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Molecular imaging of atherosclerosis with nanoparticle-based fluorinated MRI contrast agents

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Abstract

As atherosclerosis remains one of the most prevalent causes of patient mortality, the ability to diagnose early signs of plaque rupture and thrombosis represents a significant clinical need. With recent advances in nanotechnology, it is now possible to image specific molecular processes noninvasively with MRI, using various types of nanoparticles as contrast agents. In the context of cardiovascular disease, it is possible to specifically deliver contrast agents to an epitope of interest for detecting vascular inflammatory processes, which serve as predecessors to atherosclerotic plaque development. Herein, we review various applications of nanotechnology in detecting atherosclerosis using MRI, with an emphasis on perfluorocarbon nanoparticles and fluorine imaging, along with theranostic prospects of nanotechnology in cardiovascular disease.

Keywords

atherosclerosis; contrast agent; inflammation; theranostics; thrombosis

Atherosclerosis and associated acute vascular syndromes represent the underlying cause of strokes and heart attacks, which cause the greatest patient mortality in the western world [1]. Atherosclerosis is a highly inflammatory disease characterized by the development of fatty plaques in the intimal layer of arteries. Early stages of atherosclerosis are initiated by the accumulation of oxidized low-density lipoprotein (oxLDL) in the vessel wall, resulting in endothelial damage that stimulates the expression of surface adhesion molecules such as VCAM-1 and ICAM-1 [2]. Expression of these adhesion molecules promotes the

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recruitment of inflammatory cell types, for example, monocytes and T lymphocytes, which in turn promote inflammation and plaque growth through the release of cytokines that further enhance uptake of modified lipoproteins. The presence of these inflammatory mediators drives the expression and release of procoagulant factors (e.g., tissue factor, thrombin) and matrix metalloproteases that destabilize the plaque and elicit rupture or erosion, which increases thrombosis risk [3]. Many of these procoagulant factors promote endothelial dysfunction and inflammation that establishes a positive feedback loop of inflammation and coagulation resulting in more rapid plaque growth and an elevated risk for stroke and heart attack [4]. Early detection and characterization of atherosclerotic lesions is paramount in assessing thrombotic risk and for defining optimal treatment avenues.

Several techniques are commonplace in the diagnosis of cardiovascular disease, including single-photon-emission computed tomography (SPECT), positron-emission tomography, ultrasound and MRI [5,6]. Of these imaging modalities, MRI presents the unique combination of excellent soft tissue discrimination with the use of multiple imaging parameters, high penetration depth, high spatial resolution and safety due to lack of ionizing radiation [7]. Additionally, with the use of contrast agents, specific evaluation of the causes of disease may be accomplished, so called 'molecular MRI'. In this review, we will focus on the application of nanotechnology for the development of contrast agents in the advancement of MRI-based evaluation of atherosclerotic disease.

One significant advantage of nanoparticles is their ability to serve as targeted agents for imparting imaging contrast or drug delivery through passive targeting as a result of the enhanced permeability and retention (EPR) effect [8], or through surface modifications where nanoparticles can be functionalized with targeting moieties (i.e., antibodies and peptides) to accomplish ligand-directed targeting [9,10]. Additionally, surface modifications can include attachment of imaging agents or PEG chains of various lengths to impart favorable tissue half-lives. Through site-specific targeting and modulation of pharmacokinetic (PK) parameters, highly concentrated doses of drugs and imaging agents can be delivered to a disease site with diminished systemic side effects, offering the opportunity to accomplish increased efficacy of imaging and therapy, as exemplified by nanoparticulate formulations of doxorubicin that have already achieved US FDA-approval for clinical use (i.e., Doxil/Caelyx) [11].

For effective clinical translation, nanoparticles must be designed to achieve high stability, selective binding, low toxicity and favorable contrast-to-noise tissue enhancement. The design of nanoparticles must be well controlled to allow for effective, reproducible scale-up of synthesis that would preserve the purity of the formulation, while maintaining uniform potency. Additionally, several trade-offs exist in the design of nanoparticles for effective drug delivery and imaging, where surface functionalization to promote favorable biodistribution and PK parameters may interfere with ligand-targeting strategies, and thus must be designed carefully to promote efficacy of both modification strategies. Furthermore, sizing considerations are pertinent where deeper penetration into affected tissues may be advantageous [12]. Despite these considerations, the use of nanoparticles as contrast agents presents a unique opportunity to facilitate noninvasive molecular imaging of biological

processes such as inflammation and thrombogenesis through the targeting of important pathogenic biological molecules.

Contrast agents in MRI

MRI background

MRI is based on the principle of the nuclear magnetic resonance (NMR) phenomenon. When placed in a magnetic field (B_0), the spins of electrons and protons in a sample will orient themselves either parallel or anti-parallel to B_0 , corresponding to lower or higher energy states, respectively. The distribution of parallel and antiparallel spins is generally equally distributed outside of a magnetic field, resulting in a net magnetization vector of zero, but upon encountering the B_0 magnetic field, this distribution is altered, resulting in a net nonzero magnetization vector that processes at some frequency (i.e., the Larmor frequency) depending on the field strength of the magnet and the physical properties (i.e., the gyromagnetic ratio) of the particular nucleus being studied. Upon external radiofrequency excitation, these spins can absorb energy and be 'tilted away' from the direction of the B_0 field. After ceasing the radiofrequency pulses, the spins then return to their original state in a process referred to as relaxation, where different rates of relaxation are characteristic of different surrounding tissue milieus that can then produce the image contrast observed in MRI. Contrast in MRI is most often defined by 'T1' (spin-spin) or 'T2' (spin-lattice) relaxation times, depending on the exact pulse sequence used to excite and then measure the relaxing spins. Exogenous contrast agents may be employed to alter local T1 or T2 relaxation times to produce highly enhanced tissue contrast as compared with the expected background T1 or T2 signals [13].

T1 MR contrast agents

T1 magnetic resonance (MR) contrast agents consist primarily of paramagnetic ions (i.e., Gd^{3+} , Mn^{2+}) that shorten the T1 relaxation time to enhance the signal of a target area, and rely on the interactions with protons in the surrounding environment to generate a positive contrast image. The most popular T1 MRI contrast agent used clinically is gadolinium (Gd), delivered in complex with diethylenetriaminepentaacetic acid (DTPA), with DTPA serving as a chelating agent to mitigate the undesirable biodistribution of free Gd ions *in vivo*. Gadolinium-based contrast agents such as Magnevist (Bayer Schering Pharma AG) or Omnis-can (GE Healthcare), are especially useful in cardiovascular MRI for blood pool imaging, allowing for the generation of bright, positive contrast images. It is important to note that despite its common use as a T1-shortening agent, gadolinium can also be used as a T2*-shortening agent to generate negative contrast [14].

Techniques such as MR angiography with gadolinium can present a safer and less invasive alternative to x-ray angiography; however, despite the ubiquity of gadolinium-based contrast in clinical MRI and relatively safe pharmacokinetic and biodistribution profiles in healthy patients, a significant risk of toxic side effects exists in renal compromised patients. Many of these patients develop nephrogenic systemic fibrosis (NSF), which has been associated with prior administration and potential dechelation of gadolinium chelates [15]. To overcome this limitation, gadolinium chelates with increased stability (i.e., Gd-DOTA) have been explored

[16], along with alternative metal ions for T1-weighted imaging, such as manganese [17]. Furthermore, to add the potential for specific and targeted contrast, various groups have utilized nanoparticles to prolong the tissue half-life and decrease systemic dosages of Gd chelates. The types of nanoparticles used have been varied, and include perfluorocarbon nanoparticles [18–20], liposomes [21], micelles [22,23], fullerenes [24], targeted tobacco mosaic viruses [25], and polymeric nanoparticles [26], high density lipoprotein (HDL) nanoparticles [27] and low density lipoprotein (LDL) nanoparticles [28], among others.

T2 MR contrast agents

The prototypical agents for T2 contrast are super-paramagnetic iron oxides (SPIOs), which consist of nanoscaled iron oxide crystals, often coated with dextran to impart water solubility. Iron oxides function as T2 contrast agents resulting in localized signal loss that produces a negative (i.e., dark) contrast image, and have been studied extensively for their applications in MRI and drug delivery [29–31]. These particles can be characterized by their nominal hydrodynamic diameter, where the aforementioned SPIOs fall in the range of 50–200 nm, and another class known as ultrasmall SPIOs (USPIOs) fall in the range of <50 nm [32].

A number of SPIOs have been previously approved for clinical use, namely ferumoxides (Feridex) and ferucarbotran (Resovist). However, these formulations have since been taken off the market and are no longer manufactured, with the only FDA-approved SPIO on the market being ferumoxytol (Feraheme) for treatment of iron deficiency [33]. Due to their selective uptake and retention in the reticuloendothelial system, the approved use of Feridex and Resovist was specifically for liver imaging, where administered iron oxides are selectively taken up by Kupffer cells and negative contrast is utilized to determine differences between healthy and diseased tissue. The use of iron oxides, without targeting, presents several drawbacks as their action in generating a negative contrast can be ‘destructive’ to the image, where the high susceptibility of iron oxides results in distortion of the magnetic field in neighboring normal tissues. The generation of this susceptibility artifact or ‘blooming artifact’ results in distorted images that can obscure minute differences in normal versus healthy tissue [34,35]. However, with the introduction of novel pulse sequences to impart positive signal contrast images with SPIO/USPIO administration, the effects of blooming artifacts and signal destruction can largely be mitigated, and could allow for increased focus on SPIOs and USPIOs as a viable contrast agent for cardiovascular imaging in the clinic [36,37]. Despite the lack of clinical approval for iron oxides in cardiovascular MRI, many research groups have long studied the use of SPIOs and USPIOs in the detection of carotid atherosclerosis, inflammation, and myocardial infarction [38], in cell tracking [39,40] and the development of alternate coatings specific for scavenger receptors [41] and thrombi [42].

Fluorinated contrast agents

Fluorine MRI (^{19}F MRI) and spectroscopy (^{19}F MRS) utilize unique properties of fluorinated compounds that make them advantageous for NMR studies [43]. First, ^{19}F NMR exhibits a large chemical shift range of ~300 ppm that allows multiple fluorinated compounds to be detected simultaneously without risk of signal overlap. In addition, ^{19}F

NMR can be quantitative, in that the integral of a fluorine compound signature is directly proportional to the amount of compound in a sample. These properties make fluorine an attractive candidate for quantitative molecular imaging.

Our lab has pioneered the development of fluorinated nanoparticles as ultrasound [44,45] and MRI contrast agents [46–48]. These nanoparticles consist of a perfluorocarbon (PFC) core surrounded by a stabilizing lipid monolayer, resulting in nanoparticles ranging from 200–250 nm in diameter (Figure 1A). The outer lipid shell can be functionalized with drugs, targeting, or imaging agents through covalent conjugation [49,50] or insertion of compounds in the membrane [18,51–54].

Perfluorocarbon nanoparticles are ideal candidates for quantitative fluorine imaging as biologic tissue contains virtually zero endogenous fluorine content. Thus, the ^{19}F nuclei that are highly concentrated in the interior of perfluorocarbon nanoparticles represent highly specific agents for direct fluorine imaging. As previously discussed, the large chemical shift range of fluorine allows for the detection of multiple fluorinated compounds without signal overlap and as such, detection of perfluorocarbon nanoparticles with differing perfluorocarbon cores in the same sample (Figure 1B) can be accomplished as demonstrated by Morawski *et al.* [55]. Furthermore, as the relative ^{19}F spectroscopic signal is proportional to the amount of perfluorocarbon emulsion in a sample (Figure 1C), concentration maps of perfluorocarbon nanoparticle deposition can be generated, allowing for precise monitoring of molecular processes and localization of nanoparticles. (Figure 1D–F).

In addition to the distinct advantages of PFC nanoparticles for imaging and drug delivery applications, PFC nanoparticles exhibit low toxicity and favorable biocompatibility. In fact, an early use of perfluorocarbons in clinical research was in the context of artificial blood replacement, recognizing their innate ability to dissolve and carry oxygen, with some formulations having reached FDA approval in the past [56]. Adverse effects of perfluorocarbon toxicity is very low, due in part to the extremely high *in vivo* tolerance of administrated PFCs, with an LD_{50} ranging from 30–41g PFC/kg body weight. Symptoms of toxicity are relatively mild as well, characterized by ‘flu-like’ symptoms, with resolution within 12 h. In the case of PFC nanoparticles, those not bound to a specific target rapidly accumulate in the liver and spleen, with detection possible after several minutes postinjection [57] and up to 24 h, after which the lipid components are recycled by plasma carriers [48] and perfluorocarbons are cleared via the reticuloendothelial system and exhaled through the lungs [58]. It is important to note the role of species differences in the secretion and clearance of large nanoparticles especially as they relate to rodent PK models, as these differences can affect interpretation of clearance data [57]. Furthermore, the tissue half-lives of perfluorocarbons *in vivo* must be considered and potentially corrected for in instances of serial imaging where long tissue residence times [59] (i.e., PFOB) could complicate imaging studies.

Applications

Nanoparticles have been used extensively in cardiovascular imaging research, however the applications of nanoparticles to image atherosclerosis is varied based on specific disease

targets. Henceforth, we will review major targets of nanoparticles as they relate to atherosclerosis imaging, summarized in Table 1.

Thrombus imaging

Onset of occlusive thrombosis is the proximate cause of mortality in cases of heart attack and stroke, and accordingly, numerous studies have focused on the development of nanoparticle-based imaging contrast for the detection of thrombi. Destabilization of plaques due to rupture or erosion can result in disruption of the normal hemostatic barrier of the endothelium, resulting in the initiation of the coagulation cascade, which in turn generates thrombin and other coagulation factors, facilitating fibrin deposition and platelet activation [89]. The ability to image thrombi noninvasively presents immense clinical applications and implications for therapeutic management of acute ischemic syndromes [90]. Although there exist methods to characterize thrombi, for example, thrombus age [91], methemoglobin content [92], among others, the potential to image specific molecular epitopes associated with thrombi can allow for better characterization of thrombus composition and thrombus activity [93].

In 1997, Lanza *et al.* established fibrin as a target for molecular imaging of clots with MRI, demonstrating the development of a novel paramagnetic, nanoparticle-based contrast agent directed towards fibrin for T1-weighted imaging of clots. The nanoparticle used in this study was a perfluorocarbon nanoparticle imparted with various concentrations of Gd-DTPA, and conjugated with the fibrin specific antibody NIB 1H10 [46]. Initial work demonstrated the utility of these Gd-loaded PFC nanoparticles for T1-weighted imaging of plasma clots *in vitro* at 4.7T [60,61]. Later work confirmed direct binding of nanoparticles to fibrin with scanning electron microscopy (Figure 2A & B), and demonstrated the ability to image fibrin bound nanoparticles both in *ex vivo* human endarterectomy samples at 4.7T (Figure 2C), and *in vivo* using a clinical 1.5T MRI scanner (Figure 2D & E) [49]. Other fibrin-targeted nanoparticles for T1-weighted imaging include copper oleate nanocolloids (NanoQ) [63], manganese oleate lipid emulsions [64] and polymeric manganese-based ‘nanobials’ [65], which provide yet another alternative to traditional gadolinium-based MRI.

Importantly, Morawski *et al.* demonstrated the characterization of thrombi by quantitative ^{19}F MRI of human carotid endarterectomy specimens with the use of fibrin-targeted PFC nanoparticles at 4.7T, thus establishing this targeted nanoparticle platform as utile for both T1-weighted imaging and quantitative fluorine imaging [55]. Recent work from Myerson *et al.* with the perfluorocarbon nanoparticle platform has investigated the role of thrombin as a target for imaging and treating developing thrombi. In this work, perfluorocarbon nanoparticles were conjugated to a direct thrombin inhibitor, D-phenylalanyl-L-prolyl-L-arginyl-chloromethylketone (PPACK) and delivered *in vivo* in a mouse model of acute thrombosis in the carotid artery. *Ex vivo* ^{19}F MRI at 11.7T demonstrated the ability of these PPACK nanoparticles to selectively collect on the surface of clots, allowing for imaging of areas of high thrombin activity (Figure 2F–H) [50]. This antithrombin nanoparticle platform has since been translated to include bivalirudin as the thrombin-binding epitope due to increased specificity to thrombin and existing clinical approval of the drug [62].

Iron oxide-based imaging agents for thrombus detection have taken advantage of several molecular epitopes for thrombus-specific imaging. Recognizing fibrin as a potent target for thrombus imaging, Starmans *et al.* developed fibrin-specific nanoparticles consisting of iron oxide crystals encapsulated within micelles for magnetic particle imaging (MPI) [66]. Additionally, work by von zur Muhlen *et al.* has demonstrated the ability of microparticles of iron oxide (MPIOs) targeted to ligand-induced binding sites of the GPIIb/IIIa receptor to image activated platelets using *ex vivo* MRI of excised femoral arteries at 11.7T [67]. Recently, novel platelet-targeted iron oxide nanoparticles have been developed through designing a USPIO particle coated with fucoidan, a sulfated polysaccharide with a high affinity for activated platelets, as opposed to dextran or carboxymethyl-dextran coatings more commonly used in iron oxide nanoparticle synthesis [42]. Senpan *et al.* demonstrated another unique application of iron oxide colloids and coating involving the development of colloidal iron oxide nanoparticles (CIONs) comprised of oleate-coated magnetite particles encapsulated in a phospholipid emulsion targeted to fibrin. Interestingly, CIONs function primarily as a T1-weighted contrast agent by capitalizing on the relatively low doses of iron delivered with the nanoparticles, as opposed to the larger doses usually given where T2* effects dominate, resulting in signal loss and blooming artifacts generally associated with iron oxide MRI [68].

Endothelial permeability & neovasculature

In the early stages of atherosclerosis, the proinflammatory status of the vasculature affects an endothelial layer that is characterized by weakened or broken tight junctions, which increases the permeability of macromolecules through the endothelial barrier. It has been hypothesized that this increased endothelial permeability progresses to partial or complete endothelial denudation of the vascular wall due in part to endothelial cell apoptosis [94] or senescence [95], thus predisposing patients to focal atherothrombotic events [96]. Thus, the ability to selectively image areas of vascular permeability represents a distinct need, with implications for early therapeutic intervention of patients at risk of thrombotic events. In accordance with this need, Zhang *et al.* demonstrated in a rabbit model of atherosclerosis, with the use of ^{19}F MRS, the ability of nontargeted perfluoro-carbon nanoparticles to penetrate through the denuded endothelium of advanced aortic plaques. The presence of these nanoparticles following *in vivo* circulation was quantified with ^{19}F MRS, where the amount of detected nanoparticles in the aorta was increased in rabbits fed a high-cholesterol diet for 7–14 months compared with rabbits fed cholesterol for 3 months. Furthermore, imaging of retained nanoparticles in endothelial barrier disrupted aortic plaques was demonstrated with *ex vivo* ^{19}F MRI at 11.7T (Figure 3A & B) [69].

In addition to imaging strategies involving permeable plaques, other groups have focused on the imaging of neovasculature in atherosclerotic plaques. Taking advantage of the ‘leaky’ nature of developing vasculature, several albumin-binding paramagnetic agents have been utilized for imaging of permeable neovasculature. Cornily *et al.* evaluated the use of gadocoletic acid trisodium salt, or ‘B-22956/1’ which binds with high affinity to albumin, and evaluated its use in atherosclerotic rabbits. They demonstrated the ability of their albumin-binding agent to produce increased enhancement of plaques over and above enhancement produced by Gd-DTPA alone, using T1-weighted MR sequences [71]. More

recent work by Phinikaridou *et al.* involved the use of gadofosveset, a clinically approved albumin-binding T1 MRI agent for the evaluation of permeable endothelium in plaques [72] and the tracking of interventional therapies (minocycline, ebselen) in reducing plaque burden [73].

Additionally, beyond the realm of albumin binding contrast agents, Winter *et al.* demonstrated the use of a targeted T1 agent using Gd-loaded perfluorocarbon nanoparticles targeted to the $\alpha_v\beta_3$ integrin that is associated with angiogenesis. The results of this study indicated the potential for *in vivo* assessment of angiogenesis and neovasculture development using a 1.5T clinical MR scanner (Figure 3C) [20]. Furthermore, molecular targeting of nanoparticles to $\alpha_v\beta_3$ has allowed for the tracking of antiangiogenic therapy in the treatment of peripheral vascular disease using MRI as a readout [70].

Collagen imaging

Collagen is a major contributor to the structural integrity of the extracellular matrix and has been implicated as a biomarker for plaque instability [97]. There are several types of collagen that are expressed differentially based on cell type and disease state. In the case of arterial walls, the predominant types of collagens are type I and type III collagens. In atherosclerosis, collagen production is dysfunctional, resulting in a decreased amount of collagen, as demonstrated by impaired type I collagen assembly in *in vitro* studies of lipid-loaded vascular smooth muscle cells [98]. In view of the importance of collagen in plaque stabilization, the ability to noninvasively image collagen specifically with MRI has led to the development of several collagen specific MRI contrast agents. One such contrast agent is the conjugate EP-3533 by Caravan *et al.*, consisting of a 16-amino acid peptide specific for type I collagen conjugated to 3 Gd-DTPA moieties to allow for MRI [99].

Chen *et al.* recently described the development of HDL-nanoparticles containing Gd-DTPA for MRI tracking of atherosclerotic plaque regression with EP-3533. *In vivo* application of EP-3533 in a Reversa mouse model of atherosclerotic plaque regression demonstrated increased binding of EP-3533 HDL-nanoparticles following 28 days of plaque regression, in line with previous reports of increased collagen deposition in animal models of atherosclerotic plaque regression [27]. Similarly, van Bochove *et al.*, demonstrated with the use of paramagnetic Gd-loaded micelles conjugated with the collagen binding fragment protein, CNA35, the possibility of specific collagen imaging with micelles [75] and also the ability to differentiate between vascular lesions based on the relative amounts of collagen content, in accordance with previous histological reports of variable collagen content in plaques [100]. Other avenues of targeted collagen imaging involve the use of Gd-DTPA loaded per-fluorocarbon nanoparticles conjugated to an anticollagen III antibody for targeted T1-weighted contrast. In this particular study by Cyrus *et al.*, anticollagen PFC nanoparticles were successfully utilized for delineation of vascular wall injury patterns in a porcine model of carotid overstretch injury (Figure 4A–C) [74].

Macrophage & scavenger receptor imaging

Other strategies have focused on imaging of macrophages within atherosclerotic plaques. Macrophages play an important role in plaque development and serve as commonly used

indicators of plaque inflammatory status [101,102]. Additionally, several studies have identified increased macrophage content as a key characteristic of rupture-prone plaques [103,104]. The propensity of macrophages to internalize foreign bodies has made them especially attractive targets for imaging using nanoparticle contrast agents. USPIOs were among the first to be used for this purpose [76]. These nanoparticles accumulate in macrophages via receptor-mediated endocytosis [40,77] and have been used in several studies to characterize the effects of clinical interventions on plaque inflammation (Figure 5A & B) [78,79]. More recent techniques for macrophage imaging involve ligand-directed targeting of scavenger receptors on the macrophage surface. These receptors are implicated in the uptake of oxidized lipo-proteins and are highly expressed by macrophages in the lipid-rich plaque environment [105]. Gadolinium-containing micelles functionalized with antibodies to the macrophage scavenger receptor demonstrated increased plaque accumulation (Figure 5C & D) and enhanced MR signal intensity compared with untargeted micelles in a mouse model of atherosclerosis [22–23,80]. Similar results were obtained using USPIOs bearing peptides targeted to scavenger receptor A1 [41].

Imaging of inflammatory mediators

The role of inflammation in atherosclerosis has been recognized with markers of inflammation being investigated as potential targets for therapeutic intervention [106]. The early phases of atherosclerosis involve the attachment and recruitment of inflammatory cells, which contribute to later stages of plaque development. The mechanism of inflammatory cell recruitment involves the expression of cellular adhesion molecules on the inflamed endothelium, such as VCAM-1, ICAM-1 or P-selectin, among others [2].

Various studies have addressed the importance of cellular adhesion molecules as targets for MRI-based evaluation of vascular inflammation. The use of targeting peptides addressing adhesion molecules that are conjugated to iron oxide nanoparticles have proven useful in this field. Multiple studies have been reported utilizing VCAM-1 specific targeting ligands for *in vivo* T2* imaging of endothelial activation [83–85]. T2* imaging of ICAM-1 upregulation has been accomplished with the use of MPIOs functionalized with anti-ICAM-1 antibodies [86]. The use of dual-targeted nanoparticles has proven beneficial in increasing the affinity and specificity to areas of vascular inflammation. McAteer *et al.* demonstrated the use of MPIOs functionalized with antibodies targeting both VCAM-1 and P-selectin [87]. In this study, dual-targeted MPIOs bound to endothelium in atherosclerotic ApoE^{-/-} mice was increased 5–7 fold over single-target MPIOs functionalized with only P-selectin or VCAM-1. In addition to iron oxide nanoparticles, T1-weighted nanoparticle contrast agents have been used for detection of areas of vascular inflammation. ICAM-1-targeted nanoparticles loaded with gadolinium have been used to detect ICAM-1 upregulation following stroke [86] and activation of endothelial cells [88].

Fluorine imaging also has been utilized to image VCAM-1 upregulation in atherosclerosis, with the use of PFC nanoparticles as carriers for VCAM-1 targeting peptides previously developed by Kelly *et al* [83]. Initial work in this area was accomplished by Southworth and Kaneda, *et al.* using this anti-VCAM-1 nanoparticle for the imaging of renal inflammation in hyperlipidemic mice, demonstrating significant amounts of VCAM-1 targeted nanoparticles

in kidneys from fat-fed ApoE null mice over control wild-type mice (Figure 6A–C) [81]. Pan *et al.* accomplished further work in this area, in the demonstration of a novel system for rapidly functionalizing PFC nanoparticles. In this work, VCAM-1 targeting peptides were linked to a peptide based on the membrane inserting peptide, melittin, for rapid functionalization into perfluorocarbon nanoparticle membranes. These VCAM-1 targeted nanoparticles were utilized once again in the ApoE null model of atherosclerosis, where VCAM-1 targeted particles were preferentially detected in the aortas of ApoE null mice over aortas from wild-type mice (Figure 6D) [82].

Theranostic capabilities

Along with the potential for MR contrast with the use of the aforementioned nanoparticle-based agents for diagnosis of atherosclerosis and molecular imaging of biological processes of inflammation and thrombosis, there exists the potential for dual-functional aspects of both diagnostics and therapy, termed ‘theranostics’. Perfluorocarbon nanoparticles have been used extensively for drug delivery, while still allowing the option for diagnostic imaging through ^{19}F -MRI or T1-weighted contrast through membrane-incorporated gadolinium epitopes.

One such application of perfluorocarbon nano-particles has been the detection and treatment of angiogenesis [107], through targeting of the $\alpha_v\beta_3$ integrin that is highly expressed in neovasculature. In this study, $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles were loaded with the antiangiogenic agent fumagillin and tested for efficacy in atherosclerotic rabbits. Administration of fumagillin-loaded $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticles resulted in a reduction of plaque neovasculature by 50–75% following a single pulsed dose, where the treatment effect was detected up to 3 weeks following therapy [108].

Additionally, targeting of the $\alpha_v\beta_3$ integrin has proven to be useful in targeting therapeutic agents in animal models of restenosis, wherein Cyrus *et al.* demonstrated that intramural delivery of rapamycin-loaded $\alpha_v\beta_3$ -targeted nanoparticles were effective in the inhibition of restenosis following balloon overstretch injury in rabbits [18]. The importance of nanoparticle targeting is underlined with this study as inhibition of restenosis was only significant for the targeted delivery of the drug-loaded nanoparticle, but statistically insignificant for the nontargeted version of the drug loaded nanoparticle. This study once again demonstrated the ability of perfluorocarbon nanoparticles to accomplish multifunctional activity-targeting of the nanoparticles allowed for localized, specific delivery of rapamycin through ‘contact-facilitated drug delivery’ [109] and the addition of gadolinium on the nanoparticle surface allowed for effective T1-weighted imaging and location of treatment area.

Recent advances have been made utilizing per-fluorocarbon nanoparticles as carriers for antithrombotic and thrombolytic agents. A perfluorocarbon nanoparticle system utilizing direct thrombin inhibitors (PPACK [50] and bivalirudin [62]) not only accomplishes imaging of active thrombi as previously discussed, but succeeds in presenting a localized antithrombotic effect at sites of acute and active clotting, with minimal side effects. The same mechanism of thrombin binding that allows for successful imaging of clots with high

field strength fluorine MRI also allows for the establishment of an ‘anticoagulating surface,’ where nanoparticles presenting with thousands of inhibitor molecules can associate and inhibit clot-bound thrombin. This effect allows for the exposure of multiple inhibitory epitopes to the clot periphery, preventing or delaying further growth of clots. This effect has been observed in both PPACK and bivalirudin perfluorocarbon nanoparticles, along with proof-of-concept demonstrations with liposomal conjugates as well [110]. These mechanisms of nanoparticle associations with thrombi also suggest the potential for thrombolytic therapy with nanoparticles, and work by Marsh *et al.* demonstrated this concept with fibrin-targeting of streptokinase-loaded perfluorocarbon nanoparticles. *In vitro* demonstrations of thrombolysis illustrated rapid clot dissolution within 60 minutes of treatment, as tracked by ultrasound [111,112].

Other relevant applications of perfluorocarbon nanoparticles broadly related to cardiovascular imaging pertain to diagnostic kidney imaging where recent work by Hu *et al.* has demonstrated the utility of perfluorocarbon nanoparticles in assessing the extent of perfusion in the renal microvasculature following acute kidney injury [113]. Perfluorocarbon nanoparticles represent a potentially useful alternative to conventional gadolinium-based contrast agents for diagnosis and evaluation of renal compromised patients, because perfluorocarbon nanoparticles are not cleared through the kidney, but rather the liver and spleen. Additionally, perfluorocarbon nanoparticles do not suffer the complications from nephrogenic systemic fibrosis that may limit the use of gadolinium agents in these cases.

Conclusion

Specific imaging and detection of molecular processes in the pathogenesis of atherosclerosis has benefitted from a number of different nanoparticle-based formulations as reviewed above. These formulations feature directly targeted nanoparticle carriers interrogating an epitope of interest with the use of various targeting ligands to target clots, macrophages, platelets and other markers of inflammatory and thrombotic processes in atherosclerosis. Many of these formulations rely on gadolinium-based agents for T1-weighted imaging, or iron oxide-based agents for T2-weighted contrast. In addition to these well-established methods of paramagnetic MR contrast, perfluorocarbon nanoparticles have emerged as an interesting method of molecular imaging with fluorine MRI and may prove advantageous due to their favorable biocompatibility and opportunity for quantitative detection of nanomolar concentrations of PFC nanoparticles in biological samples.

Future perspective

The application of nanotechnology to the imaging of atherosclerosis is anticipated to grow overtime as this method of image contrast in MRI remains advantageous for the detection of diseases in the vasculature. Targeting and retention of many of the aforementioned imaging agents requires little to no cellular uptake or active transport for action, and thus exposure of targets to the vasculature is sufficient in most cases for adequate localization. This unique advantage allows for simplification of formulations, and future research will focus on the application of nanotechnology to image novel targets and biomarkers of atherosclerosis, along with the potential for dual function, ‘theranostic’ applications; that is, delivering a

more effective method of detection, therapy and tracking of disease progression in atherosclerosis.

Along with future considerations in dictating research in this field, it is important to evaluate potential regulatory challenges to the approval of nanoparticle-based therapeutics and contrast agents for applications in cardiovascular drug delivery. As many nanoparticle-based strategies, including those mentioned in this review and others rely on complex, multiplexed designs that combine numerous materials for imparting favorable PK parameters (PEGs and nanoparticle size considerations), drugs and imaging agents, much of the preclinical challenge beyond treatment effects is reproducibility of a stable, active formulation within a small margin of error [12,114].

Based on prior regulatory approval, liposomes and iron oxide-based nanotechnology has experienced the greatest success in regulatory approval, and as such much of the aforementioned literature reviewed involves the use of liposomes and iron oxides as carriers for drugs/targeting/imaging agents, (i.e., Gd-liposomes and coated iron oxides). In terms of fluorine imaging, perfluorocarbon nanoparticles remain a unique candidate for regulatory approval due to favorable biodistribution, compatibility and safety considerations, where elimination of materials (exhalation of PFCs through the lungs, recycling of phospholipids, *etc.* [48]) may be safer than metal-based contrast agents. However, the use of PFC emulsions as drug delivery agents beyond the preclinical phase is largely an unknown field as no PFC-based agent has yet achieved regulatory approval. As the field of fluorine imaging grows, the ability to dictate pathways to regulatory approval will also increase, with targeting strategies providing a viable method of increasing efficacy of the agent in question, and potentially limiting side effects.

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Executive summary

Imaging of atherosclerosis

- There exist several avenues for imaging of atherosclerosis, with MRI presenting a safe, and noninvasive method for diagnostic imaging.
- Early diagnosis of inflammation and thrombosis in atherosclerosis could be aided with the use of nanotechnology to generate targeted contrast agents.
- Numerous types of nanoparticles have been developed to accomplish T1- and T2-weighted imaging, along with the development of perfluorocarbon nanoparticles that allow for either T1-weighted imaging or imaging of fluorine, using ^{19}F MRI.

Targeting strategies & theranostics

- Nanoparticles can be targeted to various different epitopes for molecular imaging in atherosclerosis.
- Specific targeting has been utilized for direct imaging of active thrombi, endothelial permeability, angiogenesis, macrophage content, collagen deposition and the expression of inflammatory markers.
- These targeting strategies have also been utilized to develop so-called 'theranostic' nanoparticles that allow for simultaneous imaging and treatment of disease.
- Future work in this field will involve the further expansion of targeting to biomarkers of atherosclerosis and development of theranostic strategies to combat the development of atherosclerosis.

Figure 1. Fluorine MRI with perfluorocarbon nanoparticles

(A) Schematic of perfluorocarbon nanoparticles. (B) Representative ^{19}F spectra for perfluoro-15-crown-5 ether and PFOB. (C) Fluorine signal measured through ^{19}F MRS is proportional to the volume of emulsion in a sample. (D–F) Optical image of a human carotid endarterectomy sample (D) with corresponding ^{19}F magnetic resonance image (E) acquired at 4.7 T of targeted perfluorocarbon nanoparticles bound to fibrin epitopes in the vessel lumen. (F) Using external standards with known perfluorocarbon concentrations, it is possible to generate concentration maps of perfluorocarbon nanoparticle deposition. MRS: Magnetic resonance spectroscopy; PFOB: Perfluorooctylbromide; ppm: Parts per million.

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Figure 2. Imaging with thrombus-specific nanoparticles

Scanning electron micrographs depict fibrin clot (A) without nanoparticle treatment, with arrows depicting fibrin fibrils and (B) with addition of fibrin-targeted perfluorocarbon nanoparticles, with arrows depicting fibrin-bound nanoparticles. (C) T1-weighted gradient echo images of human carotid endarterectomy specimens treated with fibrin-targeted nanoparticles (left) demonstrating contrast enhancement with nanoparticle treatment compared with control (right). (D) *In vivo* demonstration of T1-weighted contrast enhancement (arrow, left panel) at thrombus site with fibrin-targeted nanoparticles compared with corresponding phase contrast image demonstrating flow deficit due to the presence of thrombus (arrow, right panel). (E) Control thrombus in the contralateral external canine jugular vein demonstrates no T1 enhancement (arrow, left panel) with corresponding phase contrast image to demonstrate flow deficit (arrow, right panel). (F–H) *Ex vivo* ^{19}F imaging at 11.7T of thrombin-targeted PFC nanoparticles bound to mouse thrombi. (F) Proton scan of excised mouse carotid artery with occlusive thrombus, with (G) overlay of ^{19}F magnetic resonance image collected in (H). (A–E) Reproduced with permission from [49]. (F–H) Reproduced with permission from [50].

Figure 3. Imaging of endothelial permeability and neovascularity

(A) Sagittal 3D rendering of nontargeted NP deposition measured at 11.7T in a rabbit aorta excised following 12 months of cholesterol feeding and 12 h of nontargeted NP circulation *in vivo*. (B) Cross-sectional ^{19}F image of nontargeted NP deposition in atherosclerotic plaque. Concentration mapping illustrates depth of penetration and local concentration in the rabbit atherosclerotic plaque following 9 months of cholesterol feeding and 2 h of *in vivo* nontargeted NP circulation. (C) Localization of $\alpha_v\beta_3$ -targeted NPs to areas of plaque angiogenesis in atherosclerotic rabbits. Top panel shows region of interest, with cross-sectional images of aorta prior to administration of $\alpha_v\beta_3$ -targeted NPs (pre), following $\alpha_v\beta_3$ -targeted NP administration (post) and the application of a segmented aortic wall mask (segmented) used to quantify T1-weighted contrast enhancement (enhancement). mo: Months; NP: Nanoparticle.

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(C) Reproduced with permission from [20]

Figure 4. Collagen imaging with perfluorocarbon nanoparticles

(A) Time-of-flight magnetic resonance angiogram images of porcine carotid arteries following balloon overstretch injury, either left as control (right) or following exposure to perfluorocarbon nanoparticles (left). T1-weighted black blood imaging of porcine arteries after exposure to either (B) $\alpha_v\beta_3$ -targeted nanoparticles or (C) collagen-III-targeted nanoparticles, demonstrating accumulation of collagen targeted nanoparticles on the lumenal surface of the arteries following injury.

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Figure 5. Macrophage imaging strategies with nanoparticles

In vivo T2*-weighted gradient echo MRIs of the internal (lower) and external (upper) human carotid artery (A) prior to administration and (B) 24 h after administration. Circled areas correspond to region of interest where signal loss is observed due to iron oxide uptake in the vessel wall. *In vivo* T1-weighted imaging of mouse atherosclerotic plaques (C) prior to administration of scavenger receptor targeted immunomicelles, demonstrating contrast enhancement (D) 24 h after administration

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(C & D) Reproduced with permission from [23].

Figure 6. Imaging of inflammatory mediators

^{19}F MRI of atherosclerotic mouse kidneys (top row) or wild-type mouse kidneys (bottom row) with (A) proton scan for anatomical detail, (B) ^{19}F image and (C) overlay of ^{19}F image on proton image demonstrating increased accumulation of VCAM-1 targeted perfluorocarbon nanoparticles in atherosclerotic kidneys over wild-type kidneys, corresponding to differences in VCAM-1 expression in atherosclerotic versus wild-type subjects. (D) Targeting of perfluorocarbon nanoparticles using VCAM-1 targeting peptides increases binding of nanoparticles in atherosclerotic mouse aortas, as measured with ^{19}F magnetic resonance spectroscopy at 11.7T.

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(D) Reproduced with permission from [82]

Table 1

Applications of various nanoparticles in atherosclerosis imaging.

Imaging application	Nanoparticle type	Ref.
Thrombosis	Perfluorocarbon	[46,49,50,55,60–62]
	Copper	[63]
	Manganese	[64,65]
	Iron oxide	[42,66–68]
Endothelial permeability and neovasculature	Perfluorocarbon	[20,69,70]
	Albumin	[71–73]
Collagen	Perfluorocarbon	[74]
	HDL	[27]
	Micelles	[27,75]
Macrophages	Iron oxides	[40,41,76–79]
	Micelles	[22,23,80]
Inflammatory mediators	Perfluorocarbon	[81,82]
	Iron oxides	[83–87]
	Liposomes	[86,88]