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Atherosclerosis and Nanotechnology: Diagnostic and Therapeutic Applications

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Abstract

Over the past several decades, tremendous advances have been made in the understanding, diagnosis, and treatment of coronary artery disease (CAD). However, with shifting demographics and evolving risk factors we now face new challenges that must be met in order to further advance are management of patients with CAD. In parallel with advances in our mechanistic appreciation of CAD and atherosclerosis, nanotechnology approaches have greatly expanded, offering the potential for significant improvements in our diagnostic and therapeutic management of CAD. To realize this potential we must go beyond to recognize new frontiers including knowledge gaps between understanding atherosclerosis to the translation of targeted molecular tools. This review highlights nanotechnology applications for imaging and therapeutic advancements in CAD.

Keywords

Atherosclerosis; Molecular imaging; Inflammation; Nanotechnology; Macrophage; Endothelial cell

Introduction

Our understanding of atherosclerosis has greatly advanced over the last several decades. We now appreciate that atherogenesis is centered around the complex interplay between lipoproteins, endothelial cells, smooth muscle cells, and the immune system. Because of advances in the cellular and molecular understanding of atherosclerosis and parallel advances in nanotechnology, the possibility now exists to leverage this knowledge for diagnostic and therapeutic application. Clinical challenges including the identification of “vulnerable” plaques that predispose to acute coronary syndrome (ACS) and cell-specific immune-modulating therapeutics that work in concert with anti-platelet and anti-lipid agents

are within reach. However, as these possibilities become more tangible, we must not only recognize the potential but also the hurdles limiting these translational approaches.

Several excellent reviews have summarized the potential applications for nanomedicine in cardiovascular disease [1–3]. This review builds upon recent advances in nanotechnology to outline imaging and therapeutic approaches in atherosclerosis. The first part of this review summarizes our current understanding of the molecular and cellular drivers of atherosclerosis. The second part highlights molecular imaging applications with respect to atherosclerosis. The last part uses our conceptual understanding of atherogenesis to highlight nanotherapeutic approaches with a particular focus on endothelial and macrophage-specific approaches.

Molecular Basis of Atherosclerosis

Atherosclerosis is a chronic inflammatory disease affecting all arteries throughout the body leading to arterial wall thickening and luminal narrowing. It is the primary cause of acute coronary syndrome (ACS) and cerebral vascular accident and is responsible for 23.3 million deaths annually [4, 5]. The atheroma is the framework of atherosclerosis where multiple inflammatory signals and receptors result in both the acute and chronic drivers of the disease. Underlying risk factors lead to subtle injury of the endothelial surface with increased deposition of cholesterol-containing lipoproteins (ie. LDL) and inflammatory cells into the extracellular matrix. Biochemical modification, specifically oxidation, triggers an innate immune response to enhance leukocyte adhesion with site-specific recruitment of monocytes, mast cells, neutrophils, as well as natural killer and dendritic cells [6]. This process is mediated by multiple mechanisms including the increased expression of adhesion molecules that enhance leukocyte recruitment [7]. Following leukocyte adhesion a proinflammatory cascade of cytokines, chemokines, eicosanoids, proteases, reactive oxygen species, and costimulatory molecules results in the expansive inflammatory reaction.

The macrophage plays a major role in the progression of vulnerable atherosclerosis [8]. The maturation of macrophages includes the upregulation of pattern-recognition receptors allowing for the uptake of modified LDL with conversion to foam cells [9]. The differentiation of macrophages remains with great plasticity guided by the local microenvironment [10, 11]. Growth factors and cytokines released by foam cells signal vascular smooth muscle cell migration from the media to the intima resulting in extracellular matrix secretion and fibrous cap formation [12]. One role of the M2 macrophage is lipid clearance by its own endoplasmic reticulum. However, in the setting of a high lipid burden this regulatory process fails. This leads to the release of lipid, tissue factor, and metalloproteinases further propagating the prothrombotic state and triggering acute coronary syndrome [13]. The dynamic interplay between specific immune cells, the endothelial barrier, and lipoprotein particles underscore our molecular understanding of atherosclerotic disease. To better characterize atherosclerosis, a new framework is evolving with novel biological targets for modulating this complex inflammatory cascade [14].

Nanotechnology and Molecular Imaging in Atherosclerosis

Molecular imaging approaches have the potential to not only better identify patients at risk of ACS, but also may provide mechanistic insights into CAD and ACS, an understanding that will serve as the foundation for novel therapeutic approaches. Here we highlight both novel applications of nanotechnology in imaging within the atheroma (Fig. 1) as well as challenges to applying these molecular tools to biologic systems.

Fluorescence-Imaging

Nanoparticles have been extensively applied in preclinical studies to monitor atherogenesis and inflammation using fluorescence technologies. For example, the use of fluorophore-labeled nanoparticles has expanded our understanding of post-MI remodeling and the role of inflammatory monocytes [15]. While it is clear that nanoparticle-based fluorescence imaging enabled mechanistic preclinical studies, the accessibility of these approaches to clinical patient populations is limited. Because the detection of fluorescence necessitates the absorption and emission of light, the wavelength of light is critical in dictating the depth of penetration. In the clinical setting, this is a significant limitation except in scenarios where a catheter could be placed in close proximity to the biological target decorated by a fluorescent nanoparticle. Additionally, traditional in vivo fluorescence imaging also suffers from interference from autofluorescence from molecules and structures common to the vasculature (ie. hemoglobin and elastin). Advances in fluorophore technologies including the more widespread use of near-infrared molecules has the potential to overcome some of these limitations.

Another fluorescence-based imaging technology that is emerging is fluorescence molecular tomography (FMT) [16]. FMT relies on collecting photons that are propagated three-dimensionally through the tissue of interest with image reconstructed by combining these three-dimensional measurements to obtain a distribution of the fluorescent molecules in the tissue of interest [17]. The spatial resolution of this technique ranges from approximately 1–3 mm. By increasing the source-detector pairs and nanoparticle contrast agents, resolution and quantification can be expected to improve. With respect to atherosclerosis, one example of FMT application is the use of nanoparticle “smart” probes targeting activated macrophages and macrophage-specific protease activity which contributes to atherosclerosis [18, 19].

Contrast Enhanced Ultrasound (CEUS)

Microbubble enhancement media (ie. albumin, lipid, synthetic polymer) has been applied in the clinical setting to enhance reproducible intravascular tracings [20]. Building on these advancements, echogenic liposomal agents have been developed as novel ultrasound contrast agents that can be used to probe the molecular details of atherogenesis and endothelial biology [21]. Liposome size can vary from 20 nm to 10 μ m and can take on many secondary structures depending on dispersion. These characteristics are important in designing cell-specific acoustic agents as they all affect the biological properties of the contrast agent [22]. Specifically, acoustic liposomes conjugated with antibodies to vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), or

fibrin have been used in preclinical studies of atherosclerosis [23–25]. Emerging studies have targeted VCAM-1 allowing for direct molecular visualization of the endothelial and vasa vasorum receptors with reversible VCAM-1 expression observed following initiation with statin therapy [26, 27]. Because many of these agents have been used clinically, the potential to translate cell-specific liposomal ultrasound agents especially with respect to identifying high-risk plaques or intraplaque neovasculature is high [28].

Positron Emission Tomography (PET)

One of the most fundamental techniques of “molecular” imaging is the use of PET-tracer fluorodeoxyglucose (FDG) to capture metabolically active cells with correlations in underlying atherosclerosis [29]. PET offers enhanced resolution of 250 μM –5 mM with sufficient resolution for diagnostic utility. Studies have correlated FDG uptake with macrophage infiltration and circulating inflammatory biomarkers in arteries with clinically significant CAD [30]. Despite these findings, several fundamental questions hinder clinical application of FDG to the coronary system. These include inconsistent glucose uptake with proinflammatory mediators and non-specific glucose uptake due to tissue hypoxia [31]. Alternative radiolabeled tracers include ^{11}C -PK11195, a selective ligand of translocator protein (TSPO) that correlates with multi-fold enhancement of monocyte activation [32]. Carotid plaques with associated neurologic symptoms (i.e., stroke or transient ischemic attack) evaluated with both PET/CT and with ^{11}C -PK11195 revealed enhanced tissue to background ratio and lower CT attenuation [33]. Uptake of ^{11}C -PK11195 appears more focal and localized compared to FDG uptake, suggesting that it may better interrogate intraplaque inflammation although further studies are needed for cardiovascular application [34]. One major barrier to the application of this technology includes the need for an on-site cyclotron facility.

Magnetic Resonance Imaging (MRI)

MRI offers high-resolution (25 μM –1 mM) characterization of plaque structure with multiple sequences to enhance characterization of the atherosclerotic plaque including the fibrous cap and necrotic core [35, 36]. Recent work has evaluated the use of ultra-small super-paramagnetic iron oxide (USPIO) particles to activated macrophage to further risk stratify plaques in regards to risk of rupture [37]. USPIOs have been developed with biocompatible coatings such as dextran with uptake resulting in reduced relaxation times and T2 signal. Initial studies of USPIO-MRI in vivo revealed significant reduction in plaque uptake with high-dose statin therapy, although follow-up studies failed to associate signal changes with a clinically significant decrease in major adverse cardiovascular events [38, 39]. These studies remain limited with unclear characterization of non-specific enhancement due to local hemorrhage or focal calcification.

Initial preclinical studies by Nahrendorf et al. included functionalized magnetic nanoparticle VINP-28 with cyclic peptides targeted to VCAM-1 expressed on endothelial cells and macrophages with reductions in VCAM-1 expression found to be a function of statin pharmacotherapy in apolipoprotein E-deficient mice [40]. Utilizing a different targeting moiety, gadolinium-loaded immunomicelles were targeted to the macrophage scavenger receptor with cell-specific delivery for imaging of plaque-associated macrophages within the

atheroma [41]. Oxidative stress has been directly monitored using a myeloperoxidase sensor bis-5HT-DTPA (Gd) with localized polymerization at sites of inflammation [42]. Paramagnetic gadolinium nanoparticles targeted to $\alpha_v\beta_3$ -integrins have been shown to image angiogenesis as well as track therapeutic activity in animal models [43, 44]. Iron oxide nanoparticles have been extensively studied due to enhanced biocompatibility afforded by dextran-coating [45]. Through time, preclinical studies of nanoparticle-enhanced MRI have revealed insight into neovascularization, expression of adhesion molecules, plaque macrophage inflammation, and inflammatory leukocytes. Despite the preclinical utility of MRI based nanoparticles, many limitations exist in scaling this technology for generalized use including the requirement for baseline imaging with prolonged acquisition times versus other techniques, complex synthesis schemes, and the need for further understanding of the interaction between synthetic polymers and biologic systems.

Computed Tomography Angiography (CTA)

CTA offers resolution of 25–250 μM , although this technology does not offer the fine soft tissue differentiation afforded by MRI sequence enhancement. Although CTA has been applied for the direct monitoring of stenosis many limitations remain in characterizing high risk plaques given diverse plaque composition [46, 47]. Multicolor spectral CT has been developed to overcome this limitation through hot-spot imaging to differentiate specific elements such as calcium versus iodine [48]. CTA can detect several plaque characteristics which are associated with acute ischemic events, such as larger vessel areas, positive remodeling, and a higher proportion of non-calcified and mixed plaques [49]. Initial animal studies of the macrophage targeted nanoparticle N1177 have co-localized CT enhancement with that of FDG uptake [50, 51]. Gold-labeled high density lipoprotein is also under investigation for localization to activated macrophages in intra-plaque inflammation [52].

Multi-Modality Applications

Limitations of single imaging techniques can be overcome by using multiple different modalities to confirm and normalize signals. To overcome the limitation of non-specific FDG uptake using PET, studies using dextran have shown specific uptake by macrophages instead of the surrounding myocardium [53]. Radiolabeled ^{64}Cu as well as ^{89}Zr based nanoparticles have utilized dextran specificity to monitor macrophage activity using PET-CT and PET-MRI, respectively [54, 55]. Using a dextran ^{64}Cu -labeled, magnetofluorescent nanoparticle, trimodal vascular surveys were performed on animal models with interrogation by PET, MRI and cross-referenced with optically monitored CD68 [54]. The degree of cross modality application also proves to be a barrier to the synthesis and biocompatibility of these technologies.

Nanotechnology: Therapeutic Opportunities in Atherosclerosis

Nanoparticle therapeutics were first developed for the treatment of cancer [56]. These initial applications leveraged properties of the tumor microenvironment whereby nanoparticle extravasation is promoted by both enhanced local vascular permeability and impaired lymphatic drainage. This combination of microenvironment properties is termed enhanced permeability and retention (EPR), the most common mechanism of passive targeting [57].

As identification of specific molecular receptors in atherosclerosis, new cell-specific targets are emerging that allow for active targeting of specific receptors on specific cells or tissue structures. Although multiple cell lineages have been identified in atherogenesis, here we will focus on the specific targeting of endothelial cells and macrophages in atherosclerosis (Fig. 1).

Endothelial Cell-Specific Applications

Studies of the retina have demonstrated that deficiency of very low-density lipoprotein receptors results in WNT activation and neovascularization [58]. Recently, a plasmid expressing an extracellular domain of the very low-density lipoprotein receptor was synthesized in an encapsulated poly (lactide-co-glycolide acid) nanoparticle (VLN-NP). In murine models of neovascularization, VLN-NPs were found to inhibit Wnt3a-induced cell proliferation, migration, and tube formation, resulting in reductions in vascular endothelial growth factor and neovascularization that also has potential benefits for atherosclerosis including plaque regression and stabilization.

Most therapeutic approaches have focused on mitigating the initiation or progression of inflammation. However, in the recent past, an appreciation that resolution of inflammation is also critically regulated revealed another potential therapeutic opportunity with respect to atherosclerosis. One mediator of inflammatory resolution includes a glucocorticoid-regulated protein called Annexin A1 (ANXA1). The activity of ANXA1 can be mimicked by its amino-terminal peptide containing amino acids 2-26 (Ac2-26). Collagen IV nanoparticles (NP) targeted with Ac2-26 were recently evaluated for their therapeutic activity in Ldlr $-/-$ mice [59]. This targeted NP was shown to increase collagen (with corresponding decrease in collagenase activity) with decreasing oxidative stress and ultimately reduce plaque necrosis. Importantly, these physiologic improvements were not observed in mice lacking the FPR2/ALX receptor further demonstrating the therapeutic potential of targeted therapeutics. Together, this work highlights both a new anti-inflammatory pathway in addition to a targeted nanotherapy designed against this pathway with evidence to protect against advanced atherosclerosis. This work of selective activation of ANXA1 highlights how nanotherapeutic targeting holds potential to understand new frontiers in inflammation across pathologic states.

Targeted molecular imaging and therapeutics have been developed using different perfluorocarbon nanoparticle carriers and molecular payloads that have shown great promise in models of myocardial infarction and atherosclerosis [60]. As one example, an arginine-glycine-aspartic acid (RGD) per-fluorocarbon (PFC) nanoparticle selectively delivered both imaging and therapeutic payloads to endothelial cells overexpressing the $\alpha v \beta 3$ -integrin receptor [44]. Through this design, molecular payloads were targeted to cells critical in atherogenesis that could subsequently be utilized to monitor biologic response.

Macrophage-Specific Applications

Given the role of monocytes in atheroma instability, pathways to decrease monocyte recruitment and proliferation offer potential therapeutic targets. For example, the local environment including tissue hypoxia is known to modulate macrophage responses through

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differential activation of STAT3 and HIF1 α with resulting impact on other components of atherogenesis such as cholesterol biosynthesis [61]. To directly target the monocyte, Lobatto et al. developed a liposomal glucocorticoid carrier that relied on prolonged circulation for the delivery of potent anti-inflammatory hormones [62]. Utilizing dual labeled paramagnetic GD-DTPA-DSA lipids, therapy was localized to the inflamed vasculature with corresponding reductions in arterial wall inflammation confirmed by 18-FDG PET. In addition, there were anti-angiogenic effects found by permeability studies of the target vessel using dynamic contrast-enhanced MRI without a complete understanding into the complex mechanism of macrophage localization.

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Work by Leuschner et al. has provided insight into the precise targeting of inflammatory monocytes using an in vitro model [63]. Through administration of a lipid nanoparticle to deliver siRNA against the proinflammatory chemokine receptor CCR2, attenuation of the monocytic inflammatory cascade was achieved. This therapy was found to reduce the number of monocytes in atherosclerotic plaques as well as infarct size following coronary occlusion. Histologic evaluation confirmed an 82 % reduction in monocyte and macrophage population, a 46 % reduction in CD11b with resultant 38 % reduction in lesion size.

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Monocyte populations were uniquely deployed by Choi et al. as a component of a nanodevice coupled with gold nanoshells to target monocytes within destroyed in vitro hypoxic tumor regions [64]. This technique utilized dual therapy of initial phagocytosis of gold nanoshells followed by photo-induced cell death of monocytes/macrophages within tumor spheroids. Their approach highlighted a unique utilization of differentiated macrophages in combination with a nanoparticle system. This technique, when coupled with advances in interventional cardiology, may offer a localized technique for selective targeting in atherosclerosis as well highlighted the versatility of monocytes within tissue models.

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Getts et al. recently reported on the in vitro activity of negatively charged immune modified microparticles (IMPs) [65]. Following administration, these particles were found to localize to inflammatory monocytes in a pathway shared with senescing leukocytes. Following uptake, monocytes were found to arrest trafficking to localized sites of inflammation, rather undergoing sequestration to the spleen with apoptotic caspase-3-mediated clearance. Administration of IMPs in mouse models of myocardial infarction markedly reduced monocyte accumulation at inflammatory foci, reduced disease symptoms, and promoted tissue repair. The role of these agents in reducing localized inflammation was studied in models including autoimmune encephalomyelitis, dextran sodium sulfate-induced colitis, thioglycollate-induced peritonitis, and lethal flavivirus with generalized anti-inflammatory properties. Together, this study highlights an intricate interplay between scavenger receptors, the spleen, and inflammatory monocyte function and supports the translation of IMPs for therapeutic use in monocyte potentiated disease.

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The combination of nanotechnology and current medical therapies has recently been shown to be an effective therapy for suppression of atherosclerotic plaque inflammation. An injectable formulation of reconstituted HDL containing statins was recently synthesized and evaluated for activity in apolipoprotein E-knockout mice [19]. This study confirmed co-localization of plaque macrophages and associated plaque inflammation. While current

statin therapy relies upon cholesterol reduction and systemic anti-inflammatory effects including the emigration of macrophages from plaques, these targeted statin nanotherapeutics have been suggested to inhibit monocyte recruitment [66, 67]. Targeting in this case allowed for high-dose therapy while avoiding common clinical toxicities (ie. hepatotoxicity, myopathy). Using a 1-week nanotherapy of high dose simvastatin loaded on a HDL-based nanoparticle, Tang et al. reported enhanced antiproliferative isoprenoid activity with inhibition of NF- κ B mediated proinflammatory monocyte recruitment [68]. At a time where basic science has revealed an underlying inflammatory state of high-risk plaques, the potential to deliver targeted anti-inflammatory therapy at time of myocardial infarction has potential to stabilize high-risk plaques and prevent recurrent events.

Conclusion

Although clinical outcomes in CAD has greatly improved over the last several decades, the incidence of myocardial infarction and stroke continues to remain high. The recent past has seen tremendous advances in our understanding of atherogenesis, uncovering cellular and molecular networks between endothelial cells, lipoproteins, and the immune system. This understanding of distinct stages of atherosclerosis has provided opportunities for novel therapeutic approaches. Nanotechnology, as a multidisciplinary field of chemistry, biology, engineering and clinical medicine, offers a unique approach to the development and application of new diagnostics and therapies for inflammatory drivers of atherosclerosis. Although the list of targets, delivery platforms and therapeutics continues to grow, challenges in their advancement have resulted in a paucity of approved clinical agents. Through future development of cell-specific, multifunctional nanoparticles from imaging to therapeutics, nanomedicine can work towards new frontiers in diagnostics and therapeutics by targeting the inflammation driving atherosclerosis, thus holding the potential for further improving cardiovascular outcomes.

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Fig. 1. Nanotechnology approaches targeting endothelial and macrophage populations in atherosclerosis. Within the atheroma nanotechnology has been applied for both imaging and therapeutic delivery to the endothelial cell (1) and macrophage (2). **(a)** Imaging applications in atherosclerosis including both nanoparticle description and imaging technique. **(b)** Therapies applied with both description of nanoparticle design and therapeutic payload