM-siRNA uptake by PC-3 cells (A) and 22Rv1 cells (B) after incubation with liposomes in the presence or absence of exogenous PSA at 37 °C for 2 h. The concentration of FAM-siRNA was 75 nM. The effect of α1-antichymotrypsin (ACT) on liposomal FAM-siRNA uptake by 22Rv1 cells (C) after incubation at 37 °C for 2 h. The concentration of FAM-siRNA was 75 nM. *P < 0.05 (n = 3). CLSM images of 22Rv1 cells incubated with liposomal FAM-siRNA (D) at 37 °C for 3 h. The concentration of FAM-siRNA was 225 nM. Cells were fixed with 4% paraformaldehyde and treated with Hoechst 33258 for nuclei staining (blue). FAM-siRNA fluorescence (green) was recorded. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
antibody [46], suggesting that uptake of folate-linked carriers into cells is mediated by PSMA through a similar entry route. Overall, trafficking of PSA-pretreated AF-L into 22Rv1 cells is anticipated to be complicated based on the complexity of the liposomal formulation. The properties of the selected CPP and attached cargo suggested macropinocytosis as the major contribution to entry, whereas the studies on the membrane receptor, PSMA, were more likely to support the involvement of clathrin-mediated endocytosis. Our studies confirmed that two endocytic pathways, macropinocytosis and clathrin-mediated endocytosis, can act concurrently when the indicated vehicle was exposed to 22Rv1 cells.

3.6. Intracellular trafficking of AF-L containing siRNA

As presented above, the liposomal formulation we designed enter 22Rv1 cells partially via clathrin-mediated endocytosis, which lead to delivery of the transported cargo to the lysosomes and the degradation of the subcellular compartment [47]. Accordingly, lysosomal escape of siRNA after trafficking into the cell is essential for post-transcriptional gene silencing in the cytoplasm [48]. As shown in Fig. 4B, both 0.5 h and 2.5 h of routine culture showed that the AF-L carrying FAM-siRNA (green) was not colocalized with the LysoTracker Red labeled organelles, suggesting that FAM-siRNA efficiently escaped from the endosomal or lysosomal vesicles.

Cationic lipoplexes trigger endosome release, which is attributed to the ion pairs formed by cationic lipids with anionic lipids in the endosome membrane and the subsequent destabilization of the endosomal membrane by exclusion of the surface bound water [49]. In addition to cationic lipids, the ion-pair effect was shown to be active in the protein transduction domain such as oligoarginine (R8, R9) peptides [50]. The presence of multiple guanidinium cations proved important to their ion-pair formation in the endosome, leading to efficient endosome escape. Altogether, the above-mentioned aspects may explain how subcellular trafficking of FAM-siRNA contained AF-L escapes acidic organelles, endosomes and lysosomes.

Fig. 4. Cell uptake and intracellular trafficking of FAM-siRNA encapsulated in AF-L. Flow cytometric measurement of FAM-siRNA uptake by 22Rv1 cells after incubation with AF-L in the presence of PSA at 37 °C for 2 h (A). *P < 0.05 (n = 3). The concentration of FAM-siRNA was 75 nM. Untreated cells served as the negative control. Chlorpromazine (20 μM), chloroquine (100 μM) and EIPA (50 μM) were added to the cells 0.5 h prior to AF-L. Intracellular trafficking and distribution of FAM-siRNA in 22Rv1 cells undergoing 0.5 h or 2.5 h of routine culture following 3 h of incubation with AF-L in the presence of PSA at 37 °C (B). The FAM-siRNA concentration was 225 nM. Cell nuclei and endosomes/lysosomes were counterstained with Hoechst 33258 (blue) and LysoTracker Red (red), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3.7. In vitro gene silencing and cell apoptosis assay

A key regulator of mitotic progression, PLK-1 is overexpressed in many human solid tumors, including PC [6]. Accordingly, we studied the gene as a therapeutic target against PC and evaluated the downregulation of the target gene by siRNA-loaded liposomes.

As exhibited in Fig. 5A, siN.C.-loaded AF-L did not downregulate PLK-1 expression, whereas the cells exposed to various liposomes carrying siPLK-1 presented reduced PLK-1 gene expression, indicating sequence-specific gene silencing. The folate-modified liposome expressed more powerful gene silencing than N-L, which correlated with the facilitated internalization of siRNA (Fig. 3B), suggesting PSMA-mediated cell uptake of the folate-modified vector, as demonstrated in previous studies [28,29]. In comparison to cells treated with AF-L and A-L under the absence of PSA, much lower levels of PLK-1 mRNA was detected in the cells incubated with AF-L and A-L under activation of PSA. This was attributed to the improved entry of siRNA into the cells (Fig. 3B and D) and the effective escape of siRNA from the endosome (Fig. 4B) caused by effective cleavage of the PSA-responsive peptide. Additionally, the silencing effect of siPLK-1 loaded AF-L in the presence of PSA on the target gene was inferior to that of C-L, which was not consistent with previous cell permeation. This discrepancy might be associated with the more efficient endosome escape following the translocation of C-L into cells due to the absence of folate-coupled DSPE-PEG, a barrier to penetration of the endosome membrane [50].

To investigate whether this reduction in PLK-1 mRNA was followed by a decrease of PLK-1 protein, we conducted western blot analyses in 22Rv1 cells. As shown in Fig. 5B, in contrast to the untreated control group, the delivery of siPLK-1 by the liposomes exclusively knocked down PLK-1 protein expression. However, both siN.C. contained AF-L and free siPLK-1 did not effect PLK-1 protein expression, indicating that no nonspecific gene silencing took place and that naked siRNAs could not readily translocate across the cell membrane due to their large size and highly negative charge [50]. Furthermore, siPLK-1 loaded F-L exhibited markedly improved silencing of PLK-1 protein expression compared to the N-L, and both A-L and AF-L presented strengthened silencing response under the activation of PSA, which was in agreement with the above observation in qRT-PCR, providing further support for the anticipated specificity and sensitivity. In addition, similar to the mRNA expression profiles, the lowest PLK-1 protein expression was observed in cells treated with C-L carrying siPLK-1, which would result from the beneficial intracellular trafficking mediated by the CPP, di-octaarginine.

PLK-1 represents a valid gene target in oncology. This gene’s inhibition induces apoptosis in proliferating tumor cell cultures [51]. We determined whether the indicated liposomes induced apoptosis in treated cells and whether modifications of the liposomes enhanced their apoptotic activity. As shown in Fig. 5C, the exposure to every type of liposome containing siPLK-1 resulted in an abundance of apoptotic (both Annexin V-positive and propidium iodide-positive) cells (30–55%). In contrast, only a slight effect (<3% apoptotic cells) was observed in the untreated cells and the siN.C. contained AF-L treated cells, indicating that the apoptosis response originated from the downregulated expression of PLK-1 in 22Rv1 cells. Approximately 33% of cells undergo apoptosis after transfection with siPLK-1 loaded N-L; however, a higher percentage of cells (44%) displayed apoptosis after treatment with siPLK-1 loaded F-L. As expected, the delivery of AF-L and A-L containing siPLK-1 under activation of PSA resulted in a higher percentage of apoptotic cells (54% and 43%, respectively) than AF-L and A-L without PSA (48% and 38%, respectively). Cells exposed to siPLK-1 loaded C-L and AF-L under activation of PSA presented the highest percentage, reaching 50%, of induced apoptosis, which was consistent with the PLK-1 protein expression assay described above.

The reduction in PLK-1 mRNA is responsible for the decreased PLK-1 protein expression in the 22Rv1 cells and the increased cell apoptosis. These results demonstrated that the combination of the folate ligand and the PSA-responsive peptide introduced into the liposomal formulations markedly facilitated the RNAi-mediated gene silencing and growth inhibition.

3.8. Biodistribution of siRNA-Loaded liposomes after systemic administration

For effective RNAi-based cancer treatment, siRNA must be accumulated within tumors. After the establishment of a 22Rv1 xenograft tumor model and feeding on a folate-deficient diet (to maintain a plasma folate level within the physiological range of humans [52]), various samples were administered and the tissue distribution was recorded. Judging from whole body imaging (Fig. 6A), naked siRNA was almost eliminated from the body after 12 h, and tumor accumulation did not occur. In contrast, significant tumor accumulation was observed for AF-L and F-L carrying Cy5-siRNA, with the tumor fluorescence intensity at a high level during the entire period (4 d). Moreover, the most intense distribution in tumors was displayed in the AF-L treated mice and was further confirmed by the strongest fluorescence identified in isolated tumors (Fig. 6B). The phenomenon indicated that the introduction of folate induced tumor-targeting in Cy5-siRNA, and the incorporation of PSA-responsive peptide further enhanced its accumulation in tumors. For Cy5-siRNA-loaded N-L and A-L, the duration of fluorescence in tumors also reached 4 d; however, a decrease in fluorescence intensity was found on day 2 and day 3 post-exposure. It was confirmed that the normal liposomes resulted in relatively limited targeted delivery, and the selective distribution was improved by the incorporation of PSA-responsive peptide. The tumor fluorescence intensity in C-L treated mice decreased significantly after 1 d and almost disappeared at the third day. The coupling of polyarginine did not benefit the selectivity of Cy5-siRNA in 22Rv1 tumors due to the propensity of oligoarginine peptides in cell uptake as reported previously [53]. Even in other tumor models, more specific tumor distribution has been detected for fluorescently labeled 8 compared with other CPPs composed of a different number and configuration of arginines [54]. C-L possessed a lower tumor-targeting ability in vivo than A-L, which could be attributed to CPP’s lack of selectivity, whereas the higher uptake of FAM-siRNA in C-L treated cells (Fig. 3B) derives from the promoted entry of liposome into cells via CPP.

All mice presented comparable fluorescence intensity in the kidneys, submandibular gland and pancreas (Fig. 6B), which was consistent with another study reported recently [55] and indicated that the in vivo fate of all siRNA contained samples involved renal excretion. Similarly to the publication of Liang et al. [56], the strongest fluorescence observed in excised kidneys was extremely shielded in whole-animal imaging, which resulted from the deep location of the kidney in the body and the optical impedance by soft tissues. As illustrated in Fig. 6B, there was a fluorescence signal observed in isolated testis only for the F-L treated mice, implying an association with the expression of PSMA in normal mouse testis as published previously [57,58]. In another study [53], the distribution of Cy5-siRNA complexed in non-folate-modified vehicles was also found in normal testis; the related mechanisms warrant further study.

These initial data resulting from in vivo imaging provided substantial evidence that AF-L has potential to accomplish specific targeting of siRNA to PCs.
Fig. 5. The level of PLK-1 mRNA determined by qRT-PCR (A), PLK-1 protein expression determined by western blot analysis (B). Cell apoptosis following exposure to different formulations (C). 22Rv1 cells were individually treated with various formulations carrying siPLK-1 or siN.C. (100 nm) at 37 °C for 5 h, followed by 48 h (A) or 72 h (B, C) of regular culture. *P < 0.05 (n = 3). Early apoptotic cells are shown in the lower right quadrant, and late apoptotic cells are shown in the upper right quadrant.
Fig. 6. Biodistribution of Cy5-siRNA contained in various liposomes in mice bearing 22Rv1 tumor xenograft. Whole body imaging at different time points after systemic administration (A). Fluorescence detection of isolated main tissues and organs from mice at the end point of observation (B).
Fig. 7. Antitumor activity (A) and body weight changes (B) in 22Rv1 tumor-bearing mice after treatments with 5% glucose and various liposomes carrying siPLK-1 or siN.C. Arrows represent drug administration. Data represented as the mean ± SD (n = 6–7). *P < 0.05 for AF-L versus F-L. Expression of PLK-1 mRNA (C) and protein (D) in tumors was detected 24 h after the last administration. *P < 0.05 (n = 3). The exposure was performed at a 1.5 mg/kg siRNA equivalent dose once every other day for a total of 10 d.
3.9. *In vivo* therapeutic efficacy against tumor-bearing mice

The selective distribution and prolonged retention of siRNA-formulated AF-L in prostate tumors could potentially benefit the antitumor activity of this therapeutic nucleic acid *in vivo*. To verify this, we monitored the effect of each sample carrying siRNA (siPLK-1 or siN.C.) on the growth of 22Rv1-derived tumors in nude mice following systemic administration. The growth curves of 22Rv1 tumors are shown in Fig. 7A.

Treatment with the naked siPLK-1 did not show increased tumor growth inhibition in comparison to 5% glucose, with the consequent tumor exceeding a 5-fold initial mean tumor volume, likely due to the transitory retention in systemic circulation and poor permeability into tumor cells. In contrast, various

![Fig. 8. TUNEL detection of apoptotic cells in tumor tissues following treatment with different formulations. The tumor tissues were collected 24 h after the final administration.](image-url)
formulations of siPLK-1 exhibited tumor suppression activity, particularly after 1 week post-exposure. Furthermore, one week after administration, the maximal reduction in tumor growth was noted in the group treated with AF-L carrying siPLK-1 with a 2-fold decrease compared to the control group at the end of test. This correlated with above-mentioned data revealing the advantage of AF-L over the other carriers we tested in apoptosis induction in vitro (Fig. 5C) and tumor-specific distribution in vivo (Fig. 6), indicating the combined processes of PSA activation and PSMA receptormediation. Compared with N-L and C-L, exposure of siPLK-1 with either A-L or F-L retarded tumor progression. This result revealed that the incorporation of either folate or PSA-responsive peptide into the carrier enhanced the antitumor therapeutic effects in vivo. In addition, the injection of 5% glucose and AF-L carrying siN.C. did not delay tumor growth, indicating that the antitumor effect of liposomes carrying siRNA was siPLK-1 sequence-specific. These results indicate that the therapeutic efficacy of the siPLK-1 loaded AF-L is significantly superior to that of free and other formulations of siPLK-1 in 22Rv1 xenograft models.

Additionally, despite multiple injections by various siPLK-1 or siN.C. contained formulations over the duration of treatment, no pronounced change in mouse body weight (Fig. 7B) was noted compared with the control, which suggests that there is negligible acute or severe toxicity related to the indicated treatment at the test dose [59].

To evaluate whether reduced tumor growth as exhibited above was associated with PLK-1 gene silencing in tumor cells, PLK-1 expression at the mRNA and protein levels in the tumors was assayed by qRT-PCR and western blot analyses, respectively. The expression of both mRNA and PLK-1 protein, in either the naked siRNA group or siN.C. contained AF-L group, showed no alterations compared to the 5% glucose treated tumors (Fig. 7C and D). Conversely, pronounced inhibition was noted in the siPLK-1 contained AF-L group at both the level of mRNA (~30% of the 5% glucose control, Fig. 7C) and protein (Fig. 7D). In comparison to the N-L and C-L group, much lower expression was detected in the tumors of mice treated with F-L and A-L encapsulating siPLK-1. Taken together, these results were consistent with the antitumor therapy mentioned above, supporting the direct causality between delayed tumor progression and the silenced PLK-1 gene.

By analyzing DNA strand breaks, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was extensively employed in the assessment of tumor cell apoptosis [60]. As seen in Fig. 8, neither 5% glucose nor naked siPLK-1 pronouncedly induced cell apoptosis as demonstrated by the absence of detectable TUNEL-positive tumor cells (red). Contrarily, the exposure to siPLK-1 entrapped AF-L and F-L exhibited significant apoptosis compared to other treatments with formulated siRNAs. The trend observed for apoptotic analysis was consistent with the results of the antitumor efficacy in vivo.

Overall, these results strongly supported our hypothesis that the combination of PSA-responsive and PSMA-mediated multifunctional liposomes enhances PC cell recognition and uptake and reduces nonspecific uptake. The superior antitumor activity of AF-L is due to a combination of PEGylation, folate-oriented binding to the PSMA-positive PC cells, PSA-responsive proteolytic cleavage and enhanced specific cellular uptake through CPP by selective unmasking of the polyamionic inhibitory peptides in tumor tissues in which PSA is overexpressed. Subsequently, siRNA escapes from endosomes into the cytoplasm to mediate RNAi-related gene silencing.

4. Conclusions

In the present study, we successfully designed and synthesized two functional conjugates, DSPE-PEG2000-ACPP and DSPE-PEG5000–Folate, for the surface modification of PC-targeted liposomes. A combination of PSA stimulus-responsive and PSMA ligand-mediated liposomes, AF-L, was developed with the incorporation of these two conjugates. In response to the exogenous enzymatically active PSA, the multifunctional liposome exhibited significantly enhanced uptake, inhibited PLK-1 expression and facilitated apoptosis in 22Rv1 cells when compared with other control liposomes, including singlemodified and non-modified liposomes. Additionally, we observed the favorable escape of loaded siRNA from the endosome into the cytoplasm following AF-L internalization, in which both clathrin-mediated endocytosis and macropinocytosis played an important role. Based on a 22Rv1 xenograft murine model, in vivo studies indicated that AF-L underwent elevated tumor accumulation, augmented PLK-1 silencing, delayed tumor progression and reinforced tumor cell apoptosis. Taken together, these results indicate that the multifunctional liposomes offer a strategy for effective targeted delivery of siRNA to PSMA/PSA-positive prostate tumors.

Acknowledgments

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.05.055.

References


