Minireview

Targeting and therapeutic peptides in nanomedicine for atherosclerosis

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Abstract

Peptides in atherosclerosis nanomedicine provide structural, targeting, and therapeutic functionality and can assist in overcoming delivery barriers of traditional pharmaceuticals. Moreover, their inherent biocompatibility and biodegradability make them especially attractive as materials intended for use *in vivo*. In this review, an overview of nanoparticle-associated targeting and therapeutic peptides for atherosclerosis is provided, including peptides designed for cellular targets such as endothelial cells, monocytes, and macrophages as well as for plaque components such as collagen and fibrin. An emphasis is placed on recent advances in multimodal strategies and a discussion on current challenges and barriers for clinical applicability is presented.

Keywords: Peptides, nanoparticles, atherosclerosis, micelles, inflammation, biomaterial

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Introduction

Cardiovascular diseases are the leading causes of death globally, and atherosclerosis is a major contributor.[1] Atherosclerosis is a chronic disease and a specific type of arteriosclerosis, or a condition when arteries become thick and stiff. Atherosclerosis is characterized by a dysregulated lipid metabolism and plaque build-up. Ultimately, the expansion narrows and hardens the arterial wall and can restrict blood flow to the rest of the body.[2,3] If a plaque ruptures, this can trigger a blood clot which can stop blood flow and lead to lethal events such as myocardial infarctions and heart failure, and ischemic stroke in arteries located within the brain.[4,5] In addition to the accumulation of lipids, atherosclerosis is considered to be an inflammatory disease with considerable immunological impact as well.[6,7]

The initial step of atherosclerosis onset and progression is the subendothelial retention of low-density lipoprotein (LDL) cholesterol particles and endothelial cell dysfunction, as well as leukocyte adhesion and platelet activation. Endothelial cells express cytokines/chemokines (i.e. monocyte chemotactic protein 1 (MCP-1)) and adhesion molecules (i.e. intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (VCAM-1), as well as E-selectin and P-selectin), resulting in monocyte maturation into macrophages and T-cell recruitment.

In addition to the accumulation of macrophages, smooth muscle cells (SMCs) proliferate, migrate, and synthesize matrix proteins including collagen, contributing to the fibrous cap surrounding the plaque. Plaque progression via lipid accumulation, neovascularization, and SMC, and macrophage apoptosis can continue over a period of several years or decades. When plaques rupture, proteinases released from apoptotic cells degrade the fibrous cap, exposing the highly procoagulant lipid core, and triggering thrombosis.[8] Due to the multifactorial and chronic nature of atherosclerosis, plaques most prone to rupture, called vulnerable plaques, are often discovered only after a major clinical event. This presents an opportunity for nanomedicine to intervene in advancing the current state of atherosclerosis diagnosis and treatment.

Nanomedicine

Nanomedicine is the ''application of nanotechnology for treatment, diagnosis, monitoring, and control of biological systems.''[9] Nanoparticles are typically 1–100 nm in size and can be made of various materials (i.e. biomacromolecules, thermoplastic polymers, metals, etc.) with a range of physiochemical properties (i.e. shape, charge, stability, etc.) that can be optimized to dictate their behavior *in vivo* (i.e. circulation half-life, biodistribution, clearance, etc). One notable feature of nanoparticles is their high surface-to-volume ratio which allows the surface to be exploited for avidity and efficient homing and/or multifunctionality. Initially, nanoparticles were explored as drug delivery carriers for cancer applications and were found to enhance the drug's bioavailability, while limiting adverse side effects.[10] This avenue of research continues to be a major venture in nanomedical research.

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In addition to their therapeutic use, nanoparticles can be combined with imaging agents (i.e. fluorophores, chelated ions, metals, etc.) for diagnostic imaging. Moreover, by attaching antibodies, sugars, peptides or other ligands, a nanoparticle can home to particular cell types or structures within an atherosclerotic plaque.

Peptides in nanomedicine

Peptides are instrumental in directing the use of nanoparticles for medicine because biological specificity, therapeutic function, as well as structural properties can be incorporated through rational design. Moreover, their inherent biocompatibility and biodegradability provide additional advantages as biomaterials. The combination of peptides and nanoparticles provides a powerful strategy in overcoming delivery barriers of many traditional pharmaceuticals because unlike antibodies, peptides are small enough to fit into a shallow or hydrophobic binding pocket without compromising specificity or affinity. Antibodies are approximately 10×15 nm and on the order of many nanoparticles, making it difficult to attach multiple molecules onto a single nanoparticle which is typically ideal to maximize function.[11] As a result, a wide collection of peptides of varying lengths and amino acid sequences have been reported for several biomedical applications, including diagnosis and therapy for diseases such as atherosclerosis.

Peptides are defined as molecules that consist of 2–50 amino acids joined by peptide bonds. Unlike proteins, peptides that do not normally exist in nature can be synthesized in the laboratory using standard methods such as liquid- or solid-phase synthesis. Through variations in the amino acid sequence, or the primary structure, chemical design versatility is achieved, leading to diverse secondary, tertiary, and quaternary structures via folding and hydrogen bonding. One example is the emerging beta-sheet forming peptides that use intermolecular hydrogen bonding for nanostructure assembly.[12]

In addition to peptides as structural building blocks of the nanoparticles themselves, in general, nanomedicine makes use of peptides in several ways:[11]

1. to target nanoparticles which carry drugs or diagnostic agents into diseased tissues and cells. Peptides used in this category are often based on ligands of receptors or markers of cells or structures including clots and neovasculature. In addition, random peptide libraries selected based on interaction with specific epitopes that are discovered via phage display are also quite common;
2. to act as the therapeutic entity upon conjugation to nanoparticles. Peptides used in this category have therapeutic function themselves.

These two categories of nanoparticle-associated peptides in the context of atherosclerosis will be the focus of this review. For a more detailed overview of nanocarriers for therapeutic, diagnostic, and theranostic applications, we refer the reader to additional reviews.[5,13–22] An overview of recent advances and future applications is provided, as well as a discussion on the current challenges that present barriers for clinical applicability. Particular attention is given to multimodal strategies.

Peptides for nanoparticles in atherosclerosis

Differences in cell types and tissue between normal and diseased states are at the core of therapeutic and diagnostic strategies for atherosclerosis. By targeting unique markers of atherosclerosis, nanomedicine has the potential to enhance the delivery and activity of therapeutic and diagnostic agents, while minimizing toxicity and harmful side effects to patients. To date, a variety of targeting peptides for atherosclerosis have been tested preclinically, including those that target cells such as endothelial cells, monocytes, macrophages, SMCs, and platelets, as well as characteristics of plaques including collagen and fibrin (Figure 1).[5,23,24] Although of less frequency, therapeutic peptides have also been proposed for atherosclerosis nanomedicine, intended for use in early-to-late stages of the disease.

Endothelial cells

Targeting peptides for endothelial cells

Vascular cell adhesion molecule-1. The majority of peptides used in atherosclerosis nanomedicine target inflamed endothelial cells and utilize peptides that specifically target endothelial cell adhesion molecules, namely VCAM-1. A wide range of nanoparticles have used the VHPKQHR (VHP) motif that has homology to very late

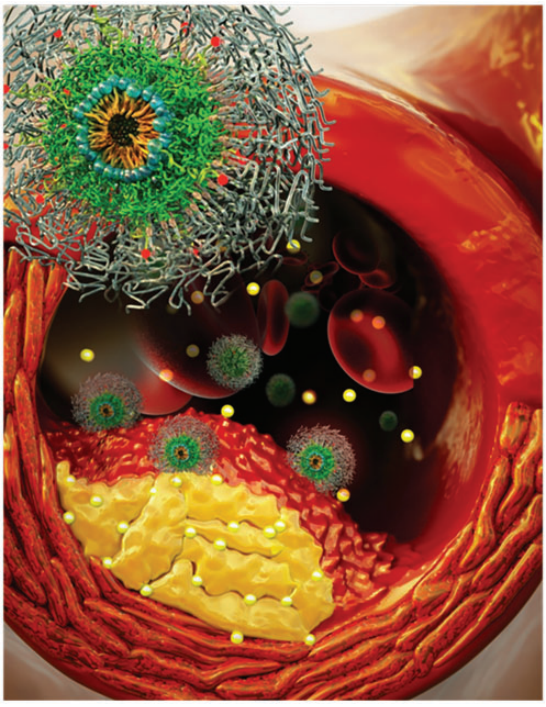

Figure 1 An example of peptides used in atherosclerosis nanomedicine. Schematic of peptide amphiphile micelles (PAMs) targeting plaque components adapted from Chung et al.[25] (A color version of this figure is available in the online journal.)

antigen-4 (VLA-4), a known ligand of VCAM-1 (Table 1).[26,27] In particular, by combining VHP-nanoparticles based on various structures and materials including dendrimers, micelles, liposomes, iron oxide, and perfluorocarbon, a variety of diagnostic agents have been reported for fluorescence and magnetic resonance imaging (MRI).[28–33]

Notably, recent investigations report on VHP-modified nanoparticles that deliver micro RNA (miR) inhibitors for targeted, drug delivery applications. Kheirolomoom et al.[34] used the shortened version of the peptide, VHPK, to coat cationic lipoparticles (CCL) containing anti-miR-712 within its core. miR-712 is a pro-atherogenic, mechanosensitive miRNA that is upregulated by distributed flow in endothelial cells. VHPK–CCLs containing miR-712 inhibitors downregulated the miR and rescued the expression of its target genes, preventing atheroma formation in Apo $E^{-/-}$ mice. Through incorporation within endothelial cell-targeting nanoparticles, 80% lower dose of the miR inhibitor achieved the same therapeutic effect compared to the naked anti-miR-712. Moreover, off-target expression of miR-712 was unchanged. In addition, Kuo et al. developed VHP-polyelectrolyte complex micelles (PCM) that formed via electrostatic complexation of polyethylene glycol (PEG)-lysine30 and nucleic acid inhibitors of miR-92.[29] miR-92 has been demonstrated to contribute to the inflamed endothelial phenotype, and when its inhibitors were combined within VHP-PCM micelles, fluorescently-labeled nanoparticles accumulated in human aortic endothelial cells (HAECs), while upregulating the anti-inflammatory transcription factor Kruppel-like factor 2 (KLF-2) *in vitro* (Figure 2).[53] The authors also incorporated the fibrin-specific peptide (REKA) and inhibitors of miR-33a to enhance macrophage reverse cholesterol transport (RCT).[54] This system provides an example of a multi-targeting nanoparticle system with imaging and therapeutic functionalities.

Besides the VHP peptide, additional VCAM-1 targeting peptides have been used for atherosclerosis nanomedicine. Kelly et al.[35] developed monocrystalline iron oxide nanoparticles with VHSPNKK, a peptide found through *in vivo* phage display that also has homology to VLA-4. Notably, compared to the monoclonal antibody, the peptide had a 12-fold higher target-to-background ratio. Moreover, the peptide displayed preferential affinity to VCAM-1 expression on inflamed endothelial cells versus on macrophages. *In vivo*, VHSPNKK-modified magnetofluorescent nanoparticles accumulated in aortic plaques detected via MRI and fluorescence imaging, and colocalized with VCAM-1 expression in Apo $E^{-/-}$ mice. In another study, Michalska et al. conjugated cyclic CNNSKSHTC to ultrasmall superparamagnetic iron oxide particles (USPIO) and demonstrated its *in vivo* capability to recognize VCAM-1 in early and advanced atherosclerotic plaques by MRI in the Apo $E^{-/-}$ mouse. In addition to endothelial cells, the targeted nanoparticles also colocalized with VCAM-1 expressing macrophages and SMCs, confirming targeting potential to both the plaque and intima.[36]

Other peptides. Like VCAM-1, through incorporating peptides that home to stabilin-2 and interleukin-4 (IL-4), multiple cell types have been shown to be targeted concurrently. Park et al.[37] recently decorated hydrophobically modified glycol chitosan nanoparticles

Table 1 Targeting and therapeutic peptides used in atherosclerosis nanomedicine

	Target	Target or name	Peptide sequence	Reference
Targeting peptides	Endothelial cell (EC)	VCAM-1	VHPKQHR	24,26–34
			CVHSPNKKC	35
			CNNSKSHTC	36
	EC, macrophage, SMC	IL-4 receptor	CRKRLDRNC	37
		Stabilin	CRTLTVRKC	38
	Monocyte	CCR2	YNFTNRKISVQRLASYRRITSSK	25
		CCR5	ASTTTNYT	39
	Macrophage	Lyp-1	CGNKRTRGC	40
		Apo A-I mimetic	CGVLESFKASFLSALEEWTKKLQ	41,42
		Apo A-I mimetic (18A)	DWLKAFYDKVAEKLKEAF	43
		Apo A-I mimetic (37pA)	DWLKAFYDKVAEKLKEAFPDWLKAFYDKVAEKLKEAF	43
		Apo A-I mimetic (4F)	FAEKFKEAVKDYFAKFWD	44
		Apo E	LRKLRKRLLRLRKLRKRLLR	45
	Platelet	Integrin GPIIb-IIIa	RGD	23
	Collagen	Type I	GKWH(CTTKFPHHYC)	46
		Type IV	KLWVLPK	47,48
	Fibrin		CREKA	49–51
			RWQPCPAESWT-Cha-CWDP	52
Therapeutic peptides	Myeloid	ALX/FPR2	AMVSEFLKQAWFIENEEQEYVQTVK	29,47,48
	Thrombin		FPRPGGGGNGDFEEIPEEYL	49

EC: endothelial cell; VCAM-1: vascular cell adhesion molecule-1; SMCs: smooth muscle cells; CCR2: C–C chemokine receptor type 2; CCR5: C–C chemokine receptor type 5; APO A-I: Apolipoprotein A-I; RGD: Rat Genome Database.

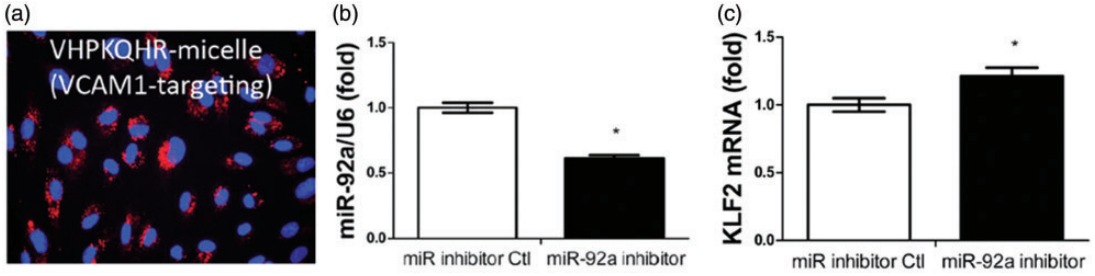

Figure 2 VCAM-1-targeting polyelectrolyte complex micelles (PCM). PCMs carrying the mir-92a inhibitor (a) bind to HAECs with specificity, (b) downregulate the miR, and (c) upregulate KLF-2 *in vitro*. Adapted from Ref. 29 with permission from The Royal Society of Chemistry. (A color version of this figure is available in the online journal.)

with CRKRLDRNC, a peptide discovered via phage display screening that selectively binds to the IL-4 receptor on endothelial cells, macrophages, and SMCs. Such was also the case with CRTLTVRKC, a peptide that binds to stabilin-2, a membrane phosphatidylserine (PS) receptor involved in the engulfment of apoptotic cells.[38]

Monocytes and macrophages

Targeting peptides for monocytes and macrophages. Monocytes and macrophages are early cell recruits to lesions making them appropriate targets for the beginning stages of the disease. However, these cell populations have also been shown to differentially accumulate in vulnerable plaques which provides an opportunity for multi-stage targeting. In the Apo $E^{-/-}$ mouse, monocyte accumulation increases with the onset of the disease,[55] and in humans, rupture-prone plaques in patients have been reported to contain a higher number of monocytes.[56] Recent investigations by Chung et al. tested varying monocyte accumulation using fluorescently labeled peptide amphiphile micelles (PAMs) with the C–C chemokine receptor type 2 (CCR2)-binding peptide motif of MCP-1. In their study, MCP-1 PAMs bound to late-stage aortas to a greater degree than in early-stage aortas of Apo $E^{-/-}$ mice, demonstrating the feasibility of this molecular imaging tool and strategy to detect ranges of plaque progression.[25,56]

Additional chemokine receptors, such as CCR5, are also upregulated on monocytes and targeted nanoparticles for positron electron tomography/computed tomography (PET/CT) imaging of the CCR5 receptor has been recently investigated as a diagnostic tool for atherosclerosis.[39] Luehmann et al. developed poly(methyl methacrylate)-core/PEG-shell amphiphilic comb-like nanoparticles with the CCR5 peptide ligand, D-Ala_1-peptide T-amide (DAPTA, D-A_1STTTNYT-NH_2), and ^{64}Cu-DOTA (DOTA-DAPTA-comb). The peptide originates from the envelope glycoprotein, gp120, of the human immunodeficiency virus (HIV), which acts at endogenous receptors to alter normal ligand signaling in the pathogenesis of HIV-induced disease.[39] In lesions of a vascular injury model in the Apo $E^{-/-}$ mouse where CCR5 is upregulated, DOTA-DAPTA-comb nanoparticle uptake was significantly higher and displayed superior imaging capabilities compared to the ^{64}Cu-DOTA-DAPTA peptide alone. Peptide sequences specific to proteases of macrophages have also been used to image plaques with a high risk of thrombotic complication via PET/CT.[58]

In addition to nanoparticles consisting of synthetic polymeric components, nanoparticles found in nature are also used as a tool for nanomedicine. Uchida et al.[40] modified heat shock protein (Hsp) cages with the Lyp-1 peptide, or CGNKRTRGC, for imaging atherosclerosis. The LyP-1 peptide is a peptide found via a phage-displayed peptide library and has been previously implicated in homing to tumor lymphatic vessels, endothelial cells, and macrophages.[59] When macrophages were incubated with Lyp-Hsp nanoparticles, enhanced affinity was found compared to monocytes, and in mice with ligated carotid arteries, Lyp-Hsp nanoparticles selectively accumulated in macrophage-rich areas in an atherosclerosis model, demonstrating the utility of the Lyp-1 peptide for targeting cardiovascular disease as well.

Apolipoprotein A-I: targeting and therapeutic. Apolipoprotein A-I (Apo A-I, 243 amino acids) is an amphiphathic α-helical apolipoprotein that is the primary structural protein (~70%) in high-density lipoproteins (HDLs). HDLs are natural nanoparticles, 6–13 nm in diameter, composed of multiple biological macromolecules including Apo A-I, cholesterol, and phospholipids and can be discoidal and spherical in shape.[60,61] Plasma HDL levels are inversely correlated with the development of atherosclerosis and is associated with anti-atherogenesis.[62–64] Apo A-I has anti-inflammatory properties and promotes RCT, leading to the removal of cholesterol from lipid-laden macrophage-foam cells to the liver for elimination.[65] The ability to bind to lipids and its association with clearance of cholesterol has led to the use of HDL and Apo A-I as targeting and/or therapeutic strategies for atherosclerosis.

Often, synthetic HDL nanoparticles with mimetic Apo A-I peptides are proposed. Ghadiri and colleagues developed HDL-like vesicles with (*R*)-(+)-1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and a peptide derivative of the helix 10 of Apo A-I (residues 221−241), which is required in the native protein for lipid binding and cholesterol efflux.[41,42] Nanolipid particles containing trimers of this 23-amino acid sequence (CGVLESFKASFLSAL EEYTKKLQ-$CONH_2$) decreased plasma total cholesterol

levels by 30–40% compared to the PBS control in low-density lipoprotein receptor knock out mice (LDLr$^{-/-}$) upon daily intraperitoneal injections for 2 weeks. Surprisingly, plasma total cholesterol levels were reduced by 43% upon oral administration of the therapeutic nanoparticles as well. Lesion volumes correlated with IP or oral administration of nanoparticles.

Additional Apo A-I mimetic peptides including 4F (DWFKAFYDKVAEKFKEAF), 18A and 37pA have been combined with synthetic HDL particles for targeting and diagnostic applications of atherosclerosis.[43,44] The 37pA peptide (DWLKAFYDKVAEKLKEAFPDWLKAFYDKVAE KLKEAF) is a dimer of 18A (DWLKAFYDKVAEKLKEAF) covalently joined by a proline residue. 37pA has a high affinity towards lipidic particles and by combining into HDL particles containing gadolinium-labeled phospholipids, imaging macrophage burden in the abdominal aorta via MRI was made possible. Cormode et al.[43] extended this study by comparing the efficacy of HDL-like particles with either 37pA or 18A. Both nanoparticles were similar in size and approximately 8 nm, with longitudinal relaxivities close to 10 (mM s)$^{-1}$. Although 37pA has superior lipid binding properties, in the Apo E$^{-/-}$ mouse, both contrast agents produced enhancements in atherosclerotic plaques (~90%) and were macrophage-specific. Comparisons such as those conducted in this study are especially meaningful when considering nanomedicine for large-scale production and accessibility for the patient population; the shorter peptide sequence offers a cost-savings opportunity for scaling up a molecular contrast agent.

In addition to Apo A-I, peptides derived from other apolipoproteins, such as Apo E, have also been incorporated into HDL particles for targeting macrophages. Chen et al.[45] recently used the A2 peptide which has the amino acid sequence (LRKLRKRLLR)$_2$, a tandem dimer (141–150)$_2$ derived from the LDLr binding domain of Apo E. In combination with gadolinium, these Apo E-HDL particles displayed pronounced and significantly higher signal enhancement of the atherosclerotic wall in the Apo E$^{-/-}$ mouse, showing that the peptide can enhance the intraplaque macrophage uptake of HDL.

Other targets

Collagen. While the vast majority of peptides in nanomedicine for atherosclerosis target cellular markers, other targets include intraplaque components such as collagen and fibrin. The compositional changes of collagen type I is an important indicator of plaque progression, correlating the thickness of the fibrous cap and the stability of plaques to atherosclerotic vulnerability. In the Reversa mouse model, Chen et al.[46] developed HDL particles for MRI with the collagen type I binding peptide GKWH[CTTKFPHHYC], and found plaque regression can be monitored by a change from low collagen to high collagen.

In another study, Fredman et al.[47] tested targeted PLGA-PEG nanoparticles with a collagen IV heptapeptide ligand (KLWVLPK) that was previously identified by phage display (Figure 3). While collagen IV is not a major component in plaque, collagen IV makes up 50% of the vascular basement membrane, and the authors hypothesized that collagen exposure will occur at sites of vascular inflammation and injury, enabling targeted delivery of therapy. The authors also incorporated the Ac2-26 peptide, an annexin A1 N-terminal 25 amino acid mimetic peptide (AMVSEFLKQAWFIENEEQEYVQTVK) that acts on the G-protein-coupled formyl peptide receptor ALX/FPR2, which is also a receptor lipoxin A4. Ac2-26 exerts anti-inflammatory actions *in vivo* and was shown to be protective in several disease models, including myocardial ischemia-reperfusion injury, allergic inflammation, and endotoxin-induced cerebral inflammation, and the zymosan-induced peritonitic model.[48,66,67] Upon administration of collagen IV-Ac2-26 nanoparticles in an LDLr$^{-/-}$ mouse, targeted nanoparticles increased collagen I production and fibrous cap thickness, while decreasing metalloproteinase activity in lesions of the aortic root compared to free Ac2-26 or therapeutic nanoparticles with a scrambled collage IV peptide. Moreover, lesion area, necrotic core, and oxidative stress decreased in plaques within the brachiocephalic artery.

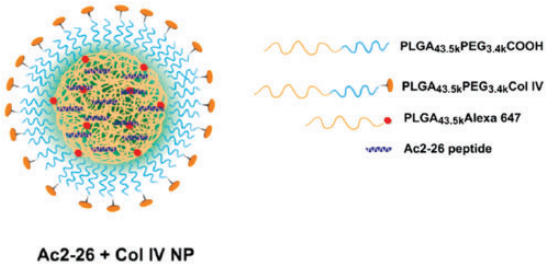

Figure 3 Collagen IV-targeting PLGA-PEG nanoparticles encapsulating the Ac2-26 therapeutic peptide. Adapted with permission from Ref. 66 from the National Academy of Sciences. (A color version of this figure is available in the online journal.)

Fibrin. Mature plaques are characterized by fibrin-containing blood clots, making fibrin a target for site-specific delivery of imaging and anticlotting agents to thrombi of atherosclerotic lesions.[49–51] Fluorescently labeled CREKA-PAMs were reported to effectively deliver the antithrombin peptide, hirulog-2 (FPRPGGGGNGDFEEIPEEYL) to late-stage plaques in the Apo E$^{-/-}$ mouse, demonstrating the theranostic capability of PAMs. In addition to the CREKA peptide, RWQPCPAESWT-Cha-CWDP, another fibrin-binding peptide identified via phage display, has also been incorporated into nanoparticles for imaging clots.[52,68] Starmans et al.[52,68] developed fibrin-binding, iron oxide nanoparticle-micelles (ION-micelles) containing this peptide and confirmed their ability to bind to human blood clots *in vitro*, while demonstrating its effectiveness as a contrast agent for magnetic particle imaging (MPI) and MRI.

Conclusion: current limitations and future outlook

Nanomedicine emerged in an effort to more efficiently and safely diagnose and treat diseases, and nanoparticle design continues to evolve as our understanding of the molecular pathogenesis of atherosclerosis is better understood.

Nanoparticles can easily incorporate multifunctionality and peptides are a biocompatible mechanism to achieve targeting and therapeutic effects with precise structural control.

While the number of nanoparticles for atherosclerosis continues to rise, there are challenges that must be addressed before commercialization and widespread patient distribution. While many types of nanoparticles exist, comparisons between nanoparticles and how small modifications change *in vivo* efficacy are needed for identification of worthy products for further development. Moreover, as atherosclerosis is a chronic inflammatory disease, targeting peptides are used to home to inflamed cell phenotypes, and whether this can in turn aggravate and heighten the inflammatory response is yet to be addressed. In addition, an accelerated development of atherosclerosis induced in the Apo $E^{-/-}$ or $LDLr^{-/-}$ murine models, the two most commonly used models of atherosclerosis preclinically, creates lesions that vary from those found in humans, which brings into question the comparability of the study.[17,69] And while atherosclerosis in the porcine model most closely resembles human atherosclerosis, cost and space associated with large animal studies hinder long-term studies with large numbers of animals, which are necessary for translation into the clinic. Nonetheless, while still a work in progress, nanomedicine continues to make strides for the management of atherosclerosis by targeted strategies of therapy, which limit off-target side effects and toxicity. Moreover, through stage-specific targeting and theranostic studies, personalized medicine continues to be a striving theme in atherosclerosis nanomedicine.

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DECLARATION OF CONFLICTING INTERESTS

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