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Potential roles of hyaluronic acid in *in vivo* CAR T cell reprogramming for cancer immunotherapy

To overcome the complexity of conventional adoptive cell transfer procedures in which T cells are collected and modified for CAR expression *ex vivo*, viral- and polymeric-based nanoparticles containing CAR transgenes have been developed to target circulating T cells. Injectable nanoparticles that incorporate targeting ligands can deliver the loaded genes specifically to T cells for CAR expression. The nanoparticle gene delivery system enables the *in vivo* genetic modification of T cells for cancer immunotherapy.

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Potential roles of hyaluronic acid in *in vivo* CAR T cell reprogramming for cancer immunotherapy

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Chimeric antigen receptor (CAR) T cell therapy has recently shown unprecedented clinical efficacy for cancer treatment, particularly of hematological malignancies. However, the complex manufacturing processes that involve *ex vivo* genetic modification of autologous T cells limits its therapeutic application. CAR T cells generated *in vivo* provide a valid alternative immunotherapy, “off-the-shelf”, for cancer treatment. This approach requires carriers for the delivery of CAR-encoding constructs, which are plasmid DNA or messenger RNA, to T cells for CAR expression to help eradicate the tumor. As such, there are a growing number of studies reporting gene delivery systems for *in vivo* CAR T cell therapy based on viral vectors and polymeric nanoparticles. Hyaluronic acid (HA) is a natural biopolymer that can serve for gene delivery, because of its inherent properties of cell recognition and internalization, as well as its biodegradability, biocompatibility, and presence of functional groups for the chemical conjugation of targeting ligands. In this review, the potential of HA in the delivery of CAR constructs is discussed on the basis of previous experience of HA-based nanoparticles for gene therapy. Furthermore, current studies on CAR carriers for *in vivo*-generated CAR T cells are included, giving an idea of a rational design of HA-based systems for the more efficient delivery of CAR to circulating T cells.

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1. Introduction

Chimeric antigen receptor (CAR) T cells offer a paradigm shift in clinical cancer immunotherapy using genetically modified T cells with synthetic receptors that recognize target antigens to mediate tumor rejection.^{1,2} CAR T cell products have been

commercially available since 2017 for the treatment of blood cancers, including tisagenlecleucel (Kymriah®, Novartis), axicabtagene ciloleucel (Yescarta®, Gilead), brexucabtagene autoleucel (Tecartus®, Gilead) and mostly recent lisocabtagene maraleucel (Breyanzi®, Juno Therapeutics).³ All products share the same characteristics in which the patient's T cells are collected, genetically programmed and expanded in a lab, before being reinfused into the patient, so-called adoptive cell transfer (ACT).^{4,5} Individualized approaches with complex and labor-intensive manufacturing processes and the requirement for accredited GMP laboratories to control and ensure the quality of the products lead to a costly treatment course that limits accessibility for some patients.^{4,6} In addition, cytokine release syndrome could occur up to 25% of patients after receiving ACT, leading to multiple organ failures, neurologic disorders, or severe immune reactions.^{7,8} To address these limitations, a novel concept of *in situ* or *in vivo* programming of CAR T cells has been introduced in which CAR constructs are administered to patients, allowing *de novo* genetic modification, expansion, and then attack of tumor cells.^{4,6} Due to this ‘off-the-shelf’ approach, individualization can be overcome, and the products can be manufactured in a large batch and delivered to a large number of patients. The comparison scheme between adoptive CAR T cell transfer and *in vivo* CAR T cell programming is illustrated in Fig. 1.

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Fig. 1 Comparison between adoptive cell transfer and *in vivo* cell programming in CAR T cell therapy. For adoptive CAR T cell transfer (upper left), T cells are isolated from patients, so-called leukapheresis, using an apheresis machine. The obtained T cells are then transfected to express CAR (lower left), before expanding, controlling the cell quality in a certified laboratory, and re-administration to the patients. In the case of *in vivo* programming of CAR T cells (upper right), the CAR constructs are loaded into nanoparticles, which can be either viral or non-viral vectors, and the nanoparticles are administered to the patients. The nanoparticles are designed to specifically bind to circulating T cells and release CAR constructs to the cytosol or nucleus of T cells. Transduced T cells express CAR to target and eliminate cancer cells (lower right).

Expression of the CAR transgene in primary T cells can be achieved by the cellular delivery of CAR constructs, which can be plasmid DNA (pDNA), messenger RNA (mRNA) or CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein). Entrapment of loaded genes into a complex using polymers is required to protect them from endogenous enzymes and facilitate cellular internalization. CAR-loaded nanoparticles are typically administered intravenously, which means that the nanoparticles should enable specific delivery to circulating T cells. A rational carrier design can prolong the retention time in the bloodstream by preventing the early elimination of nanoparticles.^{9,10} After cell uptake of the nanoparticles through receptor-mediated endocytosis,

delivery systems could aid in cell trafficking within the cells by releasing payloads from endosomes into the cytosol before CAR translation and expression (Fig. 2).

Typically, *in vivo* applications of nanoparticles should be aware of the biodistribution and accumulation in the major organs, such as the lungs, liver, spleen, and kidneys, which are dictated by the physical properties of the nanoparticles, including their size, surface charge, and shape.¹¹ For T cell reprogramming, nanoparticle delivery could be limited by the innate characteristics of T cells, including the small cell size, the high nucleus-cytoplasm ratio, and the non-phagocytic nature.¹² Internalization through receptor-mediated endocytosis could be limited due to the low expression of surface recep-





Fig. 2 Cellular uptake and CAR expression of CAR after nanoparticle administration. Step 1: The nanoparticles are injected intravenously and are specifically recognized by circulating T cells. Step 2: Cellular binding and internalization of nanoparticles occurs via receptor-mediated endocytosis, and the nanoparticles are entrapped in endosomes. Step 3: The nanoparticles mediate the disruption of the endosomes to release the loaded gene cargos into the cytosol. The remaining nanoparticles are packed in lysosomes as a cellular waste. Step 4: The loaded genes are taken up by the target organelles. Step 5: The mRNA is translated to proteins by the ribosomes. Step 6: The expression of the CAR receptor on the surface of T cells.

tors in a naïve state.¹³ More importantly, circulating T cells, which are the main target of CAR programming, are present in very small numbers in the blood,¹⁴ resulting in a low population of reprogrammed cells.

In this paper, nanoparticle-based delivery systems for gene delivery targeting T cells are reviewed, focusing on polymeric nanoparticles. Hyaluronic acid (HA) is one of the polymers that has potential as a component of delivery systems for targeting T cells. An addition of HA to the formulations of gene complexes can reduce the cytotoxicity, improve transduction activity *in vitro* and *in vivo*, guide the nanoparticles to the target sites, and prolong the stability of the gene products. Additionally, HA is a versatile biomaterial that allows a rational design of nano-formulations with desired physicochemical and biological properties.¹⁵ Although to our knowledge, there are no HA-based carriers designed specifically for T cell delivery, especially for *in vivo* CAR T cell immunotherapy, the roles and functions of HA as a gene carrier, as well as the strategies

to apply HA to enhance T cell targeting properties, will be discussed. Studies relating to nucleic acid-loaded HA-based nanoparticles, as well as the delivery systems serving *in vivo* CAR T cells, will be mentioned to give an idea of the development of an efficient carrier for *in vivo* programming of T cells.

2. Immunotherapy based on T cells for cancer treatment

The concept of cellular immunotherapy is to use host immune systems in tumor eradication. Typically, immune cells are responsible for the elimination of foreign bodies, including pathogenic microorganisms, as well as cells with abnormal proliferation, termed cancer immunosurveillance or cancer immunoediting. The fundamental concept is that the expression of cancer cells' antigens is different from normal cells, serving as rejection antigens in provoking CD8⁺ effector



Instead of expressing the full length α and β chains as TCRs, the antigen binding domain of CAR presents only a single chain variable fragment (scFv). Therefore, the binding of CAR-engineered T cells is independent of the MHC molecules. This could be beneficial for the treatment of resistant tumors in an escape phase, where immune evasion develops due to the loss of presentation of the MHC-associated antigen.²³ As shown in Fig. 1, the composition of CAR can be simply divided into 3 elements: (1) extracellular domains, which are responsible for antigen recognition and binding, linked to (2) transmembrane domains and (3) intracellular domains, which impart intracellular signaling and T cell activation.

To overcome the limitations of virus-based delivery systems, non-viral approaches, namely cationic lipids and polycations, offered a safer alternative for gene transfer. Most of the non-

viral cargos comprise positively charged moieties, such as protonated amines, which are capable of interaction with the negative phosphate groups of nucleic acids, resulting in a condensation of nanosized complexes. The positive surface charge of the complexes also enhances cellular uptake through electrostatic binding to the negatively charged cell membrane. Furthermore, the vectors can be chemically modified with targeting ligands to improve their specific recognition. However, the relatively low transfection efficiency, nonspecific binding to serum proteins, and cytotoxicity of cationic molecules are the major issues in the development of an effective non-viral-based delivery system.²⁹ BNT162b1 and mRNA-1273, the SARS-CoV-2 vaccines developed by Pfizer/BioNTech and Moderna, respectively, are mRNA-based vaccines encapsulated in lipid nanoparticles, and can serve as examples of non-viral gene delivery systems. Lipid nanoparticles not only improve the cellular internalization of mRNA, but also act as a shelter for mRNA, allowing the practical use of very labile mRNA and extending its shelf life.³⁰

Shielding the cationic complexes of DNA or RNA and lipids (lipoplexes) or polycations (polyplexes) with uncharged or counter-charged polymers is an approach for a reduction of surface charge density, resulting in a lower non-specific binding, longer retention time in the bloodstream, and reduced toxicities. The most common polymer for complex shielding is polyethylene glycol (PEG), so-called PEGylation, and the obtained nanoparticles are referred to as stealth nanoparticles. Even though PEG does not possess a specific ligand of biological receptors, the introduction of targeting groups by a chemical conjugation of PEG chains could enable the targetability of nanoparticles.³¹ In addition to PEG, other negatively charged polymers, namely hyaluronic acid (HA), chondroitin sulfate, polyesters, and polyglutamic acid, are utilized as shielding polymers by either simple electrostatic interaction or chemical conjugation with cationic lipids or polymers. Among these polymers, HA-based DNA or RNA complexes have been widely explored for their *in vitro* and *in vivo* efficiency and safety,³² confirming a promising non-viral polymeric gene delivery system for clinical uses.

4. Designing HA-based nanoparticles for gene delivery

HA is a naturally derived polysaccharide with a disaccharide monomeric unit that contains *N*-acetyl-D-glucosamine and D-glucuronic acid residues. Biological HA serves as a component of the extracellular matrix in mammals, and is highly present in synovial fluid, vitreous humor, and skin. Its physiological functions include supporting cell proliferation, differentiation, migration, and tissue repair.³³ The lists of studies that involve HA in gene delivery are presented in Tables 1 and 2, categorized by the forms of HA (original *versus* chemically modified formats) used for nanoparticle formation.

Because of the negatively charged characteristic of HA, it is difficult to form polyplexes with DNA or RNA unless the

polymer is formed with other cationic molecules prior to the addition of HA or there is modification of the HA structure to enable the potential for complexation. As shown in Table 1, cationic polyplexes or lipoplexes are formed before shielding with HA, which results in a lower surface charge of the complexes.^{34–38} Minimizing the positively charged complexes dramatically decreases cytotoxicity^{39–41} and immunogenicity,⁴² and prevents coagulation with serum proteins,^{43–45} avoiding complement activation and a rapid clearance from the systemic circulation. In addition, anionic shielding improves penetration through the layer of negatively charged glycoproteins, such as mucin, to the target tissues.⁴⁶ The most common polycation for polyplex formation is polyethyleneimine (PEI), a polymer containing amine groups that can be protonated over a wide range of pH, allowing electrostatic interaction with the phosphate groups of DNA or RNA⁹ and facilitating endosomal escape from the proton sponge effect.⁴⁷

However, due to the inherent cytotoxicity and non-biodegradability of PEI, chitosan, a naturally derived cationic polysaccharide with biodegradability and biocompatibility profiles, offers a safer alternative to use with HA in the formation of gene complexes.⁹ The major limitations of chitosan are the variability in molecular weight and the degree of deacetylation, which affect the affinity for the entrapped gene. Tirelli, N. and colleagues have used chitosan for the entrapment of RNA and have observed the targeted tumor treatment when the polyplexes are shielded with HA. Their findings revealed that chitosan with a low degree of deacetylation, related to a lower positive charge, possessed a lower affinity for RNA, resulting in a loose complexation and a high exposure to physiological nucleases. In contrast, a high molecular weight chitosan could better stabilize RNA, but a strong electrostatic interaction led to poor decomplexation, lowering RNA release and transfection efficiency. The size of the entrapped gene can also affect the affinity for chitosan, with long-stranded mRNA binding to polyplexes tighter than short-stranded siRNA.^{34,48} Balancing the molecular weight and degree of deacetylation of chitosan with DNA or RNA of different sizes to achieve high transfection efficiency and stability could be a major issue in designing HA- and chitosan-based delivery systems.

Nanoparticle formation using counterion complexation is a simple, one-pot approach, but the weak and transient electrostatic interaction can be easily disturbed. An early dissociation of the entrapped gene in the presence of negatively charged serum proteins leads to an exposure of naked genes to circulating endonucleases, resulting in lower gene availability at target sites.^{10,36,49} Therefore, nucleotide-loaded polymer-based nanoparticles, such as the covalent conjugation of nucleotides to polymers^{50,51} or polycation-modified HA, as shown in Table 2, result in a predictable gene encapsulation efficiency as well as a reliable transfection efficiency. Furthermore, the size of the gene-loaded modified HA nanoparticles was in a range of 100 to 200 nm, which is suitable for cell internalization and *in vivo* biodistribution to target cells,⁵² compared with larger electro-





Table 1 Lists of HA-based nanoparticles in gene delivery fabricated from the unmodified HA

Nanoparticle formation		Nanoparticle characteristics		Evaluation of transfection efficiency			Proposed applications	Ref.
Payloads	Cationic lipids/polymers	HA (MW)	Size (nm)	Charge (mV)	<i>In vitro</i> ^a	<i>In vivo</i>		
pDNA (SOX9)	Linear PEI (25 kDa)	5.8 kDa	70–150	+5.8 to +20.3	Human BMSCs	N/A	Cartilage tissue engineering	35
pDNA (GFP, Luc)	Linear PEI (25 kDa)	N/A	300–800	–46.3 to –37.3	B16	Tumor-bearing mice	Cancer treatment	43
pDNA (GFP, Luc, GM-CSF)	Linear PEI (25 kDa)	N/A	70–200	–48 to –40	B16	Tumor-bearing mice	Cancer treatment	45
pDNA (GFP, GM-CSF)	Linear PEI (40 kDa)	N/A	200	N/A	B16	Tumor-bearing mice	Cancer treatment	96
pDNA (GFP)	Branched PEI (25 kDa)	17 kDa	218–389	–18.9 to +27.0	MDA-MB-231, MDA-MB-435, MCF-7, COS-1	N/A	Breast cancer treatment	38
pDNA (IL-1 receptor antagonist)	Chitosan (5 kDa)	35 kDa	140–500	+15 to +35	Primary rat synoviocytes	N/A	Treatment of osteoarthritis	70
pDNA (Luc)	Chitosan	N/A	242.7–372.3	+39.4 to +57.8	Mouse NSCs	Rats	Gene delivery to the spinal cord	62
pDNA (GFP)	Chitosan (46.4 kDa)	400–1300 kDa	333.5–381.3	–39.6 to –44.6	COS-7, Huh-7	N/A	Solid-phase reverse transfection	120
pDNA (GFP)	Chitosan (50 kDa)	<10 kDa	115.6–364.8	–34.5 to +24.0	Rabbit chondrocytes	N/A	Treatment of osteoarthritis	128
pDNA (GFP, β -galactosidase)	Chitosan (10–12 kDa & 110 kDa)	170 kDa	152–199	+19 to +27	HCE, NIH/3T3	Rabbits	Ocular gene delivery	129
pDNA (GFP, β -galactosidase)	Chitosan (10–12 kDa & 170 kDa)	<10 kDa & 110 kDa	103–235	–30 to +28	HCE, IOBA-NHC	N/A	Ocular gene delivery	41
pDNA (GFP)	Chitosan (5 kDa)	17, 35 & 64 kDa	146–175	+15 to +32	HEK-293T	N/A	N/A	130
pDNA (GFP, TGF- β 1)	Chitosan (50 kDa)	160 kDa	155.4	+22.14	Rabbit chondrocytes	N/A	Cartilage tissue engineering	74
pDNA (GFP), siRNA (anti GFP, anti Snail1)	Chitosan-grafted-PEG	170 kDa	150.8–250.6	–20.1 to +31.9	HEK-293T	N/A	Cancer treatment	131
siRNA (anti STAT3)	PEI-PLA-lipoic acid	9 kDa	195	–23	4T1, MDA-MB-231	Tumor-bearing mice	Cancer treatment	132
siRNA (anti KRAS)	Chitosan (656 kDa), poly hexamethylene biguanide (3.2 kDa)	183 kDa	120–275	–34.5 to +52.0	HCT-116, HDF	N/A	Cancer treatment	34
siRNA (anti cyclophilin B)	Chitosan (35 & 656 kDa)	180 kDa	250	–40 to –35	AsPC-1, HCT-116, HDF, HT-29, HUVEC, PANC-1, THP-1	N/A	N/A	82
siRNA (anti PLK1)	PBA modified DGL	N/A	100–150	–16.9	N/A	Tumor-bearing mice	Cancer treatment	133
siRNA (anti PLK1)	Lipid nanoparticles	5 kDa	100.7	–8.2	T98G, U251, U87MG	Glioma mouse model	Treatment of glioma	77
siRNA (anti Luc)	Liposomes, protamine	N/A	114.3–117.9	+21.2 to +27.5	B16F10	Tumor-bearing mice	Cancer treatment	134
siRNA (anti cyclin D1)	Liposomes, protamine	N/A	144–161	–17.9 to –19.8	TK-1, mouse splenocytes	Mice	Intestinal inflammation, colitis	71
mRNA (Luc), siRNA (anti Luc)	Chitosan (685 kDa)	180 kDa	195–350	–44.0 to –36.0	HCT-116	N/A	N/A	48
miRNA (antimir-138)	Chitosan (100 kDa)	10 kDa	153.0–290.7	+21.5 to +45.5	Rat BMSCs	N/A	Osteogenic differentiation	72

Table 1 (Contd.)

Nanoparticle formation		Nanoparticle characteristics		Evaluation of transfection efficiency		Proposed applications	Ref.
Payloads	Cationic lipids/polymers	HA (MW)	Size (nm)	Charge (mV)	<i>In vitro</i> ^a	<i>In vivo</i>	
CRISPR/Cas9 (Ptpn2 KO)	PEI (1.8 kDa)	35 kDa	115.4	−32.9	B16F10	Tumor-bearing mice	37
CRISPR/Cas9 (Nanog KO)	Dexamethasone-loaded PDA and PEI (10, 25 kDa)	<10 kDa	200–550	+15 to +20	HeLa	N/A	135
CRISPR/Cas9 (KRAS KO)	β-Cyclodextrin conjugated PEI	N/A	200	−16.1	HEK-293T, SW-480	Tumor-bearing mice	76
CRISPR/Cas9 (APC and KRAS KO)	Phenylboronic dendrimer	N/A	220	−9.1	SW-480	Tumor-bearing mice	136

^a 4T1 (mouse breast cancer cells), AsPC-1 (human pancreas adenocarcinoma cells), B16 (murine melanoma cells), BMSCs (bone-marrow derived stem cells), COS-7 (African green monkey kidney fibroblast-like cells), HCE (human corneal epithelial cells), HCT-116 (human colorectal carcinoma cells), HDF (human dermal fibroblasts), HEK-293T (human embryonic kidney cells), HeLa (human cervical cancer cells), HT-29 (human colorectal adenocarcinoma cells), Huh-7 (human hepatocyte-derived carcinoma cells), HUVEC (human umbilical vein endothelial cells), IOBA-NHC (human conjunctiva cells), MCF-7 (human breast cancer cells), MDA-MB-231 (human breast cancer cells), MDA-MB-435 (human breast cancer cells), NIH/3T3 (murine embryonic fibroblasts), NSCs (neural stem cells), PANC-1 (human pancreatic cancer cells), SW-480 (human colon adenocarcinoma cells), T98G (human glioblastoma cells), TK-1 (human myeloid leukemia cells), THP-1 (human monocytic cells), U251 (human glioblastoma cells), U87MG (human glioblastoma cells), GFP (green fluorescent protein), GM-CSF (granulocyte macrophage-colony stimulating factor), IL-1 (interleukin-1), KO (knock out), Luc (luciferase), miRNA (microRNA), mRNA (messenger RNA), PBA (phenylboronic acid), PDA (polydopamine), pDNA (plasmid DNA), PEI (polyethyleneimine), PLA (polylactide acid), PLK1 (polo-like kinase 1), Ptpn2 (protein tyrosine phosphatase non-receptor type 2), siRNA (small interfering RNA).

statically formed HA nanoparticles. Polyamines, namely PEI, spermine and pDMAEMA (poly[2-(dimethylamino)ethyl methacrylate]), were popularly grafted onto HA to provide protonated amines for electrostatic binding to DNA or RNA. Furthermore, some studies revealed that the negative carboxyl groups of HA can loosen the electrostatic complexation between nucleic acids and cationic molecules.⁴⁹ Decreasing the negative charge density by introducing non-ionic moieties, such as PEG, was applied, and the findings showed an enhanced transfection efficiency and a longer retention time in the circulatory system.^{52–56}

Stimulus-responsive moieties were introduced to enhance the site-specific release of the entrapped genes. Cleavable disulfide or diselenide linkages with redox responsiveness were widely used, because of the different reductive environment between the extracellular space and the cytosolic compartment. The intracellular glutathione is in the range of 1–10 mM, which is much higher than the amount in the extracellular matrix (in the micromolar range).^{57,58} Redox-sensitive bonds can be directly linked to DNA or RNA,^{50,51} bonded between HA and polycations,^{59–61} or cross-linked nanoparticles,^{10,39} leading to bond cleavage after internalization and subsequent payload release.

5. Roles of HA in gene delivery systems

HA could play a vital role in nanoparticle-based formulae for gene delivery due to its anionic characteristics and inherit physicochemical properties. As illustrated in Fig. 3, the size of HA-based nanoparticles is tunable depending on the polymer chemistry and the fabrication process, as discussed previously. The advantages of HA in improving the safety of delivery systems and the stability of products and directing to target sites are discussed below.

5.1. Cell viability and transfection efficiency

In vitro. *In vitro* cytocompatibility tests confirmed enhanced cell viability after the exposure to HA-shielded nanoparticles, significantly decreasing the inherent cytotoxicity of polycations,^{38,62} or cationic lipid nanoparticles,⁶³ due to electrostatic neutralization of the HA. The effects on cell viability were varied depending on the cells, because of the unique characteristic of the cells and the different amounts of HA recognition moiety present on the cell surface (HA as a ligand of cell surface receptors will be described in the next section), which affect the internalization of HA. A study of Yin, H. *et al.*, in which the cell viability of two human breast cancer cell lines, MDA-MB-231 and MCF-7, exposed to HA nanoparticles was compared, revealed a different IC₅₀ (50% inhibitory concentration), even though both were known as highly CD44-presenting cells.⁴⁰ de la Fuente, M. *et al.* compared the cell viability of two highly CD44-expressed human corneal epithelium cell lines, HCE and IOBA-NHC, and the findings showed a lower IC₅₀ value for HCE; IOBA-NHC was discussed as secret-





Table 2 Lists of HA-based nanoparticles in gene delivery fabricated from the modified HA

Nanoparticle formation		Nanoparticle characteristics			Evaluation of transfection efficiency		Proposed applications	Ref.
Payloads	Cationic lipids/polymers	Modified HA	Size (nm)	Charge (mV)	<i>In vitro</i> ^a	<i>In vivo</i>		
pDNA (Luc)	N/A	PEI or PEG conjugated HA	200–400	–15 to –30	A549, HeLa	N/A	N/A	53
pDNA (IL-4 and IL-10)	N/A	PEI conjugated HA	185.9	–11.6	J774A.1	Mice	Macrophage repolarization	73
pDNA (GFP, endostatin)	N/A	PEI conjugated HA with cleavable disulfide bonds	82.26–154.80	+32.3 to +70.3	Human MSCs	N/A	Chondrogenic differentiation	59
pDNA (Luc, GFP, p53)	N/A	OEI and β -cyclodextrin conjugated HA	100–150	+25 to +40	MDA-MB-231, MCF-7	N/A	Cancer treatment	40
pDNA (Luc)	N/A	Spermine conjugated HA	200–300	–6.1	CHO	Mice	N/A	65
siRNA (anti MDR1)	N/A	PEI or PEG conjugated HA	173.3	–22.5	OVCAR-8	Tumor-bearing mice	Treatment of ovarian cancer	56
siRNA (anti survivin)	N/A	PEI or PEG conjugated HA	90–100	–15	A549, H69	Tumor-bearing mice	N/A	52
siRNA (anti Luc & anti VEGF)	N/A	PEI conjugated HA	42.7	N/A	B16F1	Tumor-bearing mice	Cancer treatment	64
siRNA (anti Luc)	N/A	PEI conjugated HA	21	–21.5 to –15.3	B16F1, HEK-293	N/A	N/A	122
siRNA (anti VEGF)	N/A	PEI conjugated HA with cleavable disulfide bonds	110	+1.8 to +12.1	B16F1	Tumor-bearing mice	Cancer treatment	60
siRNA (anti PLK1)	N/A	Polyamine (PEI, spermine) conjugated HA	85–190	–5.5 to +16.5	A549, H69, MDA-MB-468, Hep3B, B16F10	Tumor-bearing mice	Cancer treatment	54
siRNA (anti COX-2)	N/A	Alkyl chain and spermine conjugated HA	200–500	N/A	SGC-7901, GES-1	N/A	Treatment of gastric cancer	137
siRNA	N/A	Alkyl chain and spermine conjugated HA	125.6–555.1	+18.4 to +21.3	N/A	N/A	N/A	138
siRNA (anti RFP)	N/A	pDMAEMA and PDA conjugated HA	314.4	–6.02	B16F10, CV-1, NIH/3T3	Tumor-bearing mice	Cancer treatment	10
siRNA (anti TNF- α)	PBAE	Dopamine-grafted HA	150	<0	RAW 264.7, NIH/3T3	Inflammation-stimulated mice	Treatment of acute lung injury	46
pDNA (GFP, Luc)	Branched PEI (25 kDa)	HA with cleavable disulfide bonds	200	–23.4 to +5.0	HepG2, B16F10, NIH/3T3	Tumor-bearing mice	Cancer treatment	39
siRNA (anti GFP)	N/A	HA with cleavable disulfide bonds	198	N/A	HCT-116, NIH/3T3	N/A	N/A	50
siRNA (anti KRAS)	PAMAM	HA with cleavable disulfide bonds	181	–24.6	A549	Tumor-bearing mice	Cancer treatment	61
Antisense oligonucleotide (anti GFP)	Protamine	HA with cleavable disulfide bonds	166–200	N/A	HEK-293	N/A	N/A	51
siRNA (anti RFP)	2b RNA-binding protein	Cholesterol conjugated HA	190–410	–64.3 to –45.1	B16F10, HFF	N/A	Cancer treatment	42
siRNA (anti survivin)	Azide-modified liposomes with cleavable disulfide bonds	Alkyne functionalized HA	130	–26	A549	Tumor-bearing mice	Cancer treatment	36
pDNA (BMP-2), miRNA (mir148b)	Calcium phosphate	Catechol (dopamine) functionalized HA	71	–12	Human MSCs	N/A	Osteogenic differentiation	139
siRNA (anti Luc)	Calcium phosphate	Catechol functionalized HA	63–278	–20 to –70	HT29	Tumor-bearing mice	Cancer treatment	44
siRNA (PD-L1, STAT3)	Chitosan (modified with trimethyl and thiol groups)	TAT conjugated HA	110	+20	B16F10, 4T1	Tumor-bearing mice	Cancer treatment	140

Table 2 (Contd.)

Nanoparticle formation		Nanoparticle characteristics		Evaluation of transfection efficiency			Proposed applications	Ref.
Payloads	Cationic lipids/polymers	Modified HA	Size (nm)	Charge (mV)	<i>In vitro</i> ^a	<i>In vivo</i>		
pDNA (IL-12) CRISPR/Cas9 (CD47 KO) CRISPR/Cas9 (PPM1D KO)	Fluorinated PEI Histone, KALA peptide	TMSP conjugated HA AS1411 conjugated HA	220–250 134–177	–19.7 to –9.06 –18.2 to +23.5	B16F10 H1299, MCF-7, MCF-10A	Tumor-bearing mice N/A	Cancer immunotherapy Cancer treatment	141 79
CRISPR/Cas9 (β-catenin KO)	CaCO ₃ , protamine	TAT and AS1411 conjugated HA	230–320	–20 to –10	H1299, HeLa, HEK-293T	N/A	Cancer treatment	78
CRISPR/Cas9 (GFP KO)	N/A	PEI and/or mannose conjugated HA	115–118	–21.2 to –17.9	AML2	Mice	Liver targeted delivery	142

^a 4T1 (murine breast cancer cells), A549 (human non-small-cell lung cancer cells), AML2 (mouse hepatocyte cells), B16 (murine melanoma cells), CHO (Chinese hamster ovary cells), CV-1 (green monkey kidney cells), GES-1 (human gastric epithelial cells), H1299 (human non-small cell lung carcinoma cells), H69 (human small-cell lung cancer cells), HCT-116 (human colorectal carcinoma cells), HEK-293 (human embryonic kidney cells), Hep3B (human hepatoma cells), HeLa (human cervical cancer cells), HepG2 (human liver cancer cells), HFF (human foreskin fibroblasts), HT-29 (human colorectal adenocarcinoma cells), J774A.1 (murine monocytes), MCF-7 (human breast cancer cells), MCF-10A (human breast epithelial cells), MDA-MB-231 (human breast cancer cells), MDA-MB-468 (human breast cancer cells), MSCs (mesenchymal stem cells), NIH/3T3 (murine embryonic fibroblasts), OVCAR-8 (human ovarian cancer cells), RAW 264.7 (mouse macrophage cells), SGC-7901 (human gastric cancer cells). Abbreviations: AS1411 (anti-nucleolin aptamer), BMP-2 (bone morphogenetic protein 2), COX-2 (cyclooxygenase-2), GFP (green fluorescent protein), KO (knock out), miRNA (microRNA), MDR1 (multidrug resistance 1 gene), OEI (oligoethyleneimine), p53 (tumor protein 53), PAMAM (poly(amidoamine)), PBAAE (poly (β-amino ester)), PDA (2-(2-pyridyl-dithioethylamine), pDMAEMA (poly[2-(dimethylamino)ethyl methacrylate]), PPM1D (protein phosphatase, Mg²⁺/Mn²⁺ dependent 1D), RFP (red fluorescent protein), TAT (trans-activator of transcription), TMSP (tumor microenvironment-sensitive peptide), VEGF (vascular endothelial growth factor).

ing viscous mucin and lowering the accessibility of nanoparticles to the cell surface.⁴¹ However, the cytotoxicity dose in most studies was much higher than the transfection dose, indicating the *in vitro* safety of HA-based nanoparticles and the possibility of *in vivo* tests.

Higher transfection efficiency has been reported in cultured cells when the gene complexes were shielded with HA.^{39,53,64,65} Anionic shielding of the polyplexes can prevent particle aggregation or coagulation with serum proteins.^{39,43,64} Therefore, the size of the particle is uniform and suitable for uptake by cells. Indeed, the negative charge of HA can neutralize the positive charge of cationic polymers and loosen the complexes. So, the release of loaded genes from endosomes increases after internalization into the cytosol.⁶⁵ Furthermore, the uptake of HA-coated nanoparticles is mediated by cell surface receptors, such as CD44, of which the transfection efficiency depends on the amount of expression and the activation state of the receptors.^{38,66} High expression of CD44 relates to cell pathophysiology, such as tumor progression, infection, or inflammation, and HA has been used as a ligand for targeted treatment.⁶⁷ Therefore, HA-based nanoparticles have been used for broad applications, including cancer treatment,^{68,69} anti-inflammatory,^{70,71} tissue regeneration,^{59,72–74} and vaccine formulation.⁷⁵ Target cells with high expression of CD44 are efficiently transduced from receptor-mediated internalization of HA nanoparticles.

In vivo. Before translation to clinical trials and real-world applications of HA-based nanoparticles for gene delivery, *in vivo* results are needed to confirm the safety of the systems, even though they are considered as a safe cargo as previously discussed. Further results are needed because not only can biodistribution after administration affect the pharmacokinetics of payloads, but the off-target accumulation can also lead to an undesirable transgene expression in non-target tissues. Several studies related to *in vivo* applications of HA-based nanoparticles were aimed at tumor-targeting delivery, due to the high expression of CD44 in tumor cells (reviewed in the next section). Most studies confirmed the selectivity of the nanoparticles for tumors, leading to an efficient tumor treatment.^{10,36,37,52,64} An accumulation in major organs was also observed, including the lungs, spleen, kidneys, and liver, due to a high expression of HA receptors, hyaluronic acid receptor for endocytosis (HARE) and lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE-1).^{52,55,64} The study by Ganesh, S. *et al.* has raised a flag for an unusual accumulation of siRNA-loaded PEG or PEI-decorated HA nanoparticles in the heart within 24 h after intravenous administration, which was as high as those in tumor tissues, leading to possible cardiotoxicity. The authors discussed that the biodistribution behavior of HA-based nanoparticles was size dependent, where the smaller nanoparticles (approx. 150 nm) preferentially accumulated in the tumor and heart, and the larger particles (*ca.* 500 nm) accumulated in the liver, spleen, kidneys, and lungs.⁵² Consequently, pharmacokinetic studies of the developed nanoparticles are highly recommended for the prediction of potential toxicities for *in vivo* applications.





Fig. 3 The advantages of HA in nanoparticle-based gene delivery systems: (a) the size of the nanoparticles is adjustable from 50 to 500 nm based on the physicochemical properties of the HA used in the formulations or the fabrication process. (b) The anionic characteristic of HA can reduce the cytotoxicity and non-specific interactions with serum proteins. (c) HA possesses innate properties in the recognition of cell surface receptors, e.g., CD168 or RHAMM (receptor for hyaluronan-mediated motility), CD44, HARE (hyaluronic acid receptor for endocytosis), and LYVE-1 (lymphatic vessel endothelial hyaluronin acid receptor 1). (d) The carboxyl (–COOH) and hydroxyl (–OH) groups can be chemically conjugated with functional moieties, such as cationic polymers, targeting ligands, or molecules enhancing the properties of nanoparticles (e.g., stimulus-responsiveness), and (e) HA-based nanoparticle formulations can be lyophilized, by which the shelf-life of the products is extended without damaging the efficacy of the systems.

An addition of HA to the gene complexes with cationic polymers or lipids improved the *in vivo* transduction due to the anionic characteristics and receptor recognition of HA. Nanoparticles with a negative surface charge show no coagulation with proteins present in the blood or extracellular spaces. Thus, the size and shape of the nanoparticles are maintained as prepared, resulting in predictable distribution kinetics.^{43,52} The negative charge surface can also reduce early elimination by the complement system or capture by innate immune cells. Therefore, the nanoparticles can circulate in the bloodstream for a longer period, improving the transgene expression of the target cells.^{36,76} Furthermore, HA can guide the nanoparticles to cells with a high expression of CD44 and mediate endocytosis through the receptors.^{73,77} A conjugation of extra targeting ligands to HA can reduce the delivery to non-

target CD44-overexpressed cells, leading to high accumulation, and transduction activity of the target cells.^{37,71,78,79}

5.2. Targetability of HA-based nanoparticles

Cell surface receptors and HA recognition. The intrinsic properties of HA as ligands for various cell surface receptors, namely RHAMM (hyaluronan-mediated motility-expression protein receptor) or CD168, HARE, LYVE-1, and CD44, lead to the targetability of HA-based delivery systems at target sites with a high expression of these receptors.⁸⁰ CD44, the glycoprotein expressed on the surface of various types of cell, is a widely recognized receptor of HA, which has multiple physiological functions for wide biomedical applications.⁸¹ CD44 is even expressed in many cell types, and some diseased cells, e.g., tumors and viral infected cells, possess higher affinity of



CD44 for HA. A targeted cancer or viral infection therapy using HA-based nanoparticles, therefore, could be a promising gene delivery system.⁶⁷ Tirella, A. *et al.* compared the internalization of siRNA-entrapped chitosan-HA nanoparticles in two CD44-positive cells, normal human dermal fibroblasts (HDFa) and human colorectal carcinoma cells (HCT116), and the result showed a selective uptake of HCT116 over HDFa in co-culture conditions.³⁴ The expression of CD44 isoforms and a disordered receptor structure due to mutation of the HA binding domain are discussed to enhance the selectivity, binding and internalization of HA nanoparticles to cancer cells.^{66,82,83}

Expression of HA receptors in immune cells. As mentioned above, HA is a major component presenting in extracellular matrices, and most cells, including immune cells, express HA-binding CD44 surface receptors that relate to physiological functions maintaining their homeostasis. Under normal conditions, immune cells display low recognition of HA.⁸⁴ Upon an infection or inflammation, increased expression of CD44 of immune cells is noticed,^{84–86} enhancing the efficiency of HA binding, which relates to supportive functions of HA in immune responses, including guiding and homing immune cells to the defect tissues and promoting tissue regeneration.^{13,80} Focusing on T cells, the expression of HA-binding CD44 increases transiently upon a T cell activation and proliferation of T cells, confirmed by an *in vitro* induction using chemicals, *e.g.*, phorbol 12-myristate 13-acetate (PMA) or ionomycin,⁸⁴ CD3 monoclonal antibodies,⁸⁵ or inflammatory cytokines (IL-7 or IL-15).⁸⁴ The activation of CD44 after inflammation exhibits the role of CD44 in guiding T lymphocytes to inflammation sites, in which the extracellular matrix contains a substantial amount of HA.⁸⁷ CD44/HA binding can activate T cell responses as a co-stimulatory signal to specific antigens.^{88,89} Indeed, the HA binding capacity to CD44 of T lymphocytes was enhanced after antigen activation or exposure to pro-inflammatory cytokines or chemokines, and the activated state of CD44 was as early as 2 to 3 h and extended for up to 48 to 72 h.^{85,90} The interesting point of this study was that even though lymphocytes themselves highly express CD44, the receptors in the native state receptors are less efficient in mediating primary adhesion to HA, unless they are activated by chemicals or the specific antigens.⁸⁵ *In vivo* testing using pathogenic or immunized animal models revealed that activated T cells after antigen presentation possessed a transiently elevated HA-recognizing CD44, of which the expression returned to normal a few days after antigen exposure.^{84,86} These phenomena could allow the design of T cell targeting systems by conjugating the HA-based nanoparticles with guide antibodies or incorporating an adjuvant for T cell induction to elicit high CD44 expression as well as enhance the cellular uptake of the nanoparticles.

A major concern is that the upregulation of CD44 is not only limited to immune cells during the inflammation state, but elevated expression of CD44 is also evidenced in various types of tumor cell, including acute lymphoblastic leukemia, acute myeloid leukemia, and multiple myeloma,^{91,92} which are the main targets of CAR T cell treatment. HA, as a CD44

ligand, has been used as a component in the targeting of cancer delivery systems since HA can detect tumor cells with a high expression of CD44 receptors in an activated state, before the cells internalize them to deliver chemotherapeutic agents with high specificity.^{68,69} Therefore, the development of HA-based delivery systems to target circulating T cells based only on the inherent CD44 binding properties of HA could not be used and could result in undesirable transduction of tumor cells. Indeed, a modification of HA for a higher specificity for T cells could be required, in which the chemical structure of HA is feasible for chemical conjugation of the targeting ligands.

Targeting ligand conjugation of HA. In addition to the inherent targeting properties of HA to the recognized domains on the cell surfaces, the carboxyl groups of glucuronic acid units in HA allow covalent conjugation with amine groups using a simple carbodiimide chemistry for an additional targeting ligand. For instance, β 7 integrin monoclonal antibody was covalently introduced into HA-coated liposomes to selectively bind to circulatory lymphocytes to deliver siRNA for cyclin D1.⁷¹ The AS1411 aptamer, an antibody to nucleolin which is highly expressed in tumor cells, was conjugated to HA to promote tumor targetability using the bi-functionalization approach and lower the undesirable uptake by nontarget CD44⁺ cells.^{78,79} Modification of the HA carboxyl groups of HA was reported to reduce the recognition of HA binding domains.⁶⁴ Hence, the introduction of cell-specific ligands by a carbodiimide coupling reaction to HA could be a strategy to reduce an off-target binding and enhance cellular uptake, resulting in a high accumulation dose at the target tissues and potentially increased transfection efficiency.

5.3. HA as stability enhancers and cryoprotectants

Rapid clearance after a bolus injection of naked plasmid DNA or oligonucleotides was reported *in vivo*, due to the presence of circulatory nucleases as a biological barrier to prevent exogenous genetic materials,^{93,94} resulting in transfection failure unless the effective viral or non-viral vectors were applied. For non-viral vectors, the *in vitro* studies confirmed that complexation with polycations or cationic lipid nanoparticles significantly enhances stability against nucleases,^{36,44,71,95} which can be implied for *in vivo* applications.¹⁰ The addition of HA showed a greater protection effect against enzyme degradation, resulting in a higher transfection efficiency over polycations and nucleotide complexes.^{44,50} The stability of HA-coated nanoparticles, determined by physical characteristics, *e.g.*, size, charge, or morphology, or transfection efficiency, was preserved at 4 °C for at least one week in a solution state,^{34,53} and for a longer period after lyophilization.^{45,74,96} From the work of Ito, T. *et al.*,^{45,96} HA can act as a cryoprotectant, allowing the nanoparticles to be lyophilized. The reconstituted nanoparticles performed a transfection efficiency similar to that of the freshly prepared nanoparticles. Hence, the HA-shielded nanoparticles can possess a long shelf-life in a lyophilized form, enabling a practical use in the clinical setting.



To date, most registered CAR T cell clinical trials have focused on blood tumors, with few studies focusing on the treatment of solid tumors.²⁴ Since solid tumors are typically protected by a local immunosuppression environment and showed a heterogeneity of surface receptors, adoptive CAR T cells are less effective in complete elimination of the target

almost comparable to normal anatomy and physiological conditions.¹¹⁴

7. Challenges and future perspectives

The major concern of the utilization of HA-based nanoparticles in *in vivo* engineered CAR T cells is the retention time in the bloodstream after IV administration to allow a sufficient transfection period for the circulated T cells. Since a rapid clearance from the circulatory system was reported in the first few hours, the nanoparticles subsequently accumulated in tumor tissues and major organs, such as the lungs, spleen, liver, and kidneys.⁵² Furthermore, macrophages and leukocytes are known as CD44-overexpressing cells^{55,73,80} in which HA nanoparticles presented in the blood can be phagocytosed and rapidly eliminated through reticuloendothelial systems. An approach to increase the retention time is the PEGylation of nanoparticles to acquire stealth properties for higher systemic distribution and lower clearance.^{52,55} PEG can be simply mixed with HA for shielding nanoparticles⁵⁵ or conjugated to the HA backbone together with polyamine conjugation.^{52,53,56} Therefore, an extended systemic distribution can increase the exposure period to T cells, resulting in a greater chance of delivering entrapped genetic materials to engineer the targeted T lymphocytes.

With direct administration to the bloodstream, the nanoparticles undergo a rapid elimination, resulting in an insufficient retention time and a low transfection efficiency. Gene-containing nanoparticles can be immobilized to the solid substrate, and the gene-containing prosthesis can be implanted. Hence, circulating cells can be latched onto substrates and the cell can internalize the embedded gene cargos. This approach is called ‘solid phase reverse transfection’, which has been widely applied in *in vitro* assay platforms^{116,117} and tissue-engineered scaffolds.¹¹⁸ From a proof-of-concept study by Agarwalla, P. and colleagues using modified alginate scaffolds loaded with retroviral vectors containing CD19 CAR-encoding genes, *in vitro* results demonstrated an equivalent amount of CAR transduced cells compared with the conventional approach as well as a

The immobilization of the gene-delivery platform could not only reduce the clearance from the circulatory system compared with injectable nanoparticles, but the transfection efficiency was also found to be higher than that of the conventional transfection method due to higher efficiency in the nuclear uptake of loaded genes.¹²⁰ HA-coated pDNA polyplexes were grafted to the surface of metallic implants¹²¹ or embedded in resorbable scaffolds,⁷⁴ and it was found that a higher transfection efficiency can be achieved. HA can reduce the cytotoxicity of polyplexes and prevent aggregation when exposed to serum proteins. Indeed, coating the pDNA complexes on a substrate facilitated the cells to be exposed to pDNA at a higher concentration and for a longer period than an exposure to the pDNA in the solution since low cell transduction has been reported to be improved by a prolonged exposure time of cells to gene complexes.¹²² Furthermore, embedding transfection nanoparticles in biodegradable matrices could allow a sustained release of the genetic materials, avoiding a repeated administration, for extended transgene expression.⁷⁴

The clinical translation of CAR loaded nanoparticles could take a long time, as they are needed to fulfill the considerations of both nanomaterials and products containing genetic materials, which are classified as advanced therapy medicinal products, or ATMPs. In addition to confirming proposed indications of the products, including preclinical proof-of-concept efficacy, a dose-response relationship, and a level of transgene expression, as well as a controlled quality of product manufacturing,¹²³ toxicities, including infusion reactions, immune-mediated responses, and genotoxicity, which could be transient or persistent, are of great concern. In an aspect of nanomaterial considerations, *in vitro* and *in vivo* models are required to evaluate immune compatibility after administration to confirm the feasibility of the products, such as hemocompatibility tests, complement activation, oxidative stress to T cells, and the suppression of immune cells or bone marrow,^{109,124} which can refer to protocols developed by the Nanotechnology Characterization Laboratory (NCL) (reviewed in ref. 125 and 126). Furthermore, genome alteration due to the nuclear internalization of exogenous genetic materials mediated by viral or non-viral vectors should be addressed, and preclinical tests are required to confirm genotoxicity, mutagenicity, and sustained genetic modification, which could affect reproductive systems or the development of descendants.^{123,127} Hence, researchers working on the development of delivery systems for CAR constructs should consider not only proving the efficacy but also ensuring the biocompatibility of the systems, which could pave the way to accelerate the clinical translation of the developed systems.

In vivo CAR T cell engineering showed equivalent efficacy to ACT in a preclinical evaluation for the treatment of hematologic malignancies. Lentiviral vectors decorated with T cell targeting ligands were developed to abrogate their broad tropism and avoid off-target delivery of the gene cargo. However, a risk of insertional mutagenesis, a relapse of pathogenic variants, and persistent genomic alteration could raise awareness and delay the clinical translation of viral vectors. Non-viral, polymeric nanoparticles offer a safer alternative. Cationic polymers, such as PEI, PBAE, chitosan, or lipids, are used to condense DNA or RNA and facilitate the release of payloads from the endosomes to the cytosolic compartment. Typically, the complexes are electrostatically coated or chemically conjugated with anionic polymers to reduce a non-specific binding to the cell surface or serum proteins, as well as enhance the biocompatibility of the systems. To date, only CAR-loaded nanoparticles using PBAE and coated with antibody-conjugated PGA have been introduced for *in vivo* T cell engineering. On the basis of the same approach, HA can also be used for the fabrication of nanoparticles targeting T cells, due to its negative charge, which enables counterion conjugation to the cationic core polymers. The available carboxyl groups of HA allow for polymer functionalization and a chemical bonding to the targeting antibodies of T cells. More importantly, the inherent properties of HA as a ligand of cell surface receptors, such as CD44, could improve the targetability to T cells, due to elevated HA-binding CD44 receptors of T cells in correspondence to immunological activation. Hence, HA-based nanoparticles demonstrate promising platforms for the delivery of CAR constructs specifically to circulating T cells for the *in vivo* programming of CAR T cell therapy.

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