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Tumor targeting and microenvironment-responsive nanoparticles for gene delivery

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ABSTRACT

A tumor targeting nanoparticle system has been successfully developed to response to the lowered tumor extracellular pH (pHe) and upregulated matrix metalloproteinase 2 (MMP2) in the tumor microenvironment. The nanoparticles are modified with activatable cell-penetrating peptide (designated as *dt*ACPP) that's dual-triggered by the lowered pHe and MMP2. In *dt*ACPP, the internalization function of cell-penetrating peptide (CPP) is quenched by a pH-sensitive masking peptide, linking by a MMP2 substrate. The masking peptide is negatively charged to quench the cationic CPP well after systemic administration. Hence, *dt*ACPP-modified nanoparticles possesses passive tumor targetability *via* the enhanced permeability and retention (EPR) effect. Once reaching the tumor microenvironment, the pre-existing attraction would be eliminated due to the lowered pHe, accompanying the linker cleaved by MMP2, *dt*ACPP would be activated to expose CPP to drive the nanoparticles' internalization into the intratumoral cells. The studies of plasmid DNA loading, toxicity assessment, cellular uptake, tumor targeting delivery, and gene transfection demonstrate that *dt*ACPP-modified nanoparticle system is a potential candidate for tumor targeting gene delivery.

1. Introduction

Ideal tumor targeting nanoparticles for gene delivery could significantly increase the accumulation of plasmid DNA in the target tumor sites, and further internalize DNA into the tumor cells for transfection. After systemic administration, based on the satisfactory biocompatibility and appropriate nano-scale, nanoparticles mainly accumulate in the target tumor sites *via* the EPR effect that's caused by the leakiness of the vasculature surrounding tumors [1–3]. After that, the target molecules modifying on the surface of nanoparticles could recognize and bind to the tumor cells specifically, which would be extremely crucial to internalize nanoparticles into the tumor cells efficiently from the extracellular space [4–6]. However, it's contradictory that the target molecules might be unnecessary even detrimental in the circulation. For example, transferrin-modified tumor targeting nanoparticles would partly accumulate in the normal brain tissue, because transferrin receptor is not only overexpressed on the tumor cells but also highly existed in brain capillaries for the mediation of the iron delivery to the brain [7,8]. The corresponding side effects might be triggered as the non-specific distribution of nanoparticles. Therefore, it's critical to design a nanoparticle system that could always "turn off" the internalization function in the circulation, but "turn on" in the interior of tumor, which needs to utilize the unique tumor microenvironment [9].

In addition to the EPR effect, the lowered pHe and upregulated matrix metalloproteinases (MMPs) would be formed with the development of tumors. The high metabolic levels of the tumor need high glycolytic activity, which would cause increased lactic acid production and hence result in lowered pHe [10,11]. It has been reported that the tumor pHe is around 5.8–7.2, lower than the physiological pH [12]. In recent years, several new pH-sensitive approaches for tumor targeting delivery have been developed based on the lowered pHe [12–16]. MMPs are engaged in the degradation of extracellular matrices and tightly associated with malignant process of tumors; meanwhile, their expression and activation are increased in almost all tumors compared with normal tissue [17,18]. MMP2, one key member of the MMPs family, plays complex roles in the promotion of tumorigenesis by degrading the extracellular matrices and activating growth factors and angiogenesis [19,20].

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CPP has been widely investigated in the fields of gene and drug delivery for oncotherapy, which could facilitate the cellular uptake of various cargos without causing any cellular injury [21–24]. In this study, the cationic CPP was selected as a potential tumor targeting molecular, which is able to adsorb to the cell surface strongly through electrostatic interaction with the negative charges membrane [25,26]. Unfortunately, CPP can cause efficient cell internalization *in vitro* while cannot effectively concentrate in the target sites *in vivo* due to the lack of selectivity for target cells [27–30]. After intravenous administration, CPP initially bind to the local vasculature and redistribute to the liver, where more than 90% of the injected dose accumulated 30 min later [29]. That's why the systemically administered nanoparticles modified with CPP are difficult to accumulate in the target tumor sites *via* the EPR effect. In the limited efficacious *in vivo* application of CPP, they were locally injected into the tumor rather than systemic administration [31,32].

According to the tumor microenvironment, the mission of this study was to endow the non-specific CPP with targetability for tumor targeting gene delivery. It has been reported that charge neutralization of CPP substantially decreased both its adsorption and internalization with cells [33]. So quenching the cell-penetrating property of CPP by electrostatic attraction with the polyanionic masking domain might prevent the non-specific internalization in the circulation [29]. Utilizing the unique tumor microenvironment, *dt*ACPP, an activatable CPP dual-triggered by lowered pHe and upregulated MMP2 was designed, and whose design strategy was summarized as follow. Utilizing the lowered pHe, a pH-sensitive masking peptide (e_4k_4) with an isoelectric point (pI) of approximately 6.4 was engineered. Consequently, it would be negatively charged on physiological condition (pH 7.4) but was predominantly uncharged, even positively charged under lowered pHe. PLGLAG, the MMPs substrate, could be cleaved by MMPs between glycine and leucine; furthermore, MMP2 can cause nearly complete cleavage [29]. Therefore, PLGLAG was utilized to link the polycationic CPP (nonarginine) and the masking peptide, hence *dt*ACPP was structured.

Of the non-viral vector, dendrigraft poly-L-lysines (DGL) is widely investigated for gene delivery to tumor because of its ability to effectively complex and condense DNA to form nanoparticles [34,35]. *dt*ACPP was conjugated to the surface of DGL *via* α-Maleimidyl-ω-N-hydroxysuccinimidyl polyethyleneglycol (MAL-PEG-NHS) to construct the gene nanocarrier, *dt*ACPP-PEG-DGL (*dt*ACPPD). Condensed nanoparticles, *dt*ACPPD/DNA, were formed through electrostatic interactions between cationic DGL and negatively charged plasmid DNA. As shown in Fig. 1, the tumor targeting nanoparticles were endowed with steric stabilization to perform the EPR effect well, as the cell-penetrating property of CPP was quenched in the circulation. Once reaching the tumor site, the pre-existing attraction effect would be eliminated. Accompanying the cleavage of linker by MMP2, *dt*ACPP would be activated to expose CPP to drive the nanoparticles into the tumor cells.

Here, by grafting PEG and decorating *dt*ACPP on the nanoparticles, we endowed the *dt*ACPPD-modified nanoparticles with steric stabilization and tumor targetability. *dt*ACPPD/DNA could efficiently protect and deliver intact DNA to the target tumor for *in vivo* gene transfection.

2. Materials and methods

2.1. Materials

DGL G3 was purchased from Colcom, France. MAL-PEG-NHS (MW 3500) was purchased from Jenkem Technology Co., Ltd (Beijing, China). *dt*ACPP (e_4k_4-x-PLGLAG-r_9-x-c, MMP2 cleavable peptide), *uc*ACPP (e_4k_4-x-LALGPG-r_9-x-c, scrambled linker, uncleavable control), and CPP (r_9-x-c, positive control) were synthesized by Chinese Peptide Company (Hangzhou, China). Lowercase letters indicated D-amino acids and x meant aminohexanoic linker. Recombinant-human MMP2 proenzyme was obtained from EMD. YOYO-1 iodide, ethidium monoazide bromide (EMA), 4,6-dia-midino-2-phenylindole (DAPI), 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, sulfosuccinimidyl ester, sodium salt (Green BODIPY) and 6-(((4, 4-difluoro-5-(2-pyrrolyl)-4-bora-3a, 4a-diaza-s-indacene-3-yl)styryloxy)acetyl) aminohexanoic acid, succinimidyl ester (Red BODIPY) were purchased from Molecular Probes (Eugene, OR, USA). The plasmid pGL2 and pEGFP-N2 (Clontech, Palo Alto, CA, USA) were purified using QIAGEN Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany).

2.2. Preparation of nanocarriers and nanoparticles

DGL was reacted with MAL-PEG-NHS at the ratio 1:10 (DGL/PEG, n/n) in PBS (pH 8.0) for 2 h at room temperature. The resulting conjugate, PEG-DGL, purified by ultrafiltration through a membrane (cutoff = 5 kDa) and dissolved in PBS (pH 7.0). Then PEG-DGL was reacted with the peptide at the ratio 1:5 (DGL/peptide, n/n) in PBS (pH 7.0) for 24 h at room temperature. The MAL groups of PEG were specifically reacted with the thiol groups of peptide to form the corresponding nanocarriers, *uc*ACPPD, *dt*ACPPD, and CPPD.

The freshly prepared nanocarriers were diluted to appropriate concentration, and then the DNA solution (100 μg/ml in 50 mM sodium sulfate solution) was added to obtain specified weight ratio and immediately vortexed for 30 s. Freshly prepared nanoparticles were used in the following experiments.

Part of the DNA was labeled with EMA, the fluorescent photoaffinity label. DNA solution (1 mg/ml in TE buffer, pH 7.0) was diluted to 0.1 mg/ml with aqueous solution of EMA (1 mg/ml) and incubated for 30 min away from light at room temperature. The complex was then exposed to UV light (365 nm) for 1 h, and the resulting solution was precipitated by adding ethanol to a final concentration of 30% (v/v). The precipitate was collected by centrifugation and redissolved in 50 mM sodium sulfate solution.

2.3. Characterization of nanocarriers

The characteristic of *dt*ACPPD was analyzed by ^{1}H NMR spectroscopy. Basically, PEG-DGL and *dt*ACPPD were purified by ultrafiltration through a membrane (cutoff = 5 kDa), then freeze-dried, solubilized in D_2O and analyzed in a 400 MHz spectrometer (Varian, Palo Alto, CA, USA).

2.4. Pretreatment of MMP2 to cleave the MMP2 substrate

It has been demonstrated that although the tumor cells secret MMP2, the enzyme becomes notably diluted in the *in vitro* experiment, resulting in slow cleavage and negligible activation [29]. And it's been found that significant cleavage and uptake of ACPPs required pre-cleavage with exogenous protease [36]. Recombinant-human MMP2 proenzyme (5 μg in 80 μl of 50 mM Tris–HCl buffer) was activated with 2.5 mM 4-aminophenylmercuric acetate at 37 °C for 2 h. Afterward, *dt*ACPP (*uc*ACPP) and *dt*ACPPD (*uc*ACPPD) were activated before cell incubation with recombinant-human MMP2 (10 pmol) for 3 h in 50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, and 10 μM $ZnCl_2$ under pH 7.5. *dt*ACPPD and *uc*ACPPD without pretreatment of MMP2 were designated as (-MMP)*dt*ACPPD and (-MMP)*uc*ACPPD.

2.5. SDS-PAGE to verify the cleavage effect

SDS-polyacrylamide gel electrophoresis was performed in a Miniprotean III Cell (Bio-Rad, Hercules, CA, USA). The voltage was kept constant at 60 V during stacking and then increased to 120 V, and kept for 60 min. After that, the gels (20% polyacrylamide) were stained with Coomassie blue and then destained.

2.6. Gel retardation assay

Agarose gel retardation assay was carried out to determine the DNA binding ability of *dt*ACPPD. *dt*ACPPD/DNA were prepared at various weight ratios (DGL to DNA; 0.05:1, 0.1:1, 0.5:1, 1:1, 3:1, 6:1, and 10:1). The complexes were mixed with appropriate amounts of 6 × loading buffer and then electrophoresed on a 0.9% (w/v) agarose gel containing ethidium bromide (0.25 μg/ml of the gel). The location of DNA in the gel was analyzed on a UV illuminator and photographed using a Canon IXUS 950IS camera.

2.7. Characterization of dtACPPD/DNA

The mean diameter and zeta potential of *dt*ACPPD/DNA with DGL to DNA weight ratio at 6:1 were determined by Zetasizer Nano (Marvin Instruments Ltd., UK). Also, the morphology of dtACPPD/DNA was examined under bioscope atomic force microscopy (Veeco Instruments, USA).

2.8. Evaluation of cytotoxicity induced by dtACPPD

BEL-7402 cells (human hepatocellular carcinoma cells) were seeded in 96-well culture plates (Corning-Coaster, Tokyo, Japan) at a density of 1×10^4 cells/well.