Molecular imaging of macrophage protease activity in cardiovascular inflammation in vivo

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Summary
Macrophages contribute pivotally to cardiovascular diseases (CVD), notably to atherosclerosis. Imaging of macrophages in vivo could furnish new tools to advance evaluation of disease and therapies. Proteolytic enzymes serve as key effectors of many macrophage contributions to CVD. Therefore, intravital imaging of protease activity could aid evaluation of the progress and outcome of atherosclerosis, aortic aneurysm formation, or rejection of cardiac allografts. Among the large families of proteases, matrix metalloproteinases (MMPs) and cysteiny1 cathepsins have garnered the most interest because of their participation in extracellular matrix remodelling. These considerations have spurred the development of dedicated imaging agents for protease activity detection. Activatable fluorescent probes, radiolabelled inhibitors, and nanoparticles are currently under exploration for this purpose. While some agents and technologies may soon see clinical use, others will require further refinement. Imaging of macrophages and protease activity should provide an important adjunct to understanding pathophysiology in vivo, evaluating the effects of interventions, and ultimately aiding clinical care.

Keywords
Proteases, imaging, macrophage

Introduction
Innovations in imaging continue to revolutionise the practice of medicine by providing exquisite anatomical details that enhance diagnostic capabilities and enable monitoring of therapeutic interventions. In cardiovascular medicine, ultrasound, computed tomography (CT), nuclear, optical, and magnetic resonance imaging (MRI) provide high-resolution anatomical and functional images of the cardiovascular system, and x-ray fluoroscopy guides interventions to treat obstructive vascular disease and cardiac arrhythmias. Although many of these advanced imaging techniques provide excellent spatial resolution, in most cases they simply define anatomy or disease burden and provide physiologic information, rather than disclose information about the basic biological aspects of disease activity or therapies. Newer imaging strategies add to anatomic information by defining the cellular and molecular processes involved in cardiovascular disease (CVD), to improve understanding of the disease process, to obtain prognostic information, and to guide and follow cardiovascular therapies (1).

Inflammation plays a major role in CVD, and heightened inflammatory responses promote atherosclerosis, arterial thrombosis, vascular aneurysm formation, and rejection of transplanted hearts (2–4). Macrophages – mononuclear phagocytes in tissues derived from monocytes – are the major effectors of innate immunity in all of these CVD processes, often responding to signals from adaptive immune cells. Macrophage inflammatory functions that fight infection and promote tissue repair can turn against the host and promote disease progression. Thus, macrophages furnish an attractive target for the development of novel molecular cardiovascular imaging strategies, because these cells figure prominently in CVD, and hence macrophage-based imaging strategies might apply widely across the spectrum of cardiovascular disorders.

Molecular probes for macrophage imaging target several aspects of macrophage cell biology. Cellular probes specific for membrane markers on the cell surface can localise macrophages within tissues, and such surface proteins, the levels of which increase in stimulated cells, can preferentially identify activated cells. Surface targets for macrophage imaging, although not specific for this cell type, include VCAM-1 (vascular cell adhesion protein-1) (5), αvβ3 integrin (6–8), scavenger receptors (9, 10), ICAM-1 (inter-cellular adhesion molecule-1) (11), and CCR-2 (chemokine (C-C motif) receptor 2) (12). In addition to localisation by target-
ing surface proteins, internalisation of probes through phagocyto-
sis by macrophages can also detect such cells preferentially. Several
nanoparticle-based, superparamagnetic probes show promise in
this regard (13, 14). Stimulated phagocytes elaborate reactive
active oxygen species (ROS) that could permit their detection. Macro-
phage proteases also hold promise as targets for cardiovascular im-
ageing, because these enzymes (matrix metalloproteinases [MMPs]
and cysteinyl cathepsins) function as important regulators of CVD
processes and may comprise therapeutic targets for modulating
disease activity.

Proteases belong to several families of enzymes that catalyse
protein breakdown. These enzymes can exert endoproteolytic ac-
tivity, exopeptidase activity, or both. Approximately 2% to 4% of a
typical genome encodes proteolytic enzymes (15). The Degrad-
dome database, which does not incorporate protease pseudogenes
or sequences derived from retroviruses, lists 569 human proteases
and homologs classified into 68 families (16). Proteases fall into six
groups, depending on their mechanism of action: serine, threo-
nine, cysteine, aspartate, glutamic acid proteinases, and MMPs (also
known as matrixin). Mammals seem not to express glutamate
proteases.

As uncontrolled protease action could prove catastrophic,
several levels of regulation govern their expression and activity:
regulation of gene expression; activation of precursor zymogens;
blockade by endogenous inhibitors; targeting to specific compart-
ments such as lysosomes, mitochondria, or membranes; and post-
translational modification. Proteases control a variety of cellular
and extracellular processes. Within cells, proteinases can regulate
gene expression, cell differentiation and proliferation, or cell
death, often through limited proteolysis. Outside of cells, protei-
nases participate in extracellular matrix protein turnover, in diges-
tion of nutrients in the gut, and in host defenses and responses to
tissue injury and healing (17, 18).

Molecular imaging in cardiovascular research mostly has
focused on two families of proteases, produced abundantly by acti-
vated macrophages in inflamed tissues and implicated by consider-
able evidence in CVD: cysteinyl cathepsins and MMPs (19). This
review will summarise the current state of the art of in vivo pro-
tease activity imaging in this context and highlight some recent al-
ternatives for macrophage molecular imaging.

Imaging strategies and platforms for
protease activity

Molecular imaging probes typically exhibit a targeting moiety that
provides specificity linked to an imaging constituent (fluorophore,
radionuclide, magnetic moiety, scatterer/absorber such as micro-
bubbles, or photon generator such as luciferin) detectable by the
imaging platform. In the case of extracellular proteinases, report-
ing on their activity conveniently does not require delivery of the
probes to intracellular compartments.

Protease imaging has used two generic approaches: a) targeting
labelled small molecules to specific protein pockets, typically the
active site; and b) imaging substrates of proteases, which become
activated and detectable upon cleavage. The first approach, com-
monly used with radionuclides, has wide application in clinical
imaging with positron emission tomography (PET) or single-
photon emission computed tomography (SPECT) platforms, es-
pecially because of their non-invasiveness, high versatility, and
high sensitivity. Protease inhibitors (almost exclusively MMP in-
hibitors) have been labelled with ¹¹⁷⁷C (20, 21) or with ¹⁹F (22–24)
for PET imaging in cancer. SPECT-labelled MMP inhibitors have
undergone investigation in atherosclerosis (25–28), vascular in-
jury (29), and post-infarct remodelling (30). SPECT requires the
use of isotopes like ⁹⁹ᵐTc, ¹¹¹In, ¹²³I, and ¹³¹I. To define the anatomical
origin of the radioactive signals, both of these nuclear imaging
platforms often combine with CT or MRI. The major limitations
of PET and SPECT include low spatial resolution, exposure to radia-
tion, a limited stoichiometry (one imaging probe per target
enzyme), and the inability to differentiate protease-bound from unbound (circulating, sequestered, non-specifically bound) im-
aging labels (►Fig. 1).

Like PET and SPECT, MRI instrumentation has wide availabil-
ity in the clinical setting, and offers good spatial resolution and
multi-contrast capabilities. MRI used in co-registration with PET
or fluorescent molecular tomography (FMT) provides fiducial
anatomical references. MRI suffers, however, from lower sensitiv-
ity compared with radionuclide-based or optical approaches
(►Fig. 1). MRI technology relies on the interaction of water
protons’ magnetic moments in tissues with magnetic properties of
the surrounding environment. By adding contrast agents that
magnetically modify this environment, positive (T₁-targeted) and
negative (T₂-targeted) enhancements can be generated and de-
ected by MRI. The most commonly used contrast agents are based on gadolinium, which shortens the spin-lattice relaxation
time (T₁) of nearby water protons. Studies have reported potential systemic toxicity of gadolinium, however, in patients
with renal insufficiency (31). Superparamagnetic iron oxide nano-
particles, fluorine (¹⁹F)-carrying particles, manganese-based
agents, and chemical exchange saturation transfer (CEST) agents
are the current alternatives to contrast agents for increasing MRI
sensitivity. For protease imaging in particular, studies have re-
ported the development of specific inhibitors associated with mag-
netic agents (32, 33) and protease-sensitive magnetic nanopar-
nicles (34, 35).

The use of enzyme-activatable selective fluorescent or magnetic
substrates takes advantage of the enzyme’s catalytic activity. In this
approach, each protease can convert many different imaging mol-
ecules. This property of labelled substrates provides the advantage
of manifold amplification of the signal. One class of such “smart
probes,” fluorescently-labelled substrates, produces intense fluo-
rescence when cleaved due to separation from moieties that quench
their fluorescence when in close proximity. Selectivity for certain
enzymes derives from the sequence of the peptide-based substrate.
In addition to avoiding radiation exposure, the near-infrared flu-
orescence (NIRF) imaging spectrum combines low autofluores-
cence and useful tissue penetration (millimeters to centimeters).
Fluorescence signal can be acquired in surface-weighted mode by

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fluorescent reflectance imaging (FRI) or in tomographic mode (FMT), which employs mathematical algorithms that localise the origin of the fluorescence based on the NIRF diffusion in tissues (36). The tissue penetration of NIRF suffices for imaging of intact small animals such as mice, but would likely not permit external visualisation of deep arteries (> 5–10 cm) in humans with today’s technology. To overcome this limitation, a NIRF intravascular catheter approach in development could extend this technique to the imaging of proteinases related to inflammation in atherosclerotic human arteries (37).

Intravital confocal and multiphoton microscopy (IVM) also enables NIRF imaging. Despite limited application in humans due to its invasiveness, IVM provides high spatial resolution and sensitivity in experimental animals (Fig. 1). Multiphoton microscopy presents several advantages over confocal microscopy, including increased imaging depths and reduced out-of-focus photodamage. Multiphoton technology can also detect biological materials such as collagen, microtubules, and muscle myosin without any exogenous targeted agents by second harmonic generation (SHG) imaging.

Protease imaging can also be achieved with targeted microbubbles that permit imaging by contrast-enhanced ultrasound (CEU) (38, 39). Advantages of this approach include the widespread availability of suitable imaging platforms, the lack of ionising radiation, and non-invasiveness. Disadvantages include lower inherent sensitivity, resolution, and penetration depth. Applied frequencies can currently achieve a resolution of <50 μm at reduced depth, but this is not applicable when the structure of interest is located at deeper depths.

### MMP imaging

In cardiovascular research and in protease imaging, the MMP collagenases and gelatinases have undergone the most extensive study because of their participation in degradation of macromolecules of the extracellular matrix – processes integrally involved in the remodelling of blood vessels and the myocardium (Table 1). In the inflamed atheroma and myocardium, proteinase activity increases compared to healthy tissues, in large part due to production by and release from macrophages (19, 40) (Fig. 5, upper panel).

In atherosclerosis, degradation of the collagenous extracellular matrix of the protective fibrous cap covering the lipid core of the plaque can lessen the lesion’s biomechanical strength. Rupture of the fibrous cap causes the majority of fatal acute myocardial infarction...
farcts (MI) by initiating thrombus formation (40). In the infarcting myocardium that is deprived of blood flow, ventricular remodelling may involve the extracellular matrix–degrading activities of MMP-2, MMP-9, and MMP-14 (41, 42). Elastolytic activity, potentially driven in part by MMP-9 and MMP-12, may promote aneurysm formation (43).

Proof-of-concept studies in mice have imaged gelatinase activity (MMP-2 and MMP-9) in vivo using an activatable NIRF substrate (44, 45). Confirmed by ex vivo FRI and in situ zymography, IVM and FMT imaging detected higher MMP and gelatinase activity in macrophage-rich atherosclerotic plaques and in infarct zones (Fig. 2). Recent studies on aneurysm formation also have succeeded in monitoring MMP activity by multimodality imaging, with FMT co-registered with CT to provide anatomical co-localisation (46, 47); the intensity of MMP activity correlated with aneurysm development.

Several publications have reported the generation of specific or broad-range MMP inhibitors (MMPI) labelled by radionuclides for PET/SPECT imaging. With a primary goal of screening of MMPI, broad spectrum hydroxamate pharmacophore MMPI have been coupled with $^{125}$I (48), $^{99m}$Tc (30), and $^{18}$F (23, 24, 49) for SPECT or PET imaging (Fig. 3). Generation of MMP-2 and MMP-2/MMP-9 inhibitors, coupled with $^{18}$F (50) and $^{64}$Cu (51) or MMP-14 substrate radiolabelled with $^{99m}$Tc, could permit more selective imaging of proteinases (52). Preliminary studies in athromata, aneurysm, and wire-injured arteries correlated $^{99m}$Tc- or $^{111}$In-MMPI signals with macrophages and MMP-2/MMP-9 activity in lesions by multimodal SPECT/CT imaging (25, 26, 53–55).

MRI-dedicated nanosensors for MMP-2/MMP-9 in development use nanoparticles loaded with the MR contrast agent gadolinium. Upon proteolysis, the nanoparticles exhibit highly cationic molecules that trigger attachment and intake into cells (35). Initial reports indicate a potential use for cancer targeting in vivo, but no study has yet assessed the utility of such agents in CVD. An MR probe for MMP-9 activity used charged nanoparticles with an iron oxide core coupled with methoxypoly(ethylene glycol)
(MPEG) molecules, maintained in a stable state by a domain containing MMP-9 substrate (34). Upon MMP-9 cleavage, the remaining particles aggregated due to magnetic and electrostatic attraction. This study did not report in vivo MRI.

**Cathepsin imaging**

The cysteinyl proteinases included in the broad cathepsin (Cat) category also degrade matrix collagen and elastin (Table 1). Multiple reports implicate these enzymes in atherogenesis and in aneurysm formation (4, 56, 57). As in the case of MMPs, activatable NIRF probes can visualise cathepsin activity in vivo. An early study demonstrated the suitability of a Cat-B-dependent Cy5.5-labelled activatable NIRF probe for atherosclerosis imaging in vivo. In atherosclerotic mice after 24 weeks of consuming an atherogenic diet, FMT coregistered with MRI detected a strong signal in aortic plaques. In the same study, aortic aneurysms displayed high Cat-B activity assessed by ex vivo FRI. In contrast, arteries of healthy control mice yielded little signal. Histological analysis of the aorta after dissection confirmed a higher expression of Cat-B, colocalised with macrophages within lesions, as compared to healthy arterial tissue (58). A recent study demonstrated by ex vivo imaging that a similar probe could assess quantitatively the impact of anti-atherosclerotic treatments, such as statins, that reduced Cat-B activity in macrophage-rich plaques (59).

The potent elastolytic and collagenolytic protease Cat-K (57) may contribute to plaque destabilisation, aneurysm formation, and outward arterial remodelling during atherogenesis or following injury. In a study that used a NIRF imaging agent based on a Cat-K peptide substrate in vivo in mice, similar to the study described above involving Cat-B, optical imaging (IVM, ex vivo FRI) revealed more than twice as much signal in atherosclerotic mice and in human carotid endarterectomy specimens ex vivo, as compared with the control agent. The functional relevance of Cat-K was validated by fluorescent microscopy that co-localised Cat-K NIRF and Cat-K positive macrophages with fragmented elastin fibres within the media of underlying plaques (60).

Activity of the cysteinyl protease Cat-S associates with vascular calcification in mice with experimentally induced chronic renal disease (61). Injection of Cat-S activatable and osteogenesis-targeted imaging agents allowed the co-localisation of Cat-S activity and calcification in aortas and aortic valves by IVM and ex vivo FRI. Cat-S-deficient mice exhibited less calcification and no Cat-S activity, validating the specificity of the probe and the involvement of the protease in this process.

In proof-of-concept studies, a broad cathepsin-activatable probe has sensed proteolytic potential in vivo in rabbit atherosclerotic lesions by an intravascular approach to overcome the limited-depth penetration of NIRF that precludes its clinical use for interrogation of deep arteries in humans (62). The same protease probe has visualised cathepsin activity experimentally in cardiac allografts undergoing rejection, and in MI. Seven days post-transplantation of histo-incompatible hearts without immunosuppression, myocardial graft rejection associated with accumulation of macrophages that produced active cathepsins, as visualised by FMT in vivo and FRI ex vivo by co-injecting the cathepsin probe and macrophage-targeted magneto-fluorescent nanoparticles. Macrophage recruitment was also imaged in vivo by MRI (13, 32, 33). In other experiments, co-injection of these two imaging agents 4 days after coronary ligation in mice demonstrated the active recruitment of macrophages and increased cathepsin activity in healing zones of the ischaemic heart, as compared to healthy myocardium (14).

Cat-D is a lysosomal aspartyl protease that might play an important role in some cancers and in Alzheimer disease. Preliminary studies reported the development of Cat-D NIRF- and MRI-dedicated probes (63, 64).

**Other targets for cardiovascular imaging of macrophage activity**

In addition to MMP and cathepsin proteases, molecular imaging may prove useful for more extensively visualising activity of macrophages via other enzymes or molecules that are especially relevant in CVD.

The enzyme myeloperoxidase (MPO) has received considerable interest as a biomarker for inflammation in atherosclerotic plaques and for future acute coronary events. Patients with stroke have elevated plasma MPO concentrations (65), and MPO levels seem to predict major cardiac events in patients presenting with acute chest pain (66) and in apparently healthy individuals (67). Although generally most abundant in granulocytes, within atheroma, MPO derives mainly from macrophages and macrophage-derived foam cells. MPO activity results in the generation of ROS – notably, hypochlorous acid (HOCl) – that may contribute to local alterations in arterial biology. In infarcting myocardium, MPO may also impede the healing remodelling process (68). A high level of MPO in plasma predicted an increase in 5-year mortality for patients with acute MI (69). To assess MPO activity in vivo, a novel MPO sensor based on gadolinium permits detection of MPO by MRI (70). Studies in rabbits and mice reported high MPO activity, reflected by hyper-enhancement in diseased aortas and in infarct zones, when conventional gadolinium contrast would have subsided (Fig. 4) (68, 71, 72).

Because macrophages play a key role in inflammatory CVD, they have received much attention as a target for molecular imaging. Iodinated, fluorescent, and/or magnetic nanoparticles based on an iron-oxide core phagocytosed by macrophages in plaques and in injured myocardium, can be imaged by optical or MRI or CT approaches (13, 14, 73–75). 18F-fluorodeoxyglucose (18FDG) can track glucose uptake in macrophages. FDG signals co-localise with macrophage-rich inflamed sites in atheromas, as assessed in humans and in mice by SPECT/CT (76–78). Uptake of 11C-choline or specific targeting imaging agents (99mTc-anti-RAGE antibody, 18F-galacto-RGD for αvβ3 integrin targeting, 99mTc-Annexin A5, 18F-radioligand for a mitochondrial benzo-
diazepine receptor) has undergone testing in experimental models of atherosclerosis (7, 79–83).

**Conclusion: Limits and future promises**

Non-invasive molecular *in vivo* imaging, as an emerging technology, is a reality for several disease states but still requires refinement to progress into the clinical setting. In addition to the technological challenge for further increasing spatial resolution and sensitivity of the imaging hardware, a particular effort to improve or develop new imaging agents is crucial. For example, particle size and electrical charge are of great importance for the behaviour of the agents *in vivo*, and therefore need to be carefully established. Moreover, future clinical applications for molecular imaging will require optimisation of specificity, brightness (tissue penetration), and development of non-toxic agents.

Despite these current technological and cost-related limits, molecular imaging in general, and protease activity in particular, should contribute to biomedicine in the near future.

Detection of asymptomatic disease, and the prediction of severe complications, remains elusive to current diagnostic tools for many vascular disorders. Intravital imaging of protease activity promises to extend beyond anatomy and disclose biological aspects of the progress of atherosclerosis, myocardial infarct healing, heart transplant rejection, or aortic aneurysms.

Moreover, novel therapeutic strategies in CVD should emerge from molecular imaging – including non-invasive imaging markers for prevention and personalised medicine, interventional imaging combined with treatment in the acute phase, and molecular assessment of therapeutic efficacy during treatment.

Preclinical studies in mice and rabbits have demonstrated how close we are to translating molecular imaging tools to the clinic. Imaging agents specifically targeting MMPs, cysteiny1 cathepsins, or other enzymes implicated in CVD pathogenesis have demonstrated compatibility with established imaging platforms (PET, SPECT, MRI). The promising field of optical imaging will require special efforts in hardware to detect non-invasively small accumulations of active “smart probes” in inflamed or diseased tissues. Nonetheless, studies using MMPs and cathepsin-activatable probes have achieved proof of concept of the relevance and value of functional intravital imaging.

In addition to more reliable diagnosis and potential prediction of the outcome of a disease, molecular imaging for protease activity should also serve as a tremendous tool for development, evaluation, and dose ranging of novel therapeutics *in vivo*. Moreover, the coupling of imaging and therapeutic interventions with multifunctional nanoparticles opens the possibility of “theranostic” approaches. This concept involves targeting therapeutic drugs in particles carrying imaging moieties, for selective drug delivery to diseased tissues and simultaneous monitoring of not only targeting, but also possibly efficacy. In an illustration of such an approach, agents targeting scavenger receptor 1A on macrophages can detect MMP-9 activity (84). Multi-modality imaging – for example, tri-functional agents combining magnetic, optical, and radionuclide imaging functionality – promises to expand the gamut of molecular imaging (75, 85–87). Such multimodal imaging agents must contain an adequate ratio of the various contrast agents for matching the relative sensitivity of the applied imaging modalities. This
A particular requirement may lead to the development of chemical constructs to create multiple binding sites of contrast agents, from which overall charge and size remain compatible with penetration into the targeted tissues (88).

Multimodal imaging strategies could also combine non-invasive and high-resolution technologies (e.g., confocal or multiphoton microscopy) to study protease activity in vivo, in interstitial as well as in intracellular milieu, to define their roles better and to characterise more precisely the behaviour of imaging agents in the field of CVD. Current NIRF contrast agents, for example, may be applicable to combined FMT and (optical parametric oscillator-equipped) two-photon microscopy, where two-photon microscopy contributes both subcellular resolution and imaging of intrinsic emission derived from extracellular matrix components.

Protease imaging – and more broadly, molecular imaging – not only applies to CVD, but also to other diseases such as arthritis (89), asthma (90), and cancer (91–93). Non-invasive intravital assessment of protease presence and activity will likely be feasible first by nuclear imaging in clinical trials, because of picomolar dosing and its widespread use in humans. The advantages of activatable probes over labelled inhibitors and the advent of multimodal imaging should increase efforts to introduce optical imaging into the clinic. The pace of progress in the development of novel molecular probes and imaging platforms during recent years highlights the promise of enabling advances in research and clinical applications through probing physiopathologic processes and assessment of specific molecular processes in intact subjects in vivo.

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Figure 5: Macrophage protease activity imaging in CVD. Upper panel: Impact of protease activity on extracellular matrix turnover in atherosclerosis, aneurysm, heart transplant rejection, and myocardial infarction. Lower panel: Current imaging agents and strategies for protease activity imaging in vivo.
References


