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Insights into Atherosclerosis Using Nanotechnology

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Abstract

A developing forefront in vascular disease research is the application of nanotechnology, the engineering of devices at the molecular scale, for diagnostic and therapeutic applications in atherosclerosis. Promising research in this field over the past decade has resulted in the preclinical validation of nanoscale devices that target cellular and molecular components of the atherosclerotic plaque, including one of its prominent cell types, the macrophage. Nanoscale contrast agents targeting constituents of plaque biology have been adapted for application in multiple imaging modalities, leading toward more detailed diagnostic readouts, whereas nanoscale drug delivery devices can be tailored for site-specific therapeutic activity. This review highlights recent progress in utilizing nanotechnology for the clinical management of atherosclerosis, drawing upon recent preclinical studies relevant to diagnosis and treatment of the plaque and promising future applications.

Keywords

Nanotechnology; Atherosclerosis; Quantum dot; Vascular imaging; Inflammation; Macrophage

Introduction

Complications of atherosclerotic disease are a leading cause of death in industrialized countries [1]. The pathogenesis of arterial plaques starts with the inflammatory activation of endothelial cells and the accumulation of lipids within the subendothelial space [1]. Activation of endothelium and secretion of chemoattractants promotes the adherence and arrest of leukocytes, such as mononuclear phagocytes and T lymphocytes, followed by the diapedesis of leukocytes from the arterial lumen into the subendothelial space [1,2•,3]. As the lesion progresses in severity, monocyte-derived macrophages ingest large quantities of lipid and become characteristic “foam cells.” Macrophages can secrete cytokines, which contribute to

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inflammation, cell apoptosis, and activity of matrix metalloproteinases (MMPs), which can weaken the plaque's architecture through extracellular matrix degradation and activated angiogenesis [2•,3–5]. These changes may lead to the formation of a "vulnerable plaque," which is more often responsible for acute coronary events than are fully occluding lesions [1].

Strategies for interfacing with these key cellular and molecular participants throughout atherosclerotic plaque initiation and progression are needed to enable the development of diagnostic tools and to facilitate early therapeutic interventions. Detection of dysfunctional endothelium along early lesions, for example, may permit timely therapy to reduce leukocyte infiltration within artery walls [6]. Furthermore, as "druggable" disease targets within the plaque are characterized, such as the subtypes of collagen-degrading MMPs [7], the design of customized treatment strategies will be enabled for patient-specific clinical management.

Nanotechnology can be applied to components of plaque biology to enhance clinical approaches to atherosclerosis. The term *nanotechnology*, which deals with the study of material properties at the sub–100-nm scale, was originally associated with the miniaturization of semiconductor materials but has now been adapted to provide novel solutions to the clinical management of disease, giving rise to the term *nanomedicine* [8]. Nanomedicine-based approaches in the context of vascular medicine can be generally categorized into diagnostic and therapeutic devices, although multifunctional devices have been extensively reported in the literature, such as those designed for image-guided therapy [9]. As exhibited in the forthcoming discussion, many reagents within these categories have been extensively characterized using in vitro and in vivo models of atherosclerosis.

Nanoscale devices, or "nanoparticles," may be composed of multiple organic and inorganic materials. To generate contrast enhancement, nanoscale imaging agents may be composed of gadolinium or iron oxide for magnetic resonance contrast [10,11], gold colloids or radiotracer-based nanoassemblies for x-ray contrast [10], gas-filled microbubbles for contrast using ultrasound [12], and infrared dyes and quantum dot nanocrystals (QD) for optical imaging [13,14]. These contrast agents may be injected intravenously as is, or be encapsulated within emulsions, lipids, or polymer-based biocompatible matrices to increase contrast properties (ie, greater number of contrast agents per nanoparticle) or to improve in vivo half-life and targeting efficacy. Prominent examples of nanocarrier matrices include liposomes, micelles, and block co-polymer nanoparticles [10,15,16]. When designing nanocarriers for therapeutics, drugs can be encapsulated within vehicles based on similar building blocks. Polylactide co-glycolide (PLGA) nanoparticles feature controlled degradation rates for tunable drug release profiles [17]. For gene therapy applications, the cationic polymer polyethyleneimine (PEI) has been extensively utilized, primarily in the branched form [18]. To prolong persistence of the therapeutic agent in vivo, polyethylene glycol-coated phospholipid liposomes have been designed that delay clearance from the circulation [19]. Both imaging and therapeutic nanocarriers are usually functionalized with biorecognition moieties on the surface, such as peptides or antibodies, to enable in vivo delivery of the imaging agent or therapy to the intended site within the plaque, such as the macrophage, to aid in image-guided staging of the plaque [2•].

The promise of nanomedicine in vascular disease is predicated on the fact that the nanometer scale upon which biological interactions are based is the same scale at which many materials exhibit novel properties. For example, QD can be engineered on the single-nanometer scale to tune their fluorescence emission profiles from the ultraviolet to the infrared spectrum, enabling the color-coding of distinct cell populations within the plaque [14]. Polymer-based nanospheres can be coated with bioactive ligands to permit their efficient internalization into diseased cells for the site-specific delivery of antithrombotic agents [20]. These properties have led to the

development of advanced nanoscale devices that can interface with the complex biological processes underlying plaque development and progression for the implementation of novel diagnostic and therapeutic strategies.

Nanotechnology-Guided Targeting of Atherosclerosis

The most promising application of nanomedicine in vascular biology is the targeting of imaging or therapeutic agents to atherosclerotic plaques. Advances in plaque biology have identified molecular profiles of early-stage and late-stage plaques, and in conjunction with available techniques to target nanoscale carriers to specific plaque biomolecules, a framework is being developed for the stage-specific diagnosis and therapy of atherosclerosis [10,21]. Examples of important molecular targets throughout the time-course of plaque development currently investigated for targeting by nanoscale agents are listed in Table 1.

Endothelial Cells

As previously discussed, early lesions feature endothelial activation with secretion of inflammatory molecules, such as cell adhesion molecules (CAMs) and selectins, to promote the infiltration of leukocytes within the intima. This focal expression on plaque walls offers an opportunity for targeting by diagnostic and therapeutic agents at a very early stage in plaque development [22]. The selectins, such as E- and P- selectin, participate in the first leukocyte-endothelial interactions in atherosclerosis, and their direct blockade with antibodies can be used to slow lesion development [23]. This application was recently expanded by decorating the surface of polyester nanoparticles with selectin ligands, such that the constructs effectively mimic circulating leukocytes, binding to inflamed endothelium [24]. Such systems enable not only therapeutic inhibition of inflammatory biomolecules lining the plaque, but also site-directed release of therapies and imaging agents, which should enable the development of new therapies. CAMs expressed at elevated levels on endothelium lining plaques, such as intercellular adhesion molecule (ICAM) [25], not only play important roles in leukocyte transmigration into the subendothelial space, but also are endocytosed as part of surface protein recycling [6]. This activity has been exploited to internalize CAM-targeted nanocarriers bearing imaging agents (CT, MRI, optical contrast agents) or therapies (antithrombotics) into dysfunctional endothelial cells [20,26]. These types of nanomedicine-based clinical approaches may enable detection and treatment of endothelial dysfunction prior to extensive leukocyte infiltration.

Macrophages

Macrophage accumulation in the subendothelial space can be observed through all stages of lesion development, and increased macrophage density is associated with lesion progression and rupture [3]. The macrophage has potential for both reparative and destructive effects within the plaque, and is thus a significant imaging and therapeutic target for nanoscale reagents [1]. A promising emerging nanotechnology for targeting the macrophage is a multimodal nanoparticulate platform based on high-density lipoprotein (HDL) [27]. The main high-density lipoprotein (HDL) protein, apolipoprotein A-I (apoA-I), synthesized in the liver and intestine, enters the subendothelial space of plaques to remove cholesterol from local cells for delivery to the liver in a process known as reverse cholesterol transport, and thus possesses significant therapeutic relevance [28]. Furthermore, HDL efficiently engages with arterial macrophages and can be engineered to carry nanoscale contrast agents such as QD (optical), gold nanoparticles (CT), or iron oxide nanoparticles (MRI) [27]. This feature was used in animal models to image macrophage-rich lesions with high signal-to-noise ratios using several modalities. The macrophage targeting efficiency can potentially be improved by the use of peptides, such as the apolipoprotein-derived peptide P2A2 [29]. Regarding the potential for clinical translation of this technology, it may be possible to increase the benefits of native HDL

by using complexes of specific apolipoprotein peptides and phospholipids [30]. Several other approaches have been reported for targeting nanoscale reagents to macrophages in plaques. The coating of imaging agents with dextran promotes their internalization by macrophages by scavenger receptor-mediated endocytosis. Additionally, the macrophage scavenger receptor can be directly targeted using monoclonal antibodies, providing significant (up to 80%) enhancement of lesion signal intensity in T1-weighted MRI [2,31]. Example applications of nanomedicine relevant to the macrophage may include imaging of macrophage density for staging lesion progression or delivery of therapies within the macrophage to induce reverse cholesterol transport or attenuate inflammation.

Lipid Content and Apoptosis

As atherosclerotic disease progresses, lesions exhibit high extracellular lipid content and disseminated death (by apoptosis and necrosis) of resident cells caused by excess cholesterol engorgement and local inflammation [32]. These events provide ample opportunities for stage-specific targeting of atherosclerosis. By engineering the lipophilicity of contrast agents, improved targeting of lipid-rich plaque regions can be achieved for MRI signal enhancement [33]. Apoptosis can be monitored within the plaque by targeting exposed phosphatidyl serine residues on apoptotic cell membrane leaflets using the targeting protein annexin V [34]. Targeting of annexin V nanoparticles to apoptotic cells was demonstrated to aid imaging of lesions using CT and MRI, and annexin V targeting capabilities can be readily conferred onto other nanoparticulate contrast agents, such as QD [34,35]. A more direct approach for imaging apoptosis involves the conjugation of radiotracers to caspase-binding ligands [36]. Caspases are enzymes involved in carrying out apoptotic processes within cells and represent an important imaging and therapeutic target. Apoptosis of cells, such as macrophages, smooth muscle cells, and endothelial cells within the plaque, has been associated with vulnerability to plaque rupture [32], and therefore nanomedicine-guided targeting of apoptotic regions may have important clinical applications.

Angiogenesis

In advanced disease, the aberrant growth of neovessels within lesions is thought to promote plaque instability secondary to hemorrhage and further influx of macrophages within the subendothelial space. A broad spectrum of nanoparticles can be loaded with antiangiogenic drugs and coupled with targeting ligands for plaque-specific inhibition of blood vessel growth. A particularly-interesting approach for this purpose is based on liquid perfluorocarbon nanoparticles bearing the angiostatic reagent fumagillin [5]. The vehicle is coupled with a novel $\alpha_v\beta_3$ integrin-targeted peptidomimetic agent to target adventitial neovessels, and can also bear paramagnetic or optical contrast agents to enable tracking of the therapeutic. The nanoparticles exhibited a sustained antiangiogenic effect for up to 4 weeks when combined with concurrent statin therapy [5]. Perfluorocarbon nanoparticles are versatile candidates for multimodal imaging and therapy of atherosclerotic lesions, given their amenability to conjugation of ligands and encapsulation of physicochemically diverse imaging agents and drugs [37]. Several other promising targets are available for imaging and therapy of angiogenesis in lesions. The B domain of fibronectin, which is absent in normal tissue but present at readily detectable levels during angiogenesis and tissue remodeling, was imaged in lesions using x-ray and optical contrast imaging modalities [38].

Extracellular Matrix

A critical event in plaque biology, the rupture of the smooth muscle cell and collagen-rich fibrous cap, can be detected in plaques using nanoscale imaging agents conjugated to collagen-binding proteins or smooth muscle cell-specific epitopes [39]. MMPs, which contribute to plaque instability, can be visualized using optical imaging, CT, and positron emission

tomography by injecting contrast agents coupled to matrix metalloproteinase (MMP) inhibitors or MMP substrate peptides [4,40]. Given the large number of MMP subtypes known to participate in plaque development [40], an important goal is to develop tools to image multiple MMP subtypes simultaneously. A number of spectrally distinct nanoscale optical contrast agents, such as QD, may prove useful for imaging MMP subsets within plaques, provided that intravascular optical imaging of these nanoscale reagents can be demonstrated in the clinic.

Thrombosis

Plaque rupture exposes the luminal compartment to intraplaque milieu, which contains procoagulant and prothrombotic stimuli [41]. Components of thrombi, such as platelets and fibrin, have been targeted by several nanoscale platforms for imaging and treatment of the vulnerable plaque. Platelet-targeted liposomes bearing a glycoprotein (GP)IIa-IIIb integrin-binding peptide tether specifically onto platelets and can be engineered to bear optical or CT contrast agents [19]. The perfluorocarbon nanoparticle platform discussed previously for targeting of plaque neovessels has also been adapted for targeting fibrin in lesions with high avidity, and the number of clinical applications for treating the vulnerable plaque using this approach are high given the versatility of this nanocarrier for bearing imaging and therapeutic agents [37]. Several other platforms based on fibrin-targeting capability for imaging the vulnerable plaque have been described [42].

Nanomedicine-Based Surgical Devices and Adjuvants

Nanotechnology has also been applied toward improving the success of surgical procedures relevant to the treatment of atherosclerosis. A major focus has been the nano-engineering of drug-eluting stents [43]. Techniques for modifying the surface texture of stents can be utilized to improve mechanical properties, endothelial cell seeding, and the efficiency of arterial integration [43]. To improve the drug delivery range of drug-eluting stents, polymer “needles” can be coupled upon the stent surface to facilitate deeper penetration of therapeutics for reduced lesion revascularization [43]. As an adjuvant to stent angioplasty, by incorporating a magnetically sensitive alloy into steel intravascular stents, endothelial cells loaded with paramagnetic nanoparticles were guided toward the stent surface under a local magnetic field, thus enhancing re-endothelialization [44•]. In a different application, improving perfusion to cardiac tissue under ischemic conditions can be achieved through nanotechnology-assisted therapeutic neovascularization. Although several approaches can be utilized to develop collateral circulation, most involve nanoscale controlled-release devices such that the appropriate concentration of angiogenic factors are delivered at the appropriate time. PLGA nanoparticles are useful for this purpose, as the ratio of polymer blocks (polyglycolides [PGA] and polylactic acid [PLA]) dictates the rate of drug release (days to weeks) by controlling polymer scission by hydrolysis [17]. This approach was used for delivery of statins in a rodent model of hind limb ischemia, resulting in restoration of perfusion through a stimulation of therapeutic angiogenesis [45].

Next-Generation Nanoscale Devices for Atherosclerosis

Emerging nanotechnologies in early stages of evaluation may uncover novel approaches for the management of vascular disease. Two encouraging avenues of application are the development of adaptive biosensors for providing molecular-specific readouts of plaque biology and the utilization of plaque-homing cell subpopulations for long-term imaging and therapy of atherosclerosis.

Atheroma-Specific Biosensors

Nanoscale devices can be triggered by molecular components of the atheroma to facilitate more advanced, disease-customized approaches. Several reports illustrating this concept are based upon contrast agents that can be triggered to aggregate when stimulated by proteolytic activity [7,46]. The aggregation of contrast agents, such as iron oxide nanoparticles, can be exploited to increase magnetic resonance signal contrast. Using a related strategy, protease-triggered plaque uptake of nanoparticles can be achieved by the surface incorporation of polymers that inhibit nanoparticle internalization into the plaque but are cleavable by specific enzymes [47•]. Nanoscale biosensors are being developed for the detection of circulating biomarkers with value in predicting atherosclerosis and response to treatment, such as those capable of quantifying the properties of blood-borne lipoproteins. In these systems, nanoscale engineering of the device permits improved biological interactions, resulting in higher sensitivity compared with conventional approaches. Cardiac troponin T, a marker of myocardial tissue loss, was detected at unprecedented nanomolar concentrations using a fluorescence resonance energy transfer (FRET) nanoscale system [48]. In this approach, a dye-labeled anti-troponin antibody is mixed with a gold nanoparticle linked to a distinct anti-troponin antibody that binds to a different epitope on the protein. Fluorescence emission of the dye is quenched at intensities proportional to troponin T concentration. High-sensitivity nanoscale biosensors can interrogate components of plaque biology at very low levels and may facilitate early clinical detection and treatment of atheroma.

Cell-Based Imaging and Therapy

This laboratory and others are developing nanomedicine-based approaches for investigating circulating cells that have intrinsic homing capabilities toward atherosclerotic plaques and sites of arterial inflammation [14,49]. By loading cell subpopulations with nanoscale contrast agents, such as QD or iron oxide nanoparticles, their biodistribution within tissues can be evaluated in vivo using several imaging modalities. Our laboratory has developed an approach to safely and efficiently load QD into any cell type by coupling nanocrystals with the cell penetrating peptide maurocalcine [14]. To demonstrate the utility of this approach, T lymphocytes and monocyte-macrophages were color coded with spectrally distinct QD-maurocalcine tags, and the trafficking of labeled cells administered to lesionprone apoE$^{-/-}$ mice was investigated. QD-maurocalcine bioconjugates enabled the simultaneous imaging of the leukocyte subsets within lesions ex vivo with high signal-to-background ratios. QD-maurocalcine nanoparticles labeled both cell types with high efficiency and low cytotoxicity, as assessed by flow cytometric analysis. Furthermore, using cytokine release and endothelial adhesion assays, it was observed that QD bioconjugates did not adversely affect native cell functions. QD-labeled cells could be detected for up to 1 month within oil red O-positive aortic lesions of mice ex vivo using *en face* optical imaging. Additionally, QD-labeled cells co-localized beginning 2 days post-injection with endogenous matched cell types as demonstrated by immunofluorescence analysis (Fig. 1). With the advent of intravascular optical imaging approaches, QD-based strategies for investigating trafficking of cells to lesions may be useful in characterizing plaque development and progression.

In a related approach used by this laboratory, bone-marrow derived cell populations are loaded with nano-scale imaging agents, drugs, or gene therapy agents, and injected into the circulation to enable their accumulation at vascular sites prone to disease. A current goal in this laboratory based on this approach is the development of a technique for the controlled release of apoA-I within the subendothelial space using genetically engineered bone marrow-derived cells [50]. This approach has been shown to increase reverse cholesterol transport in the plaque, and may achieve additional therapeutic goals such as activating cellular egress and inducing plaque regression. It is hoped that by exploiting the naturally targeted biological process of cell homing to plaques, new methods of specific diagnosis and treatment of the plaque will be enabled.

Conclusions

The concurrent growth of the knowledge base of plaque biology and the design of nanoscale devices has resulted in the emergence of several promising avenues for improving the clinical management of atherosclerosis. Major areas of focus have included molecular targeting, stent engineering, therapeutic neovascularization, biosensors for detection of disease-specific biomarkers, and the harnessing of plaque homing cells for imaging and therapy. A future goal is the development of adaptive, multifunctional nanoscale devices capable of providing clinical feedback by interfacing with multiple stage-specific biomarkers, or the spatial and temporal release of therapies in response to molecular cues. Clinical translation of these technologies will be facilitated by the regulatory approval of clinical trials to validate the specificity, sensitivity, and biocompatibility metrics reported in preclinical models.

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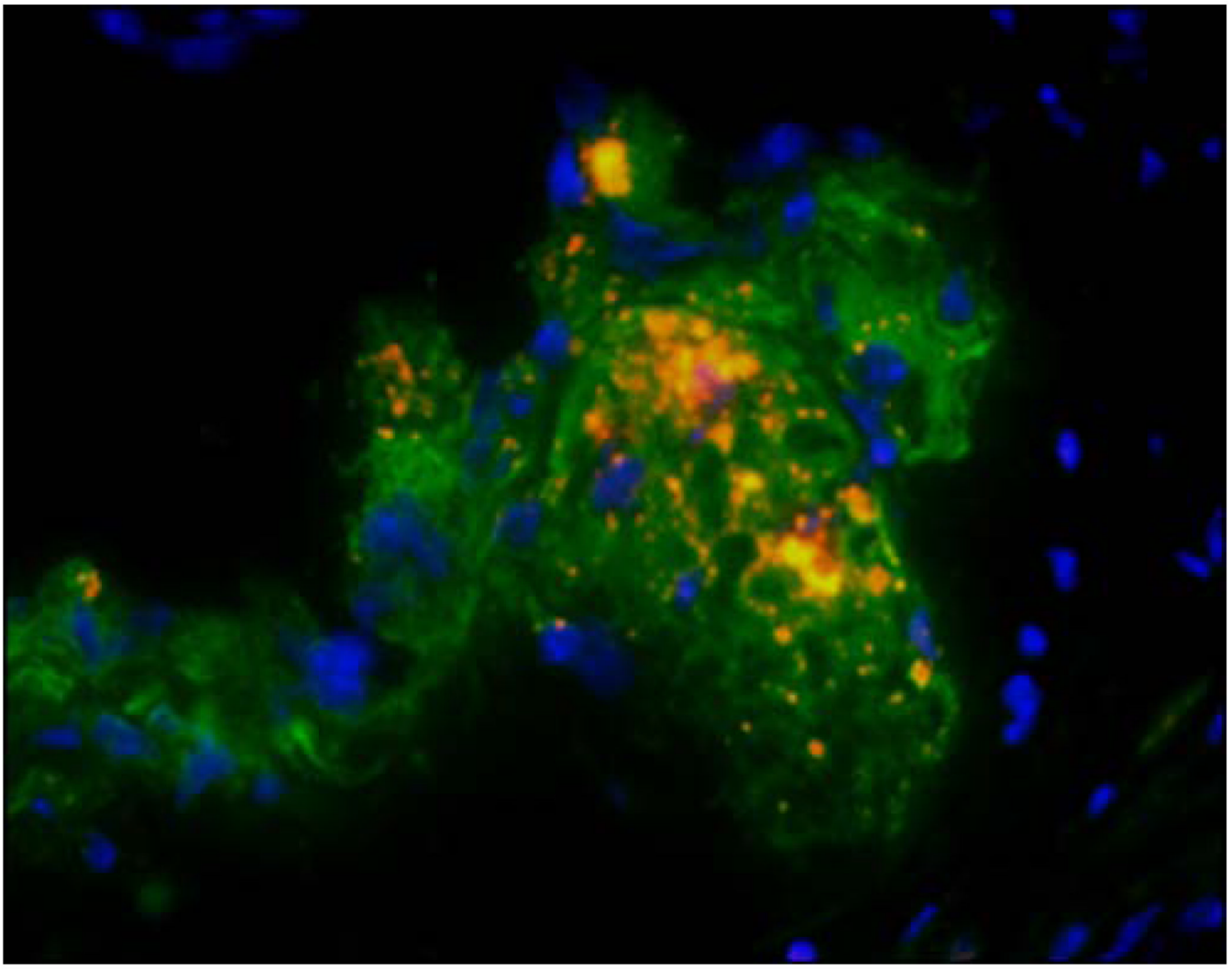

Fig. 1.

Quantum dot–mediated imaging of monocyte/macrophages in atherosclerotic plaques. Twelve-month-old apolipoprotein $E^{-/-}$ mice received an injection of monocyte/macrophages loaded with cell-penetrating quantum dots. Aortic root lesions were imaged ex vivo. Injected cells (*orange*) co-localized with the macrophage marker CD68 (*green*), indicating that homing specificity of labeled cells was preserved. This technique is being utilized to monitor changes in the atheroma after introduction of macrophages carrying the high-density lipoprotein protein known as apolipoprotein A–I. (From Jayagopal et al. [14]; with permission)

Table 1

Examples of targets investigated for nanotechnology-guided imaging and therapy of atherosclerosis

Target	Description of targeting mechanism	References
ICAM-1, VCAM-1 (endothelial cell)	Internalizes imaging agents or therapies bound to cell adhesion molecule-specific peptides or antibodies	[17,20,26]
Macrophage scavenger receptor	Internalizes dextran-coated iron oxide nanoparticles for MR contrast, or antibody-linked imaging agents and/or therapies via scavenger receptor endocytosis	[2•,31]
Apoptosis	Phosphatidyl serine residues on apoptotic cells, or the caspase family of enzymes, can be targeted by molecular imaging agents	[34–36]
Neovascularization	Neovessel-specific integrins, extracellular matrix molecules, and cell adhesion molecules enable targeted molecular imaging and therapy.	[5,37,38]
Matrix metalloproteinases	Nanoparticles featuring MMP-cleavable can be used to develop a variety of site-specifically activated imaging and drug delivery reagents. Several emerging nanotechnologies are based on functional actuation by MMPs.	[4,40]
Extracellular matrix	Collagen subtypes present in the plaque such as I, III, and IV can be imaged in the plaque using a collagen-binding protein linked to contrast agents.	[39]
Thrombus	Activated platelet integrins and exposed fibrin can be targeted by antibodies, peptides, and small molecules linked to contrast agents to image vulnerable plaques, or drug delivery vehicles for delivery of antithrombotic agents.	[19,37,41,42]

ICAM intercellular adhesion molecule, *MMP* matrix metalloproteinase, *MR* magnetic resonance, *VCAM* vascular cell adhesion molecule