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Retro-inverso D-peptide-modified hyaluronic acid/bioreducible hyperbranched poly(amido amine)/pDNA core-shell ternary nanoparticles for the dual-targeted delivery of short hairpin RNA-encoding plasmids

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ABSTRACT

The active targeting of gene carriers is a powerful strategy for improving tumour-specific delivery and therapy. Although numerous L-peptide ligands play significant roles in the active targeting of nanomedicine, retro-inverso D-peptides have been explored as targeting ligands due to their superior stability and bioactivity *in vivo*. In this study, retro-inverso D-peptide (RIF7)-modified hyaluronic acid (HA)/bioreducible hyperbranched poly(amido amine) (RHB)/plasmid DNA (pDNA) ternary nanoparticles were successfully developed using the layer-by-layer method for the CD44-positive tumour-specific delivery of short hairpin RNA (shRNA)-encoding pDNA through the combination of the Anxa1 (tumour vasculature) and CD44 (tumour cell-surface) receptors, which mediated the dual targeting. The potential of these newly designed nanoparticles was evaluated by examining the efficacy of their cellular uptake and transfection in cell monolayers, tumour spheroids, and malignant xenograft animal models. With negligible cytotoxicity, the spherical-shaped RIF7-HA/RHB/pDNA nanoparticles were the direct result of an electrostatic complex that had efficiently targeted CD44-positive tumour delivery, penetration, and cellular uptake *in vitro*. The nanoparticles showed excellent target-specific gene transfection even in the presence of serum. The *in vivo* therapeutic effect of RIF7-HA/RHB/pDNA-shRNA nanoparticle-mediated shRNA targeting of the Cyclin gene (shCyclin) was evaluated in tumour-bearing mice. The RIF7-HA/RHB/pDNA-shCyclin nanoparticles significantly increased the survival time of tumour-bearing mice and substantially reduced tumour growth due to their extremely specific tumour-targeting activity. These results suggested that the combination of HA and retro-inverso peptide RIF7 significantly increased the therapeutic effect of pDNA-shCyclin-loaded nanoparticles for CD44-positive tumours. Thus, RIF7-HA-mediated multitarget ternary gene vectors are an efficient and promising strategy for the delivery of pDNA-shRNA in the targeted treatment of malignant and metastatic cancers.

Statement of Significance

Although L-peptide ligands play significant roles in the active targeting of nanomedicine, retro-inverso D-peptides have been explored as targeting ligands due to their superior stability and bioactivity *in vivo*. Retro-inverso peptide RIF7 was designed as a ligand of Anxa1 receptor. The resultant peptide, RIF7, displayed high binding efficiency within Anxa1 receptor, which is highly expressed tumour vasculature cells and some tumour cells such as B16F10 and U87MG cells. The most important feature of RIF7 is its high stability in the blood, which is suitable and promising for application *in vivo*. Multifunctional RIF7-HA was then synthesized by conjugating the RIF7 peptide to HA, which was used to modify the surface of RHB/pDNA nanoparticles to prepare RIF7-HA/RHB/pDNA core-shell ternary nanoparticles for the dual-targeted delivery of shRNA-encoding plasmids *in vitro* and *in vivo*.

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1. Introduction

Gene therapy is widely regarded as a promising treatment for many genetic disorders as well as acquired diseases (such as AIDS or cancer) [1,2]. An efficient and safe nanomedicine-based gene delivery vector is the key issue and the most important challenge for achieving successful gene therapy. Therefore, comprehensive consideration of a delivery system is needed for overcoming the extracellular barriers (such as *in vivo* stability, rapid clearance during systemic delivery, specific targeting, and degradation of nucleic acid) [3,4] and the cellular barriers (cellular uptake, endosomal escape, nuclear entry, and nucleic release) [5–9]. Non-viral vectors are considered to be ideal gene delivery vectors, as they have low cytotoxicity, host immunogenicity, and cost; their synthesis is easy and reproducible with construction flexibility; and they can be modified with targeted ligands [10,11]. In particular, cationic polymers have attracted much interest because of their chemical and physical properties that endow multiple functions for gene delivery. However, it should be noted that high cytotoxicity and the risk of unknown long-term effects after accumulation are obstacles for the development of cationic polymer-based non-viral vectors, even though they have relatively high transfection efficiencies *in vitro* [12,13]. Biodegradable cationic polymers are potentially safer alternatives for gene delivery as the degradation of the cationic polymers is a possible advantage that could reduce their toxicity (degradation leads to non-toxic products) and avoid accumulation in cells. For instance, reducible hyperbranched poly(amido amine)s (RHBs), which contain biodegradable disulfide linkages in the main chain, are well-designed highly branched macromolecules composed of nontoxic low molecular weight monomers that are a promising class of polymers for many potential applications in gene delivery due to their biodegradability, low cytotoxicity, and high transfection efficiency *in vitro* [14]. Disulfide bonds present in the RHB structure are known to be stable in blood and are easily cleaved by glutathione (GSH) in the intracellular reducing environment [15]. The main limitation of cationic polymer-based non-viral systems is their low gene transfection efficiency *in vivo*, which greatly limits their clinical applications; however, this shortcoming has been improved using different strategies, and efforts are still ongoing [16]. Thus, preparing safe and efficient non-viral vectors is desirable for gene delivery.

The design of safe and efficient non-viral vectors depends mainly on overcoming the difficulties that occur after *in vivo* administration. The main potential obstacles of RHB *in vivo* applications are positively charged surface characteristics and nonspecific delivery. Although unshielded polycation/nucleic acid polyplexes can facilitate cellular internalization, the high positive surface charges of polyplexes may lead to low stability in serum, erythrocyte aggregation, and short circulation times *in vivo* by inducing interactions with negatively charged blood constituents, such as proteins and erythrocytes [17]. Shielding the positive surface charges of polyplexes is currently an important strategy to circumvent these problems [18]. Introducing polyanions into polycation-based gene carriers by physical electrostatic coating can provide progressive advantages [17,18]. Hyaluronic acid (HA) is a natural linear extracellular matrix polysaccharide that has pivotal roles in various biological functions [19,20]. Our previous studies showed that an HA coating could recharge the surface of polyplexes such that they are negatively charged, effectively diminishing the adverse interactions, which reduced cytotoxicity, enabled long circulation times, and increased the tumour biodistribution and anticancer activity of the polycation/nucleic acid polyplexes *in vivo*. Importantly, HA is an endogenous ligand for the cell-surface receptor CD44, which is considered to be a well-characterized biomarker that can be used for specific ligand targeting of cancer cells overexpressing CD44 [19].

Successful targeted gene therapy depends on an efficient and safe delivery system that can deliver therapeutic genes to the desired specific target site to exert their action while minimizing the exposure of normal tissues. Therefore, multimodal active targeting strategies are urgently needed. The typical targeting strategies are size-dependent "passive" targeting and/or ligand-directed "active" targeting approaches [21]. Peptides and/or proteins have been commonly utilized as ligands to functionalize the non-viral vectors to improve tumour-specific delivery and to induce the desired target cell-specific internalization [22,23]. Vasculature-targeted delivery has become an attractive strategy due to the phenotypic changes on endothelial cell surfaces that are associated with pathological conditions such as inflammation and angiogenesis. Therefore, the identification of novel ligands with high affinity and specificity that recognize pathological vasculature is of great interest. Annexin 1 (Anxa1) is a highly specific tumour vasculature surface marker found in several tumour types in mice and humans [24]. Thus, Anxa1 is an attractive molecular target, and high affinity ligands for the Anxa1 receptor could be powerful tools for the delivery of therapeutics and/or imaging agents to tumour sites. Previous studies indicated that a specific Anxa1-binding peptide (IFLLWQR), designated IF7, can bind to a 15 kDa fragment of Anxa1 [24]. We hypothesized that this peptide could serve as a highly efficient targeted tumour-specific drug delivery vehicle for delivering anticancer drugs to a tumour *in vivo*. However, medical applications of peptide ligands remain a challenge due to their lack of serum stability. After intravenous administration, the degradation of peptides is the first barrier that needs to be overcome. Peptides can be degraded by proteases in blood. Therefore, increasing protease resistance is crucial for achieving targeting effects. Retro-inverso peptide analogues, also called retro-all-D or retroenantio-peptide analogues, are obtained through assembling amino acid residues in the reverse order of the parent peptide sequence and by replacing L- with D-amino acids. Theoretically, retro-inverso peptides, unless fixed in special secondary structures, present a side chain orientation that is very similar to that of the original structure. Such peptides are resistant to protease degradation, have modified antigenicity, and may have equal or even higher bioactivities compared with their parent L-peptides. Several retro-inverso peptides, such as enkephalin, glutathione and gastrin, possess much higher stability and bioactivity than their native structures [25–27]. Thus, a popular D-configuration technology was typically employed to synthetize retro-inverso peptides to increase the stability of the peptides by replacing L-amino acids with D-amino acids and reversing the primary peptide bonds [28].

Previously, we demonstrated that HA coating (HA/RHB/pDNA ternary nanoparticles) can enhance the stability of RHB/pDNA nanoparticles *in vivo* and achieve active CD44-positive tumour targeting [29]. Here, to improve the active targeting efficiency, retro-inverso peptide of IF7 (RIF7) were conjugated to HA through amide bond formation. Then, multifunctional RIF7-HA/RHB/pDNA ternary nanoparticles were designed based on an electrostatic coating that could actively target solid tumours through two independent receptors: selective binding to the tumour vasculature Anxa1 receptor and to the cell-surface CD44 receptor. The characteristics of this system are: (1) RHB contains disulfide bonds that can be degraded into small molecules *in vivo*, thus reducing the toxicity of the cationic polymer caused by accumulation; (2) HA significantly reduces the positive charges on the surface of RHB/pDNA complexes, resulting in higher stability and lower cytotoxicity *in vivo*; and (3) RIF7-modified HA serves as a multifunctional component that responds to the Anxa1 and CD44 receptors, thus greatly enhancing the efficiency of targeted delivery to solid tumours. The rational process of this targeted delivery is presented in Fig. 1.