Bimodal imaging of molecular targets associated with plaque vulnerability

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Bimodal imaging of molecular targets associated with plaque vulnerability

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Bimodal imaging of molecular targets associated with plaque vulnerability

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One

General introduction

Most humans develop atherosclerosis to some extent during aging. It can be accelerated by, e.g., genetic predisposition, a Western diet, or smoking. Atherosclerosis is a disease in which cholesterol esters are deposited inside the vascular wall at certain sites, called atherosclerotic plaques. This deposition may lead to complete or partial occlusion of the vessel or to rupture of the plaque, causing thrombosis. Plaques usually develop at sites of disturbed laminar flow, leading to low and oscillatory shear stress on the endothelial cells ¹. Examples of such sites are the bifurcation in the abdominal aorta, the bifurcation in the common carotid artery, and the many bifurcations in the coronary arteries. Low or oscillatory shear stress induces mechanotransduction-mediated alterations in protein expression in endothelial cells ¹, which can lead to endothelial activation under the influence of risk factors ¹. Endothelial activation in turn may induce inflammation ^{2, 3}. Activated endothelial cells express adhesion molecules that allow platelets and different subsets of leukocytes to adhere to the endothelium ^{2, 4, 5}. After adhesion, leukocytes enter the vascular wall ². Once inside the vascular wall, monocytes, one of the subsets of leukocytes, differentiate to macrophages and start to endocytose lipoproteins ². In time, plaques increase in size by taking up more lipids and leukocytes. Macrophages endocytose more lipids, forming foam cells, and this eventually results in development of a necrotic core. Fibrous caps that consist mainly of collagen and elastin develop and cover the plaque. When plaques become advanced extracellular matrix degrading enzymes degrade the fibrous cap, which may cause cap rupture. In this process the necrotic core of the plaque is exposed to the blood stream, causing thrombosis. This may lead to the occlusion of the artery at this site or at a site more downstream, causing ischemia in the tissue distal to the occlusion, e.g., a myocardial or cerebral infarction.

Prevention of such ischemic events would lead to a major decrease of morbidity and mortality in the industrialized countries, and is thus one of the major research areas in biomedical science. Currently, rupture-prone sites are detected after an ischemic event has taken place by performing angiography. This is a technique in which an X-ray contrast agent is injected intravenously in the patient, visualizing the arterial lumen. The occluded artery as well as the culprit lesion is identified as a narrowed artery. Patients are treated with thrombolytic therapy, angioplasty of stenotic plaques, or both. How-

ever, the culprit lesion is not necessarily a stenotic plaque, as rupture-prone plaques may very well be (or perhaps even in most cases ^{1, 6}) outward remodeled plaques, and thus not visible in angiography. Furthermore, it would be convenient if these rupture-prone plaques were identified prior to actual events. In other words, atherosclerotic patients-at-risk should be screened for rupture-prone plaques so that preventative action can be taken.

Molecular imaging is an emerging branch of biomedical science, in which contrast agents are developed that bind molecular targets specific for a certain pathology. This concept has major advantages. First, as these contrast agents bind with molecular specificity, aspecific localization of the contrast agents is reduced, and diagnosis is more accurate. Second, as pathologies induce molecular changes prior to clinical events this disease can be detected prior to these events, and preventative measures can be taken. Third, as the aspecific binding of these contrast agents is considerably reduced, a lower dose is needed, reducing possible side-effects for the patient.

Imaging can be performed using several techniques, such as fluorescence imaging, magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), or single photon emission computed tomography (SPECT). They all have their own advantages and disadvantages in research and in clinical use. Fluorescence imaging provides a high (sub-cellular) resolution (μm range) and high sensitivity. Some anatomical information is provided as some tissue-types exhibit autofluorescence. However, fluorescence imaging is usually invasive due to the low penetration depth of light and is not often used as a clinical imaging technique. New optical, potentially clinical techniques are being developed, as discussed in chapter 2. CT offers a reasonable resolution (mm range) and sensitivity and provides some anatomical information, but is an imaging technique using radiation, and iodinated contrast agents are necessary for visualization. Thus, CT is potentially harmful to patients. PET and SPECT offer high sensitivity but low resolution (several mms) and no anatomical information. Furthermore, they are also imaging techniques using radiation and requiring radioactive contrast agents. MRI has a relatively high resolution (sub-mm range), and is a non-invasive technique providing anatomical information, but is not very sensitive to contrast agents.

In this thesis, three different nanoparticles were developed. Each nanoparticle targets a molecular epitope specific for either atherosclerosis or thrombosis, or both. All three nanoparticles were bimodal, i.e., visible with both fluorescence microscopy and MRI. In this way, advantages of both imaging techniques are combined. Two atherosclerosis-targeting nanoparticles consisted of a fluorescent, streptavidin-coated quantum dot, which is approximately 10 nm in diameter. One nanoparticle targets dying cells, the other targets collagen present in atherosclerotic plaques. MRI contrast was provided by Gadolinium-DTPA wedges, each containing 8 Gadolinium-DTPA moieties for signal amplification. The two nanoparticles contained different ligands, each with their own molecular targets. The third nanoparticle is a peptide-based, thrombus-targeting nanoparticle, and is much smaller (< 1 nm). A sequence of 12 amino acids with known binding properties to fibrin was used, to which both an organic fluorescent dye (rhodamine) and Gadolinium-DTPA were covalently attached. All three nanoparticles were first tested *in vitro* on cells and/or blood clots as a proof-of-principle. Next, mouse models of atherosclerosis and thrombosis were used to establish the feasibility of the three bimodal nanoparticles to image either pathology in carotid arteries. Arteries were imaged both *in vivo* with MRI and *ex vivo* with two-photon laser scanning microscopy (TPLSM), in order to allow both non-invasive imaging with (relatively) high resolution and sub-cellular resolution for validation of the molecular localization of the nanoparticle.

Chapter 2 is an introductory chapter, discussing the structure of arterial walls. Next, atherosclerosis in mice is discussed, explaining the difference between atherosclerosis in mice and men. Then, biochemical processes of cell death and blood coagulation are discussed. Furthermore, the physical principles of two-photon laser scanning microscopy (TPLSM) are reviewed. Also, it is illustrated how plaques were imaged using TPLSM. In chapter 3, optical imaging techniques and novel optical en hybrid (bimodal) nanoparticles are reviewed, followed by the development of atherosclerosis in humans and appropriate molecular targets and corresponding targeting ligands. Furthermore, the clinical potential of these imaging techniques and nanoparticles is discussed. In chapter 4, the proof-of-principle of a bimodal quantum dot for the detection of apoptosis and activated platelets is provided in an *in vitro* and *ex vivo* carotid artery study. Chapter 5 describes an *in vivo* mouse study using the bimodal nanoparticle from chap-

ter 4, functionalized with two different molecular targets, to visualize atherosclerosis in mice. Here, it is determined if this bimodal contrast agent is applicable to atherosclerotic mice, imaged *in vivo* with MRI and *ex vivo* with TPLSM. In chapter 6 a peptide-based bimodal nanoparticle is described, that is tested in an *in vitro* study providing the proof-of-principle, followed by an *in vivo* mouse study of fresh thrombi in carotid arteries using *in vivo* MRI and *ex vivo* TPLSM. Finally, chapter 7 is the general discussion of this thesis.

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Two

Imaging of atherosclerosis in murine arteries

In this chapter, several subjects will be discussed as an introduction to this thesis. These subjects include vascular wall structure, differences in atherosclerosis development between mice and humans, cell death in atherosclerotic plaques, the blood coagulation cascade, the physical principles of two-photon imaging, and plaque imaging with TPLSM.

Arterial wall structure

The mammalian arterial wall consists of three different layers; tunica intima, tunica media, and tunica adventitia (figure 1). Tunica intima is the most luminal layer, consisting of the endothelium, underlying connective tissue (the basal lamina), and the internal elastic lamina. The endothelium consists of a single layer of endothelial cells, and provides an antithrombotic surface, preventing blood from coagulating under normal circumstances. Furthermore, endothelial cells monitor and regulate many processes, such as vasoconstriction and vasodilatation by releasing regulatory factors, permeability to specific molecules, and, in case of inflammation, capturing and allowing trans- and paracellular migration of inflammatory cells ^{1, 2}. The luminal side of endothelial cells is covered by a matrix of proteoglycans and glycoproteins called the glycocalyx. This matrix also regulates vascular permeability and may induce mechanotransduction ³. The basal lamina connects the endothelial cells to the vascular wall; it is a structure of extracellular matrix components such as collagen. Collagen is a fibrillar protein that provides the strength of the vascular wall. The internal elastic lamina separates the tunica intima from the tunica media. It consists of elastin, an extracellular matrix protein that provides the elasticity of the vascular wall.

The second layer is the tunica media, which consists of several layers of smooth muscle cells. These smooth muscle cells are enclosed by a basal lamina and connective tissue (collagen types I, III, and IV, and elastin). Furthermore, in between these layers of cells elastic laminae are present ². The number of elastic laminae depends on the size and type of the artery: large arteries, such as aorta and carotid arteries, are more elastic and contain more elastic laminae. This is because these arteries have to deal with large pulsations in arterial pressure. Muscular arteries are present further downstream, and

Figure 1. Schematic representation of transverse view of arterial wall of a murine carotid artery. '1' indicates tunica intima, '2' tunica media, and '3' tunica adventitia. Color figure on page 178.

contain fewer elastic laminae. Unlike the name suggests, muscular arteries actually have a lower smooth muscle cell density than elastic arteries ⁴.

The external elastic lamina separates the tunica media from the tunica adventitia, the outer most layer of the vascular wall. The adventitial layer consists of collagen type I, elastin fibers, and fibrotic cells ², and embeds the artery in the surrounding tissue.

With increasing lumen diameter, vascular wall thickness generally also increases. When arteries are larger than, roughly, 0.5 mm in lumen diameter, transmural blood supply to the outer vascular wall layers is not sufficient, and vasa vasora are necessary ⁵. These are small vessels supplying the tunica adventitia with nutrients and oxygen. Generally, murine arteries do not have vasa vasorum under normal circumstances as most arteries have a luminal diameter smaller than 0.5 mm. However, when there is a large plaque present, it may be supplied by a vasa vasorum. For example, in ApoE/LDL double knock-out mice, vasa vasora were observed in adventitia in case of a large plaque in combination with a lumen diameter larger than 0.4 mm ⁶.

Besides endothelial cells, smooth muscle cells, and fibroblasts, other types of cells may also be present within the vascular wall, such as dendritic cells, T-cells, and endothelial progenitor cells, but it is outside the scope of this thesis to discuss these.

This study investigates murine carotid arteries. In healthy conditions, they consist of an intimal layer, a media consisting of several layers of smooth muscle cells and three elastic laminae, and an adventitium (figure 1).

Atherosclerosis in mice

Mouse models of atherosclerosis are widely used to understand biology of atherogenesis. Normal mice do not develop atherosclerosis, since mice hardly have circulating LDL (the 'bad' cholesterol) as humans do, but mainly have circulating HDL (the 'good' cholesterol) ⁷. Therefore, genetic modifications have been introduced to disturb their lipid metabolism. The most common mouse model for atherosclerosis research is the Apolipoprotein E knockout mouse (ApoE ^{-/-} mouse) ⁷. Apolipoprotein E is a ligand involved in lipoprotein clearance, and ApoE ^{-/-} mice have a delayed lipoprotein clearance ⁷. Especially when fed a high-fat diet, ApoE ^{-/-} mice have increased levels of circulating chylomicron and VLDL remnants, leading, over the course of approximately 40 weeks (a mouse's lifetime), to the development of fibrous lesions that closely resemble human plaques ^{7,8}. These plaques are located mainly at sites of oscillatory shear stress, such as at branching arteries (e.g. at the branch points of the carotids at the aorta, and at the bifurcation of the carotids into the internal and external carotids), but also in the curvature of the aortic arch ^{7,8}. When ApoE ^{-/-} mice are fed a high-fat diet, these plaques start to develop after 6 weeks of age with monocyte adhesion to the endothelium. This is presumably preceded by the adhesion of platelets to activated endothelium ⁹. After about 8 weeks the first foam cells appear in lesions. At 10 weeks, intermediate lesions containing more foam cells and smooth muscle cells start to develop, followed by progression to advanced lesions containing necrotic cores at 15 weeks of age. Lesions progress further by increasing in size and advancing occlusion of the artery ⁸. This shows great resemblance with human plaque formation (see chapter 3), except on a time scale of weeks instead of years.

To increase the rate of plaque development, Von der Thüsen *et al.*¹⁰ developed a modified mouse model of atherosclerosis. They used the ApoE $-/-$ mouse on a high-fat diet in combination with a silicon collar placed around the common carotid artery, proximal to the bifurcation. This collar is mildly constrictive (30% stenosis)¹⁰, and induces plaque formation proximal to the collar, presumably due to disturbance of laminar flow proximal to the collar¹⁰. In this mouse model plaque progression is accelerated; already 6 weeks after collar placement advanced lesions are present. This model was used in chapter 5 of this thesis. These plaques resemble those in spontaneous atherosclerosis in ApoE $-/-$ mice; heterogeneous in composition, an acellular necrotic core, a well-defined fibrous cap, significant extracellular matrix composition, intra- and extracellular lipids, lipid-rich foam cells, and smooth muscle cells in the intima after 6 weeks of collar-placement¹⁰.

Murine plaques are usually of a stable, fibrous phenotype that ruptures only sporadically¹¹. In humans, plaque rupture is the most common cause for acute events such as myocardial infarction and stroke. Spontaneous rupture in ApoE $-/-$ mice does occur, however at a far too low frequency to make it a reliable and reproducible model for studying plaque rupture¹². Furthermore, murine plaque morphology differs from human plaque morphology. The latter has a well-defined fibrous cap preventing contact between blood and the underlying necrotic core. The fibrous cap can rupture which results in exposure of the necrotic core to the blood and a subsequent thrombotic response. Murine plaques do not have a well-defined fibrous cap¹¹, although Von der Thüsen *et al.* did describe plaques in mice that had a collar for 6 weeks as having a well-defined fibrous cap¹⁰. 'Rupture' described in these plaques may refer to either the disintegration of endothelium or deep erosion of the necrotic core^{11,12}, both of which may occasionally lead to thrombosis. Several mouse models have been developed in an attempt to resemble human plaque rupture¹³⁻¹⁶. Schwartz *et al.* state in their review discussing plaque ruptures in mice and humans¹¹ that an animal model of plaque rupture should presuppose the existence of a fibrous cap overlying a necrotic core, as this is required in human plaque rupture. This has so far not been achieved in mice.

In chapter 5, the collar model was used in order to determine effectiveness of new bi-modal contrast agents, because: i) the collar model has a rapid rate of plaque progres-

sion, and ii) the collar determines the location of plaque formation, facilitating visualization of the plaque by MRI. This model also has disadvantages. First, due to the invasive nature of collar placement, the survival rate of these mice is lower. Furthermore, due to collar placement, fibrous tissue develops around the collar, hindering optical imaging.

Cell death in atherosclerosis

Several types of cell death occur in living organisms. The two best defined types of cell death are necrosis and apoptosis. Necrosis is a process of cell death in which the cell increases in size and eventually bursts, inducing inflammation¹⁷. Apoptosis is a form of programmed cell death, in which the cell shrinks, divides into multiple apoptotic bodies, and is cleared by macrophages without leading to inflammation¹⁷. Apoptosis is a process necessary for homeostasis in any multicellular organism, and also plays an essential role in several pathologies. In tumor development, increased cell division as well as decreased apoptosis leads to excessive tissue growth, while after myocardial infarction apoptosis increases. Other, less well-defined types of cell death also exist¹⁸, but are not discussed here.

A common phenomenon in many types of cell death is the exposure of phosphatidylserine (PS)¹⁹ on the cellular membrane, which, in viable cells, is only exposed on the cytoplasmic membrane leaflet. In atherosclerotic plaques PS-exposing cells are present. Smooth muscle cells²⁰, macrophages²¹, and presumably also endothelial cells²² may undergo apoptosis in plaques, contributing to plaque vulnerability. Furthermore, activated macrophages²³ may expose PS, as well as activated platelets²⁴. The presence of apoptotic cells, macrophages, and platelets in plaques are all a measure of plaque vulnerability. In other words, PS imaging is a potentially appropriate tool for vulnerable plaque detection²⁵.

Annexin A5 (AnxA5) is a ligand well-known for its high-affinity binding to PS²⁶. AnxA5 is a member of the Annexin-family, the members of which share structural and functional features²⁷. The primary structure of an annexin is composed of a divergent N-terminal tail and a conserved C-terminal core that consists of 4 or 8 homologous domains. The conserved domains harbor the Ca²⁺ and phospholipid binding sites. The tertiary structure of an annexin has a bent shape with a convex and a concave side containing the

phospholipid binding sites and the N-terminal tail, respectively ²⁷. When bound to a phospholipid surface the concave side with the N-terminal tail faces away from the phospholipid surface and stays in contact with the medium surrounding the membrane. The annexins differ in their affinities for binding various phospholipid species. To date AnxA5 is the annexin with the highest reported affinity for PS binding. AnxA5 can be coupled to several contrast agents to allow imaging with different techniques, such as radiographic or fluorescent imaging. AnxA5 has been used in multiple pre-clinical and clinical studies. Examples of pathologies imaged with AnxA5 are endocarditis ²⁸, thrombosis ²⁹, atherosclerosis ^{30, 31}, abdominal aortic aneurysm ³², ischemic tissue after myocardial infarction ³³⁻³⁵, heart failure ³⁶, liver apoptosis ³⁷, and radiation-induced apoptosis in anti-tumor treatment ^{38, 39}.

Thrombosis

Thrombosis is the major event leading to infarctions after plaque rupture. Thrombi bound to the vascular wall (mural thrombi) are thus also a suitable target for detection of vulnerable plaques. The pathway leading to thrombus formation is the coagulation cascade. The initiation phase of the cascade encompasses the complexation of cell-bound tissue factor (TF) with factor VII. In vascular surroundings, TF may be expressed on smooth muscle cells, fibroblasts, and in case of inflammation, also on endothelial cells and monocytes ⁴⁰. Also necessary for initiation is binding of platelets to collagen or other extracellular matrix components after vascular wall damage, leading to platelet activation ⁴¹. Platelet activation leads to PS exposition, and coagulation factors are activated. In the amplification phase, small amounts of thrombin are exposed by TF-bearing cells, resulting in an amplification of the activation of coagulation factors by activated platelets ⁴¹. Finally, in the propagation phase, activation of more coagulation factors occurs, resulting in the conversion of large amounts of prothrombin into thrombin ⁴¹. Thrombin converts circulating fibrinogen into fibrin, which is followed by cross-linking of fibrin fibers under the influence of activated factor XIII, forming a stabilized fibrin clot. α_2 -antiplasmin is also cross-linked to fibrin under the influence of activated factor XIII ⁴², making the clot more resistible to fibrinolysis. Eventually, over the course of

hours, a stabilized clot develops consisting of activated platelets, erythrocytes, and leucocytes, in a matrix of cross-linked fibrin.

This coagulation cascade gives rise to many molecular targets of thrombosis. Fibrin is perhaps the easiest target within a clot, and has been used in several studies⁴³⁻⁴⁸. In chapter 6 of this thesis, an α_2 -antiplasmin-based contrast agent is used to target fibrin. Also activated platelets are a potential target^{49, 50}, for example by targeting PS³².

Two-photon laser scanning microscopy

Two-photon laser scanning microscopy (TPLSM) is a type of fluorescence imaging in which two photons instead of a single photon are used to excite a fluorescent molecule (figure 2). These photons each have about half the energy and a wavelength that is about double that of the photons used in single photon imaging, as the wavelength is inversely related to its energy:

$$E = \frac{hc}{\lambda}$$

with E the energy, h Planck's constant, c the speed of light and λ the wavelength.

These two photons have to arrive at the molecule within 10^{-18} s in order to induce excitation⁵¹. This means that a high density of photons is needed at the focal point of the laser beam in order to achieve a measurable amount of excitation, and thus also emission. This density needs to be approximately 10^{31} photons/cm²⁵², 10^6 times higher than for single-photon excitation⁵¹. For this reason, photons are concentrated both in spatial and temporal dimensions. Temporal concentration of photons is achieved by a pulsed laser beam instead of a continuous one. Pulsation of the laser beam leads to high-intensity pulses, in turn leading to sufficient excitation, while the average laser power remains low⁵¹. A pulse duration of about 100 femtoseconds ($1 \text{ fs} = 10^{-15} \text{ s}$) and a repetition rate of 80 MHz⁵³ (1 pulse per 12.5 nanoseconds; $1 \text{ ns} = 10^{-9} \text{ s}$) are required for a measurable amount of two-photon excitation (figure 3). Spatial concentration is achieved by tight focusing of the laser beam. The laser has to have a very high intensity; an incident laser power in the order of 50 mW is required⁵³. This is large compared to the incident laser light of confocal microscopy: $\approx 3 \text{ mW}$ ⁵³.

Figure 2. Simplified Jablonski diagram showing single (blue) and two (red) -photon excitation. Color figure on page 178.

Figure 3. Schematic representation of photons in a continuous (A) and pulsed (B) laser beam.

As the absorption of photons by fluorescent molecules depends on the square of the excitation intensity, and because the excitation intensity decreases with the square of the distance to the focal point, the two-photon excitation probability falls with the fourth power with distance to the focal point⁵². This means that, effectively, only in the focal point a measurable amount of two-photon excitation and consequent fluorescence emission occurs (figure 4). Thus, with TPLSM, no out-of-focus excitation occurs. This results in intrinsic optical sectioning making 3D reconstruction possible without the use of pinholes, as required in confocal microscopy. Also, no photodamage or photobleaching occurs outside of the focal point, since no excitation occurs outside focus. This is an important advantage over single-photon excitation, such as confocal imaging. Another advantage is that the penetration depth in tissue is larger. This is due to the

Figure 4. Schematic representation of excitation and fluorescence emission in TPLSM (A) and confocal microscopy (B). In TPLSM, near infrared laser light (indicated by red arrows) is used to excite molecules in the focal point. As excitation only occurs in the focal point, all of the green emitted light (indicated by green arrows) originates from the focal point, and can be used for reconstruction of the image. In confocal microscopy, blue/green laser light is used for excitation, as indicated by blue arrows. Molecules are excited along the laser path, not necessarily only in the focal plane. Color figure on page 179.

absence of out-of-focus absorption and due to the longer excitation wavelength. Excitation is in the near infrared range (700–1100 nm), reducing scattering of light in tissue.

Two-photon absorption and emission were first predicted by Maria Göppert-Mayer already in 1931. Two-photon excitation was not possible until the discovery of the laser in 1960⁵⁴, and the development of a femtosecond-pulsed laser in 1971⁵⁴. Denk *et al.* were the first to apply two-photon excitation fluorescence microscopy to image biological samples in 1990⁵³. They described the combination of a femtosecond pulsed laser with a laser scanning microscope, which allowed two-photon laser scanning microscopy (TPLSM). Since then, many papers have been published in which TPLSM is used to image biological tissues with high resolution and at larger penetration depth. Examples of application areas are: neurology^{55–59}, immunology^{60–62}, nephrology^{63, 64}, and vascular biology^{3, 4, 65}.

Plaque imaging with TPLSM

Atherosclerotic plaques are difficult to visualize with fluorescence imaging due to the lack of penetration depth in fat. Three different methods were employed to visualize

the plaque as much as possible. The first method was described by Megens *et al.*⁴, using flow chambers in which arteries are mounted on glass micropipettes. In this flow chamber, arteries can be visualized while under static transmural pressure, usually 80 mmHg. Visualization is performed from the adventitial side to the luminal side of the vascular wall (figures 5A and 6A). In this method, shoulders of plaques can be imaged well; however, the luminal side and the center of the plaque is usually undetectable when plaques are too thick, i.e. >100 μm ⁶⁶. Therefore, in the second method, carotids are carefully dissected in longitudinal direction (figures 5B and 6B), and placed flat on silicon gel. This way, the plaque can be imaged from the luminal side. However when plaques are too thick, again not the entire plaque can be visualized. In the third method, the artery is dissected in transverse direction and placed in agarose gel (figures 5C and 6C). Now, the plaque can be visualized from the adventitial to the luminal side, but only few tens of micrometers in longitudinal direction. In other words, all three methods have advantages as well as disadvantages.

Atherosclerotic plaques have previously been imaged with TPLSM. Megens *et al.* have performed a study in which CNA-OG488 was investigated for its ability to label fibrous caps in plaques⁶⁷. Here, mounted carotid arteries, including the bifurcation, of old ApoE^{-/-} mice were examined for the presence of CNA-OG488 in combination with a nucleic acid stain. In a follow-up study, the interaction of inflammatory cells with collagen in plaques was investigated⁶⁶. Yu *et al.* performed an *in vivo* study in which they investigated murine carotid arteries for the presence of lipids using Nile Red⁶⁸. Another study used TPLSM in combination with second harmonic generation (SHG) to investigate human aortic samples for their intrinsic collagen and elastin luminiscence⁶⁹. Yet another study combined TPLSM, SHG, and coherent anti-Stokes Raman scattering (CARS) for visualization of several plaque components (collagen, elastin, and lipids) in *ex vivo* pig iliac arteries, also without the addition of any fluorescent label⁷⁰.

Figure 5. Schematic representation of artery visualization. A) Left: Artery in flow chamber. Right: Optical sections can be made from adventitial side to luminal side. From Megens *et al.*⁴. B) Artery is dissected in longitudinal direction and imaged from the luminal side. C) Artery is dissected in transverse direction and viewed as a transverse slice.

Figure 6. TPLSM images of plaque-containing carotid arteries with three different methods. A) Artery mounted in flow chamber. In the vascular wall, smooth muscle cells are visible; an example is indicated by dashed arrow. Also inside the plaque cells are visible. Endothelial cells are not visible in this image. B) Artery longitudinally dissected and imaged while in agarose gel, with the luminal side on top. Here, only the plaque is visible. Inside the plaque, foam cells containing multiple nuclei (indicated by circles) are visible. C) Artery transversally dissected. Circle indicates border between vascular wall and surrounding tissue. IEL indicates border between vascular wall and plaque (endothelium is not clearly visible in this image). The three elastic laminae are indicated by arrows. Blue: nuclei; red in B: lipids. VW: vascular wall, EL: elastic lamina, IEL: internal elastic lamina. In A and B, bar = 10 μ m; in C, bar = 50 μ m. Color figure on page 179.

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Nanoparticles for optical molecular imaging of atherosclerosis

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Abstract

Molecular imaging contributes to future personalized medicine dedicated to treat cardiovascular disease, the leading cause of mortality in industrialized countries. Endoscope-compatible optical imaging techniques would offer a stand-alone alternative and high spatial resolution validation technique to the clinically accepted imaging techniques in the (intravascular) assessment of vulnerable atherosclerotic lesions, which are predisposed to initiate acute clinical events. Efficient optical visualization of molecular epitopes specific for vulnerable atherosclerotic lesions requires targeting of high quality optical contrast-enhancing particles. In this Review, we provide an overview of both current nanoparticles for optical imaging and targeting ligands for optical molecular imaging of atherosclerotic lesions and speculate on their applicability in the clinical setting.

Introduction

Cardiovascular disease (CVD) is the leading cause of death in industrialized countries and places a social and financial burden on Western society by contributing strongly to acute clinical events, such as myocardial infarction. Myocardial infarction typically results from exposure of the plaque's thrombogenic content upon rupture. It would be of great clinical relevance to non-invasively assess atherosclerotic plaques in the pre-symptomatic phase, including detection of vulnerable plaques and intraplaque hemorrhage, and to classify the plaque's stage of development, thus allowing stratification of patients-at-risk. Assessment of atherogenesis in the early stage of development would direct early treatment to impede this process. The majority of current clinical imaging platforms, including X-ray angiography and Computed Tomography (CT), focus on post-symptomatic detection of morphological alterations of the arterial wall that affect the lumen diameter. However, the assessment of plaque extent is obscured by outward remodeling and does not provide direct information on plaque vulnerability. Therefore, molecular imaging strategies are being developed for non-invasive diagnosis of coronary artery disease (CAD) at various stages. Most of these strategies are based on recognizing the stage-specific expression of molecules that allow binding of ligand-conjugated nanoparticles (NPs)¹, which can be detected with non-invasive clinical imaging platforms such as Magnetic Resonance Imaging (MRI), radionuclide-based imaging (PET, SPECT), CT and Ultrasound (US). The routine application of NPs is still far ahead, but it is an actively evolving field of research with high promises for clinical use.

The feasibility of molecular imaging studies is based on progress made in three scientific fields: (1) non-invasive imaging platforms, (2) molecular epitope recognition and ligand identification, and (3) design of high quality contrast agents. As far as the latter is concerned, significant progress is being made in the design of contrast-enhancing NPs for molecular imaging²⁻⁵, enabling detection of these NPs with clinical imaging platforms, such as MRI and PET/SPECT. However, these imaging platforms have either limited sensitivity or low spatial resolution and therefore lack the ability to detect sparsely expressed molecular epitopes. Moreover, discrimination between specific and non-specific binding or even between bound and circulating NPs is often difficult. In con-

trast, optical techniques combine high sensitivity with high spatial resolution (μm range, reviewed in ⁶) and therefore have gained considerable attention as techniques that allow (sub)cellular localization of targeted hybrid NPs with additional luminescent properties.

In this review, we explore the feasibility of optical imaging techniques in combination with NPs in the visualization and identification of vulnerable plaques and discuss the potential clinical applications. Common for all optical imaging techniques is the limited penetration depth, which ranges from sub-mm to several cm (see section 2). The penetration depth depends on tissue type, as each type has different absorbance and scattering properties which depend on composition and density. In order to reduce tissue absorbance, the excitation and emission light should match the optical window, which is usually in the 700-1200 nm range ⁷. Below 650 nm, absorbance of light by hemoglobin increases, while water absorbs light above 1200 nm. Scattering of light may also cause a reduction in penetration depth. Plaque tissue consists for a large part of cholesterol esters, which are known to scatter light more than other tissue components. Also calcifications are known to scatter light. In other words, plaque imaging is hampered by its scattering properties. This can partially be resolved by using excitation with long wavelengths, and by using near infrared NPs.

Several optical imaging techniques are discussed. Two-photon laser scanning microscopy (TPLSM) allows fluorescence imaging at larger penetration depths than single-photon techniques, such as confocal microscopy. Optical coherence tomography (OCT) is the optical equivalent of ultrasound, in which the backscatter of emitted light is captured into an image. Raman micro-spectroscopy (RMS) uses the vibration of chemical bonds to identify molecular composition. In photoacoustic tomography (PAT) light is converted into acoustic signals. These techniques will be more extensively discussed in section 2. This review further provides an overview of the physicochemical properties of five NPs for optical imaging that have been utilized in *in vitro* and *in vivo* studies in section 3. Additionally, in section 4 various possible targeting strategies are provided that ensure homing of the presented NPs to (vulnerable) atherosclerotic lesions. Subsequently, multi-modal NPs are presented that enable (sequential) visualization of pathological processes with complementary imaging platforms in section 5. Finally, we con-

sider the clinical perspectives of the presented NPs for optical imaging in the Summary and Outlook section.

Optical Imaging Platforms for Cardiovascular Research

Optical imaging techniques offer a spatial resolution superior to conventional clinical imaging platforms and enable simultaneous visualization of multiple molecular epitopes. With optical imaging techniques, contrast can be generated by detecting (differences in) fluorophore emission spectra (fluorescence microscopy and derivatives), fluorescence lifetime (Fluorescence Lifetime Imaging, FLIM), polarization (polarization microscopy), molecular resonances (Raman Micro-Spectroscopy, RMS), backscattering (Optical Coherence Tomography, OCT) or photoacoustic waves (PhotoAcoustic Tomography, PAT). Due to the limited penetration depth of light, non-invasive whole-body animal luminescence imaging is confined to superficial or subcutaneous events, whereas exposure of tissue is mandatory in other situations, for example atherosclerosis. However, with progress made in (near) real-time endoscope-compatible microscopic systems, including Multi-Photon Laser Scanning Microscopy (MPLSM)^{8,9}, RMS¹⁰, OCT^{11,12}, and PAT¹³, state-of-the-art intravascular and real-time optical examination of the morphology of atherosclerotic plaques is within reach. Additionally, targeted NPs for optical imaging would allow assessment of the molecular expression patterns of these lesions (see Table 1 for the specifications of the optical imaging platforms).

Two-Photon Laser Scanning Microscopy (TPLSM) is the most frequently encountered version of MPLSM and is based on the non-linear absorption of two near-infrared (NIR) photons¹⁴, i.e. photons with wavelengths ranging 700-1000 nm. The probability of excitation depends on the spatial and temporal overlap of two NIR photons at the excitable molecule. Even then absorption cross sections are extremely small and consequently excitation only occurs effectively at the objective's focal zone, the probability rapidly decreasing away from the focus. The absence of out-of-focus absorption and emission allows optical slicing and additionally limits photobleaching and -damage, allowing vital imaging of intact tissue. Furthermore, NIR photons have a wavelength approximately double that of traditional fluorescence microscopy and are thus less scattered, resulting in increased penetration depths up to 250 μm , the exact value strongly depending on

tissue characteristics. In cardiovascular research, TPLSM has primarily been applied *ex vivo*¹⁵⁻¹⁷, but *in vivo* applications are within reach due to recent developments in imaging speed and optical detection efficiency.

Raman (Micro-)Spectroscopy (RMS) is based on detecting vibrational alterations of chemical bonds in molecules upon interaction with monochromatic light and hence provides detailed information on the molecular composition of tissue. For example, *ex vivo* RMS allows quantification of cholesterol content in arterial walls¹⁸ and recognition of atherosclerotic vasculature^{19, 20}. Furthermore, *in vivo* studies have shown that RMS is suitable for characterizing arterial wall composition¹⁰ and is applicable in tumor molecular imaging²¹.

Optical Coherence Tomography (OCT) utilizes the echo time delay and intensity of back-scattered light to create cross-sectional images of sub-surface tissue structures. The penetration depth of ~2 mm is 10-fold larger than that of TPLSM; whereas the spatial resolution is 10-fold lower. OCT allows *ex vivo* intravascular imaging and characterization of plaque morphology²²⁻²⁴ and *in vivo* visualization of vulnerable atherosclerotic plaques in mice²⁵ and morphology in human coronary atherosclerotic plaques^{26, 27}.

In PhotoAcoustic Tomography (PAT), ultrasonic sources are generated by absorption of pulsed light. Though the application of PAT to cardiovascular disease is in its infancy, PAT allows vascular imaging *in vivo* with^{28, 29} and without^{30, 31} exogenous contrast agents. More interestingly, with intravascular PAT atherosclerotic plaques can be identified and characterized *ex vivo*^{32, 33}, which holds great promise for *in vivo* applications.

Taken together, RMS and OCT allow *in vivo* assessment of the molecular and structural changes of the atherosclerotic arterial wall. Examples of the *in vivo* application of TPLSM and PAT in visualizing atherosclerosis are currently unavailable in scientific literature, however, the various *in vitro* applications have shown their feasibility in atherosclerosis research. NPs for optical imaging, targeted towards molecular epitopes specifically expressed in each stage of plaque development, will increase the sensitivity in the microscopic evaluation of the plaque's stage of development.

Table 1. Optical imaging platforms suitable for intravascular applications.

Optical platform	Contrast	Penetration depth [a]	Spatial resolution [μm]		Temporal resolution [fps] [b]
TPLSM	Spectral	~ 250 [μm]	Axial	< 1	up to 50
			Lateral	< 1	
OCT	Echo delay & back-scatter	1-2 [mm]	Axial	~ 20	30
			Lateral	2-4	
RMS	Molecular resonance	450 [μm]	Axial	-	1
			Lateral	25	
PAT	Laser-induced ultrasound	several [cm]	Axial	25	1
			Lateral	100	

[a] Penetration depth: maximal depth from which an adequate signal may be acquired.

[b] Temporal resolution: maximal frame-rate (in frames per second, fps) that may be attained.

Nanoparticles for Optical Imaging in Cardiovascular Research

For *in vivo* molecular imaging, NPs for optical imaging require good physicochemical properties for efficient optical detection and functionalization by targeting ligands. In addition to NP size and shape, modifications in surface structure, e.g. by altering surface charge and attachment of bio-molecules, affect the pharmacokinetics (reviewed in ³⁴). Numerous covalent, amphiphilic and electrostatic strategies have been developed to coat NPs for optical imaging and adapt circulation half-life, organ accumulation and excretion pathways, but may have opposing effects on the NP luminescent properties. For example, organic coating of quantum dots (QDs) increases hydrophilicity, chemical stability and luminescent properties ³⁵, whereas covalent functionalization of carbon nanotubes (CNTs) decreases the luminescent properties ³⁶. Although NP surface chemistry affects their *in vivo* behavior and luminescent properties, an elaborate discussion on this matter extends the scope of this paper. We therefore refer to ³⁷ for additional information. Note that the physicochemical properties in Table 2 apply for water-soluble NPs for optical imaging without targeting ligands.

Figure 1. Schematic overview of the composition of A) quantum dot, B) gold nanoparticle, C) diamond nanoparticle, D1) carbon nanotube, D2) carbon dot (C-Dot), E1) silica nanoparticle with encapsulated organic fluorophores, E2) silica nanoparticle with fluorescent core. Several chemical methods for surface binding of bio-active compounds are mentioned in the text. See Table 2 for spatial dimensions. Color figure on page 180.

The NPs for optical imaging described here (see Figure 1 for graphical representations and Table 2 for their main physicochemical properties) are relatively new, but some have already been tested *in vivo* in preclinical disease models. Quantum dots (QDs) and gold NPs (AuNPs) have proven extremely useful in molecular imaging with optical methods, including TPLSM, OCT and PAT, but presumably have limitations with respect to long term biocompatibility. Three recently synthesized NPs, diamond (DNP), carbon (CNP) and silica (SNP), theoretically have a better biocompatibility and have been tested *in vitro* for imaging purposes and were shown to have characteristics highly suitable for *in vivo* optical imaging. These five NPs will be discussed below.

The physical principles behind the luminescence from QDs, AuNPs and CNPs are beyond the scope of this paper. Therefore light emitted by these NPs is simply referred to as luminescence.

Quantum Dots

Quantum dots (QDs) are nanometer-sized, semiconductor crystals (Figure 1A) that offer superior photophysical properties over organic dyes^{38, 39}, including high quantum yields⁴⁰, broad excitation spectra and narrow emission spectra and high photostability^{41, 42} (Table 2).

Importantly, they allow efficient two-photon excitation, which together with the aforementioned advantageous properties makes them suitable for TPLSM imaging deep in scattering tissue. Moreover, the size-dependent emission wavelength⁴³ allows simultaneous visualization of differently sized QD with a single-wavelength excitation source. The photophysical properties of QDs are strongly dependent on regularity in core structure and size-distribution and their synthesis therefore requires stringent conditions^{44, 45}. The QD quantum yield can reach 70% by encapsulating the QD core with an inorganic shell to obtain core/shell QDs^{46, 47}. The organic shell also reduces desorption and subsequent oxidation of the toxic core components⁴⁸. Moreover, additional organic coating of core/shell QDs provides both water-solubility and increased core/shell stability³⁵, which are required for *in vivo* applications.

In mice, QDs are generally cleared from the blood by accumulation in organs of the reticuloendothelial system (RES) and in some cases also the kidneys^{49, 50}, although this may vary depending on the surface chemistry and ligands attached. However, QDs are usually not excreted through urine or feces^{50, 51}, even for months after intravascular administration, and are therefore assumed to reside in these organs. It is assumed that eventually the accumulated QDs will chemically degrade and release their cytotoxic components and are therefore considered to be clinically unsafe. Therefore, several methods were recently developed to produce QDs that are composed of non-toxic components^{52, 53}, are encapsulated in a silica-shell to increase biocompatibility⁵⁴, and are cleared by the kidneys⁴⁹. These new developments will potentiate the use of QD in future clinical applications⁵⁵.

Targeted QDs are obtained by covalently binding ligands to the shell-surface⁵⁶ or via non-covalent strategies⁵⁷, including the streptavidin-biotin bond⁵⁸. The latter is frequently used in biomedical research. Thus far, QDs have been applied to cancer re-

search in animals, including sentinel node mapping^{59, 60} and *in vivo* molecular imaging of tumors^{57, 61}. The application of (targeted) QDs to cardiovascular research is advancing. QDs have been used to visualize *ex vivo* the expression of the oxLDL receptor CD36⁶², vascular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1)⁶³, the angiogenic marker CD13¹⁵, and phosphatidylserine (PS) exposing cells⁶⁴. Recently, ICAM-, VCAM-, as well as platelet endothelial cell adhesion molecule (PECAM) - conjugated QDs have been used for simultaneous targeting and imaging of the endothelial phenotype in retinal vasculature⁶⁵. In addition, the proteolytic activity of matrix metalloproteinases (MMPs) in atherosclerotic plaques, contributing to a mechanically unstable phenotype (see section 4), can be visualized using QD-based biosensors⁶⁶, which were successfully applied *in vivo* in tumors⁶⁷. However, the approaches mentioned above require external excitation, often implying exposure of the tissue of interest. By chemical excitation of infrared emitting QDs via bioluminescence resonance energy transfer (BRET)⁶⁸, true non-invasive optical imaging may be realized.

The examples above show that QDs are highly suitable for optical imaging in cardiovascular research. However, besides the optical properties, the local contrast enhancing effects of targeted NPs are also dependent on ligand-receptor interactions. Various studies have shown that QDs allow quantification of ligand-receptor binding kinetics⁶⁹ and monitoring of receptor-mediated endocytosis^{41, 58}. Therefore, QDs may be applicable to *in vivo* probing of binding affinity and subsequent processing of ligands specific for atherosclerosis-related molecular epitopes.

Gold Nanoparticles

Gold particles are used extensively in electron microscopy⁷⁰. Recent progress in the synthesis of gold nanoparticles (AuNPs, Figure 1B) has yielded highly luminescent NPs with emissions ranging from 400 nm⁷¹ to 1200 nm⁷². The near-infrared emission makes them suitable for optical spectral imaging of biological tissues, since tissue absorption at these wavelengths is limited. AuNPs require an adequate protecting and stabilizing organic shell for their luminescence and display relatively narrow excitation and broad emission spectra with a quantum yield up to 40%⁷¹. Similar to QDs, the emission spectra of AuNPs are size-dependent⁷³. Moreover, AuNPs are strongly luminescent upon

two-photon absorption⁷⁴ and display specific surface scattering upon excitation with the appropriate wavelength⁷⁵, indicating that AuNPs can be visualized by both TPLSM and OCT. Moreover, excitation with NIR photons induces ultrasonic waves, which allow detection with PAT²⁹ (see Table 2).

In vitro, organically stabilized AuNPs are internalized via endocytosis^{76, 77} without affecting cell viability^{77, 78}. *In vivo*, AuNPs predominantly accumulate in the liver and spleen of mice⁷⁹, do not initiate (hepatic) toxicity⁸⁰, and are slowly excreted via the feces⁸¹. AuNPs are clinically approved for rheumatoid arthritis^{82, 83} and are considered safe⁸⁴, although several studies showed a correlation between AuNP therapy and lung disease^{85, 86}.

Covalent attachment of amine⁸⁷ and thiol⁸⁸ groups to the AuNP surface allow easy functionalization with amino acids⁸⁹ and proteins⁹⁰ for targeted delivery, e.g. to atherosclerosis-related markers. Moreover, direct capping of the AuNP surface with certain amino acids yields stable, water-dispersible and functionalized particles^{89, 91}.

Agglutination of AuNPs enhances their detection with reflectance microscopy, including OCT⁹², thus allowing *in vitro* visualization of cancer cells^{75, 76}. This phenomenon can be exploited in molecular imaging since surface-expressed markers spatially coincide, thereby bringing targeted AuNPs in close contact with each other. Additionally, TPLSM allows visualization of non-targeted AuNPs *in vivo*⁹³ and identification of AuNP-labeled cancer cells in tissue phantoms targeted by AuNPs⁹⁴.

Diamond Nanoparticles

Diamond nanoparticles (DNPs) represent a potentially ideal optical label, combining efficient scattering properties (i.e. they can be used for OCT)⁹⁵, bright⁹⁶, and photostable fluorescence⁹⁶, low cytotoxicity⁹⁷, and chemical stability (Table 2). Fluorescence arises from so-called point defects (Figure 1C)⁹⁸, which are initiated by high-energy irradiation⁹⁹. Each of the defect centers has a fluorescence brightness comparable to a single organic fluorophore, enabling detection of individual DNPs by fluorescence microscopy⁹⁷, including TPLSM¹⁰⁰. However, current DNPs have broad emission spectra⁹⁶, which significantly hampers multicolor imaging. For functionalization, DNPs are chemically modified to yield carboxylated DNPs for the covalent attachment of bioactive

ligands^{96, 101}. Recent progress in the dispersion of DNPs in aqueous media has facilitated their use in physiological solutions¹⁰².

Preliminary results yielded DNPs as non-toxic *in vitro*⁹⁷ as well as *in vivo*¹⁰³. Long term follow-up studies on DNP toxicity however are currently lacking.

The favorable photophysical and potential pharmacodynamic characteristics of DNPs show great promise for biological applications, yet preclinical research on DNPs is still in its infancy. *In vitro*, DNPs were shown to enable single-particle tracking in the cytoplasm of HeLa cells⁹⁶ and detection of growth hormone receptor expression¹⁰¹.

Carbon Nanoparticles

Carbon nanoparticles (CNPs), which include carbon nanotubes (CNTs, Figure 1D1) and carbon dots (or C-Dots¹⁰⁴, Figure 1D2), may offer a less cytotoxic alternative to the aforementioned quantum dots. CNTs and C-Dots are photostable^{104, 105}, but display relatively broad - excitation wavelength dependent - emission spectra (Table 2)^{105, 106}. Therefore, in contrast to QDs, multi-color TPLSM imaging of CNPs in tissue is difficult. Non-aggregated CNTs display an inherent NIR luminescence with a tube-length dependent quantum yield¹⁰⁷, reaching values up to 10%¹⁰⁸. C-Dots, however, require a large surface-to-volume ratio and surface stabilization with organic polymers, such as PEG1500, to become luminescent¹⁰⁵. Several strategies were developed to obtain individualized and hydrophilic CNTs, including non-covalent functionalization with proteins¹⁰⁹, dendrimers¹¹⁰ and phospholipids¹¹¹. CNPs are internalized via endocytosis *in vitro*^{105, 112}, but both CNTs and C-Dots display no acute toxicity^{104, 113}. However, long-term effects of CNPs on cell viability strongly depend on surface-bound (bio-)molecules and dose^{112, 114} and traces of metal catalysts used during synthesis¹¹³. In addition, CNTs may penetrate the nuclear membrane physically¹¹⁵ and elicit DNA damage through ROS generation¹¹⁶. After intravenous administration of CNTs, no pathological abnormalities were found in the liver, kidneys and other organs¹¹⁷. In rodents, CNTs are cleared by the kidneys¹¹⁸, whereas excretion routes of C-Dots are currently unknown. CNPs may be functionalized for molecular imaging by coating with ligand-bound phospholipids¹¹⁹ and amino-terminated dendrimers¹¹⁰.

Until now, few studies have utilized CNPs for *in vivo* optical imaging. Nevertheless, the NIR and Raman signals from CNTs were detected in the blood pool of *Drosophila melanogaster*¹⁰⁶ and in subcutaneously implanted tumors in mice²¹, respectively. These preliminary results indicate the potential of TPLSM in visualizing CNPs in biological tissue.

Silica Nanoparticles

Fluorescent silica nanoparticles (SNPs) are synthesized by trapping fluorophores in a mesoporous silica matrix (Figure 1). The spectral properties thus depend on the entrapped fluorophores and can therefore not be specified. The quantum yield has an optimum since both a low concentration and too high concentration (inducing self-quenching¹²⁰) will affect SNP brightness¹²¹. The brightness of dye-doped SNPs (Figure 1E1) additionally suffers from dye-leakage from the silica pores, which also hampers accurate assessment of the spatial distribution of SNPs by optical imaging modalities. The leakage can be reduced by either covalent⁴² or electrostatic interactions¹²⁰ between the fluorophore and the silica precursors, or by increasing the size of the dye molecule¹²⁰, or by stabilizing the fluorophore-doped silica core with a dye-free silica shell^{42, 120} (Figure 1E2). Core/shell SNPs exhibit a relatively high brightness, but still display an approximately 3-fold lower luminescence intensity than QDs⁴².

Reactive silica precursors enable chemical modifications of the SNP surface. For example, maleimide and iodoacetyl groups can be introduced¹²² and amino groups¹²³ can be ionized to enable (covalent) attachment of bioactive ligands.

In vitro experiments show cytotoxic responses to SNPs. Upon internalization by endocytosis¹²⁴, SNPs impair proliferative activity, stimulate the release of the inflammation marker interleukin-8, and reduce cell viability *in vitro* in a dose- and time-dependent manner¹²⁵⁻¹²⁷. In mice, however, intravenous administration of colloidal core/shell SNPs at biologically relevant concentrations do not induce acute or chronic responses, even 60 days post-injection¹²⁸.

Several *in vitro* fluorescence microscopy studies have shown that SNPs allow peripheral labeling of cancer cells by targeting folate receptors^{42, 129}. In addition, antibody-conjugated SNPs allow multi-target monitoring of bacteria species¹³⁰.

Detection of nanoparticles for optical imaging

For efficient detection, NPs for optical imaging should have favorable luminescence properties, i.e. high quantum yield, broad excitation spectrum and narrow emission spectrum that matches the optical window of tissue. The presented NPs for optical imaging share the ability to be detected with TPLSM (Table 1). However, the 250 μm penetration depth of TPLSM in biological tissue limits its clinical application to early atherosclerotic lesions. The penetration depth of OCT and PAT are 1 and 2 orders larger, respectively, and may therefore be preferred over TPLSM when greater depths should be attained, i.e. intraplaque visualization of developed lesions.

Table 2. Physicochemical properties of water-soluble non-targeted nanoparticles for optical molecular imaging.

	Quantum Dot	Gold	Diamond	Carbon dot	Carbon tube	Silica
Quantum yield	<70% ^{46, 47, 131}	<40% ⁷¹	~50% ⁹⁶	~10% ¹⁰⁵	<10% ^{108, 132}	Fluorophore & concentration dependent
Fluorescence excitation spectrum [nm]	>200 [a]	300~450 [a] ⁷¹	450~550 [a] ^{97, 133, 134}	450~650 [a] ¹⁰⁵	Unknown	Fluorophore dependent
Fluorescence emission spectrum [nm]	~30 [b]	~60 [b]	~100 [b] ^{96, 133}	~100 [b] ¹⁰⁵	~500 [a] ^{106, 114, 117}	Fluorophore dependent
Hydrodynamic diameter [nm]	<20	1-50	35-100	<5	~1 nm (ϕ); 500 (length)	>15
Blood clearance	Predominantly spleen & liver	Predominantly spleen & liver	Unknown	Unknown	Kidneys	Spleen, liver & lung ¹²⁸
Excretion	None	Slowly via feces ⁸¹	Unknown	Unknown	Urine ¹¹⁸	Urine and feces ¹³⁵
Optical platform	TPLSM	TPLSM, OCT, PAT	TPLSM, OCT	TPLSM	TPLSM	TPLSM

[a] Total bandwidth; [b] Full-Width-at-Half-Maximum (FWHM)

Targeting nanoparticles to atherosclerotic lesions

The application of molecular imaging in assessing vulnerable atherosclerotic plaques is a challenging task, since these plaques are highly complex and constantly evolving structures. Consequently, these lesions display numerous molecular targets, expressed either at the endothelial surface or subendothelially by cells and extracellular matrix in the vascular wall.

The American Heart Association (AHA) has recognized six states of plaque development in humans, divided in precursor and advanced types (see Figure 2). Precursor lesions (types I, II, III) are asymptomatic and do not narrow the lumen¹³⁶; whereas advanced lesions (types IV, V, VI) are hemodynamically and clinically more relevant since they may lead to ischemic attacks¹³⁷. Type I plaques are characterized by endothelial activation, which is accompanied by luminal expression of various adhesion molecules¹³⁸; these include intercellular (ICAM), vascular (VCAM), and platelet endothelial (PECAM) cell adhesion molecules, and the lectin-like oxLDL receptor LOX-1. These adhesion molecules initiate the accumulation of macrophages and lipids in the vascular wall, causing gradual progression to type II lesions. Type III lesions contain extracellular lipid and apoptotic cells¹³⁸ and typically show extracellular matrix-degrading enzymes, such as matrix metalloproteinases (MMPs) and cathepsins¹³⁸. In type IV lesions the necrotic core develops, as large amounts of extracellular lipid accumulate in the intima. Capillaries surround the lipid core, mostly in the shoulder of the plaque, and calcifications may also be present. Macrophages, foam cells, and lymphocytes are mainly present in the periphery of the lesion, most notably in the shoulders of the plaque. Type V lesions develop when the subendothelial layer grows into more collagen-rich tissue, forming the fibrous cap. More and larger capillaries are present in these lesions. Type VI lesions closely resemble type IV and V lesions, but have had disruptions of the lesion surface, hematoma or hemorrhages, with subsequent thrombus formation. It is important to stress that vulnerable plaques do not necessarily coincide with rupture-prone plaques. More accurately, vulnerable plaques are at risk for thrombotic complications and consequent rapid progression of plaque instability, as was proposed by Naghavi *et al.*¹³⁹.

Figure 2. Schematic representation of human plaque progression. The different types of plaque morphologies are not necessarily successive and not all plaques progress to ruptured plaques (AHA type VI). Color figure on page 180.

Each stage of plaque development displays specific features, such as inflammation, activated endothelium, accumulated lipid and macrophages, collagen-rich fibrous cap, angiogenic activity, apoptosis and thrombi. Each of these features is associated with specific molecular targets that may be used for molecular imaging. Available targets and corresponding ligands, which have been used in clinical and pre-clinical studies, are mentioned in Table 3. The NPs in Table 3 are not necessarily NPs for optical imaging; however, the ligands mentioned in Table 3 can be combined with the NPs for optical imaging in section 3, to generate particles for specific and high-resolution visualization of vulnerable plaques using (endoscope-based) optical imaging techniques. Optical techniques described in Table 3 either require exposure of the tissue of interest or served as an *ex vivo* validation tool. Optical imaging techniques are currently being developed that would allow *in vivo*, endoscopic-based imaging (see section 2).

A distinction should be made between luminal (such as CD13^{15, 140}) and sub-endothelial targets (such as apoptotic cells in atherosclerotic plaques¹⁴¹). For sub-endothelial targets the hydrodynamic size of the NP should be considered, as the particle has to translocate through the endothelial layer. Furthermore, the permeability of the endothelium should be taken into account, as plaque endothelium is leakier than healthy endothe-

lium, allowing translocation of larger NPs. In advanced plaques, NPs may also reach the intraplaque targets through the vasa vasorum, an adventitial vascular network that expands into the lesion during plaque growth.

Table 3 – CH3b

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Hybrid nanoparticles for multimodal imaging

Clinically approved imaging techniques allow non-invasive evaluation of the localization of targeted contrast enhancing NPs, yet lack the sensitivity and resolution to prove target specificity. Therefore, high spatial resolution validation of non-invasive molecular imaging is mandatory and requires NPs suitable for multimodal imaging. Hybrid NPs can be detected by optical imaging methods as well as by non-invasive clinical imaging methods. These hybrid NPs may also be linked to the ligands in table 3, for imaging of atherosclerosis-related molecular markers. In such a set up, a combination of non-invasive imaging for macroscopic detection, followed by invasive optical imaging, allows determination of distribution and exact location. Hybrid NPs developed so far are mostly decorated with MRI and fluorescent labels. Of the hybrid NPs currently available, few have been applied *in vivo*.

Hybrid lipidic structures, such as liposomes and micelles, are composed of natural amphiphilic lipids and are therefore highly biocompatible. They can be easily formulated and adjusted for various purposes. The maximum payload of added moieties, e.g., the ligand or contrast agent, depends on the size of the liposomes, but may be thousands per liposome. The size of liposomes is often larger than 100 nm, which is large compared to the NPs discussed in previous paragraphs. In recent decades, though, liposomes have been investigated extensively as drug carriers^{173, 174} and two liposomal cytostatic formulations have been approved for clinical use so far¹⁷⁵. Incorporation of contrast agents allows their application in imaging purposes, e.g. for *in vivo* MRI detection of intimal thickening¹⁷⁶. When contrast agents with different functionalities are incorporated in the lipid bilayer, dual detection with for instance MRI and fluorescence microscopy is enabled. Hybrid liposomes have been developed to specifically target and visualize the expression of E-selectin¹⁷⁷ and apoptotic cells¹⁷⁸. $\alpha_v\beta_3$ targeted liposomes with surface-conjugated RGD have been used to detect angiogenic activity in tumors¹⁷⁹ and in atherosclerotic plaques¹⁵⁹ by *in vivo* MRI and subsequent *ex vivo* validation with fluorescence microscopy¹⁷⁹. Bimodal micelles targeting the macrophage scavenger receptor (MSR) by surface-bound anti-MSR antibodies visualize the uptake of macro-

phages in atherosclerotic plaques of mice with *in vivo* MRI¹⁸⁰, and by *ex vivo* fluorescence microscopy.

QDs may serve as a highly luminescent scaffold for MR probes, PET/SPECT tracers and CT contrast agents to produce multimodal imaging probes. The payload of additional contrast agents or ligands is estimated to be 10-30 per QD. QDs provided with a paramagnetic lipid-coating were applied to MR and optical visualization of $\alpha_v\beta_3$ -expression on the membranes of activated HUVECs¹⁸¹ or for PS exposure of apoptotic cells¹⁸². A different approach was chosen through silica-coated QDs with a hydrophobic phase between the QD surface and the silica-shell¹⁸³ in order to incorporate hydrophobic contrast agents. The drawback of this approach is the rather tedious and stringent synthesis protocol to produce monodisperse NPs containing a single QD. The time-consuming synthesis makes these particles less suitable for PET/SPECT applications, due to decay of radioactive tracers.

A more flexible scaffold is provided by streptavidin-coated QDs, since the QD-surface can easily be functionalized by biotinylated moieties, including ligands and non-optical imaging probes. To further increase detectability, dendritic wedges with 8 DTPA or DOTA compounds were developed to incorporate paramagnetic gadolinium or radioactive PET/SPECT tracers, respectively¹⁸⁴. Utilizing these dendritic structures, paramagnetic QDs were successfully applied for MR and optical imaging of apoptosis *ex vivo* using Annexin A5⁶⁴. Moreover, cNGR-coupled QDs were successfully utilized for *in vivo* optical detection of angiogenic activity after myocardial infarction¹⁵. Recently, a bimodal cNGR-coupled QD with a dendritic gadolinium wedge was shown to enable the detection of angiogenic activity in tumor-bearing mice by *in vivo* MRI and *ex vivo* TPLSM¹⁴⁰.

A different type of hybrid NP is a QD containing a shell doped with paramagnetic manganese¹⁸⁵. This NP was shown to be taken up by macrophages *in vitro* as shown by MRI and optical methods.

The mesoporous matrix of silica NPs and coatings allows the incorporation of probes with different functionality. By coating a luminescent ruthenium core with a silylated Gd complex, bimodal NPs were obtained for efficient luminescent and paramagnetic

labeling of monocytes *in vitro*¹⁸⁶, with the potential to extrapolate this to *in vivo* visualization of leukocyte accumulation in atherosclerotic plaques. Also, gadolinium oxide cores may be covered by a silica layer containing fluorophores¹⁸⁷. In contrast to the previously discussed NPs, these PEGylated NPs were excreted mainly via the kidney when intravenously injected in mice, as was visualized *in vivo* with both fluorescence imaging and MRI. In addition, magnetite (Fe_3O_4) can be coated with a silica shell, in which luminescent iridium is included, in order to visualize their passive ingestion by HeLa cells *in vitro*¹⁸⁸.

In contrast to gadolinium, superparamagnetic compounds, such as iron oxides generate negative contrast with MRI. Several different iron oxide based multimodal nanoparticles have been developed, but only few have been applied *in vivo* so far. Magnetite particles, to which NIR fluorophores were conjugated¹⁸⁹, were used to load macrophages for *in vivo* imaging applications. In addition, iron oxide particles covered with a phospholipidic layer containing fluorophores¹⁷⁸ were developed to specifically visualize apoptotic cells *in vitro*. Also so-called nanosponges were developed in which the magnetic and luminescent characteristics of their components are maintained¹⁹⁰. The *in vivo* experience with iron oxide based hybrids is still limited. Small (3 nm) iron oxide NPs coated with fluorescent cyanine 5.5, allowed visualization of atherosclerotic lesions in mice with *in vivo* MRI and *ex vivo* fluorescence microscopy¹⁹¹, apparently by virtue of their macrophages uptake. These results have promising implications for non-invasive assessment of inflammation, and vulnerability to rupture, of an atherosclerotic plaque.

Also, CNT conjugation to iron oxide nanoparticles resulted in a bimodal NP allowing visualization of the *in vitro* uptake by macrophages with MRI, fluorescence imaging and Raman scattering¹⁹². To increase biocompatibility, this NP was encapsulated in DNA.

Summary and outlook

In this paper, optical and hybrid contrast enhancing NPs were described that have been applied to *in vitro* and *in vivo* animal studies. As such, these particles may play a role in confirming (sub) cellular localization following non-invasive imaging in animal models. Due to fast developments, NPs may also find clinical applications in optical imaging techniques. The ideal NPs for optical molecular imaging in clinical use should bear the

following characteristics: (1) highly luminescent upon irradiation, (2) high binding affinity and specificity for the target, (3) resistance to chemical degradation and (4) clearance through kidney or liver upon intravenous injection, to prevent accumulation in vital organs. It should be kept in mind that the *in vivo* targeting behavior, as well as the level of toxicity, of the NPs highly depend on geometrical (i.e. NP size), biophysical (i.e. the availability of the target and hemodynamic forces), chemical (i.e. NP stability), and biological (i.e. ligand-target affinity and excretion pathway) parameters. As such, a thorough quantitative analysis of the *in vivo* bio-distribution and pharmacokinetics of each individual construct (i.e., NP and applied surface modifications) should be performed and should result in a well defined and uniform safety and efficacy profile prior to clinical use.

The relatively narrow emission spectra of QDs and AuNPs offer a valuable contribution to the research of complex and multifactorial diseases, including atherosclerosis, since multiple molecular targets may be visualized simultaneously. However, the current commercially available QDs consist of heavy metals such as cadmium, and are not likely to be suitable for clinical use, despite encapsulation of the core. CNPs offer a potentially non-toxic alternative to QDs, but display a low quantum yield and broad emission spectrum, both hampering multi-color imaging *in vivo*. AuNPs, DNPs and SNPs combine high luminescence with a non-toxic potential *in vivo*, although it is not yet known whether DNPs are excretable. Therefore, based on current knowledge on toxicity and optical properties of the presented NPs, AuNPs and SNPs are the most promising NPs for clinical use in optical molecular imaging of atherosclerosis. The use of AuNPs is clinically approved in treatment of rheumatoid arthritis;^{82, 83} however, both AuNPs and SNPs have been poorly characterized for *in vivo* imaging applications at this moment.

Many of the presented NPs for optical imaging hold a dual-purpose character, i.e. they may be used as contrast agent in optical imaging as well as therapeutic agent to specifically disrupt processes that contribute to plaque destabilization. These processes include MMP expression by macrophages and angiogenic expansion of the vasa vasorum that penetrates the plaque. The use of NPs for optical imaging as therapeutic agent is still in initial phase of development, but several approaches can be recognized. First, surface binding or encapsulation of photosensitizers may employ QDs and SNPs, re-

spectively, for targeted photodynamic therapy (PDT) of atherosclerotic lesions¹⁹³⁻¹⁹⁹. In photodynamic therapy, (indirect) illumination of the photosensitizers induces local formation of reactive oxygen species, which stimulates apoptosis of the surrounding cells. Second, AuNPs²⁰⁰ and CNTs²⁰¹ convert absorbed radiation into heat²⁰⁰ and hence may allow directed photothermal destruction of cells⁷⁶ to which these NPs were targeted. This is referred to as photothermal therapy (PTT). Third, AuNPs^{202, 203}, CNTs²⁰¹, and SNPs¹²³ may serve as scaffold for targeted gene therapy or, in the case of SNPs, may be filled with chemotherapeutic drugs²⁰⁴ to attenuate the progression of atherosclerotic lesions.

Research committed to finding the most suitable target, as well as the accompanying ligand for the assessment of vulnerable atherosclerotic plaques, has increased tremendously in recent years. It should be mentioned that the molecular targets used in molecular imaging and therapy of tumors show great resemblance to the targets expressed in atherosclerotic plaques, including inflammation²⁰⁵, proteolytic activity^{206, 207}, neo-vascularization^{208, 209}, and apoptosis^{210, 211}. Although both pathologies have different origins and progressions, the shared molecular markers may aid in the development and testing of ligands for specific targeting of NPs towards vulnerable plaques, as research committed to targeted tumor treatment is more advanced than targeted atherosclerotic plaque therapy.

In conclusion, current optical techniques and associated nanoparticles are promising for their application in high-resolution and high-sensitivity stand-alone imaging platforms as well as a complementary technique to clinically accepted imaging platforms.

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Optical and magnetic resonance imaging of
cell death and platelet activation using
Annexin A5-functionalized quantum dots

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Abstract

A quantum dot-based nanoparticle is presented, allowing visualization of cell death and activated platelets with fluorescence imaging and MRI. The particle exhibits intense fluorescence and a large MR relaxivity (r_1) of 3000-4500 mM⁻¹s⁻¹ per nanoparticle, due to a newly designed construct increasing the gadolinium-DTPA load. The nanoparticle is suitable for both anatomic and sub-cellular imaging of structures in the vessel wall and is a promising bimodal contrast-agent for future *in vivo* imaging studies.

Introduction

Contrast-enhancing nanoparticles are essential in the field of molecular imaging. Molecular imaging focuses on early detection of various diseases and supports personalized therapy. While conventional imaging modalities such as computer tomography and magnetic resonance imaging (MRI) allow visualization of anatomical and (patho)physiological consequences of the disease, molecular imaging is based on the molecular and cellular fingerprints of the disease, requiring disease-specific molecular targets as well as target-specific nanoparticles. Furthermore, these nanoparticles should allow visualization *in vivo* with sufficient perceptibility.

Programmed cell death (PCD) is a process of organized cell suicide, and occurs in various forms, of which apoptosis is the best known ¹. PCD plays important roles in physiology as well as pathology of multi-cellular organisms. During homeostasis a balance exists between cell proliferation and cell death. This equilibrium is disturbed under certain pathological conditions. Acute myocardial infarctions ², heart failure ³, or unstable atherosclerotic plaques ^{4, 5} show increased PCD, whereas in cancer this balance shifts towards cell proliferation ⁶. Anti-cancer therapies are being developed in which PCD is induced in cancer cells to reverse tumor growth ⁷⁻⁹. Because of the role of PCD in numerous diseases, imaging of this process would be of great value to diagnosis and guidance of therapy.

One of the most explored molecular imaging targets of PCD is phosphatidylserine (PS). This aminophospholipid is translocated from the inner to the outer leaflet of the cellular membrane in dying cells, triggering phagocytosis ¹⁰. Cell surface exposure of PS occurs during most types of cell death ⁸, but is not limited to dying cells. For example, blood platelets expose PS in an advanced stage of activation ^{11, 12}, providing a physiological surface for blood coagulation processes. Hence, molecular imaging of PS can also be of value to diagnosis and management of thrombotic diseases.

Annexin A5 (AnxA5) is a well explored molecular imaging probe to visualize cell surface exposure of PS ¹³. AnxA5 binds to PS with a K_d in the nM-range in the presence of Ca^{2+} -ions ¹⁴. It can be coupled to a variety of reporter compounds making it suitable to use in

combination with various imaging modalities including optical^{15, 16}, nuclear^{17, 18}, and magnetic resonance imaging (MRI)^{19, 20}. *In vivo* studies in animals and patients demonstrated that AnxA5 can be used to non-invasively detect the vulnerability of atherosclerotic plaques^{21, 22}, size and location of the infarct region in acute myocardial infarctions², the extent of liver apoptosis^{23, 24}, and the localization of mural thrombi in abdominal aortic aneurisms²⁵. In addition, imaging cell death with AnxA5 allows early assessment of the efficacy of anti-cancer therapy⁹. These studies utilized radioactively labeled AnxA5 in combination with single photon emission computed tomography (SPECT) or positron emission tomography (PET). SPECT and PET imaging do not yield anatomical information, and have a relatively poor resolution: 8-14 mm for SPECT and 4-7 mm for PET^{17, 26}. These limitations can be overcome by MRI using targeted contrast-agents. MRI has a better resolution ($\pm 300 \mu\text{m}$)²⁶ and provides anatomical information, enabling a more accurate localization and characterization of the target site. However, sensitivity of this imaging modality for contrast-agents is still relatively low.

In this paper a new, AnxA5-functionalized and bimodal nanoparticle is presented that is suitable for MRI and fluorescence imaging allowing analysis at the anatomical as well as sub-cellular level. The nanoparticle is based on a quantum dot (QD), which is intensely fluorescent. The emission wavelength of QDs can be varied by altering the size of the semiconductor crystal core. Emission spectra of QDs are very narrow: emission peak width is about 20 nm at half maximum. Furthermore, QDs with different emission wavelengths have the same excitation wavelength. These properties allow multicolor analysis of samples in a single procedure²⁷. A recent publication by Le Gac *et al.*²⁸ showed that QDs (Quantum Dot Corporation, now Invitrogen) resist photo-bleaching and allow therefore imaging of fast, cellular processes over a longer period of time as compared to organic fluorescent dyes.

Long term *in vivo* effects of QDs are unknown so far. QDs used in this study are potentially toxic substances because the semiconductor crystal core contains cadmium and selenium. Toxicity of QDs depends on multiple factors, e.g. size, charge, concentration, outer coating bioactivity, and stability of the QD²⁹. A recent study reported that mice tolerated intravenous injection of QDs well up to 133 days after administration. Neither

signs of necrosis at sites of deposition nor signs of QD breakdown were observed³⁰. The QDs used in that study are from the same supplier as the QDs of the current study.

The present study visualizes QDs by two-photon laser scanning microscopy (TPLSM). The laser power of TPLSM required to have sufficient QD fluorescence is considerably lower as compared to organic fluorescent dyes, due to the high quantum yield of QDs. This limits further potential photo-bleaching of the probe and photo-damage of the sample. Furthermore, high resolution images with low background fluorescence can be acquired. This is due to several factors: first, TPLSM excitation occurs only at the focal point preventing out-of-focus contribution^{31, 32}. Second, as indicated above, the required laser power is low, thereby also reducing background (auto)fluorescence. Third, because emission spectra of the QDs are narrow²⁷, stringent emission filters can be applied to reduce background light as much as possible. Hence, it is possible to image structures hidden deeper in tissues more accurately with TPLSM without provoking tissue damage, for example atherosclerotic plaque imaging in arteries³¹.

Materials and Methods

Annexin A5: In order to accommodate conjugation chemistry for coupling AnxA5 to larger structures without impairing the PS binding property, a cysteine residue was engineered in the N-terminal tail, which is located apical to the PS binding sites (cys2-AnxA5)³³. Maleimide-activated biotin (Pierce, Rockford, IL) was coupled to cys2-AnxA5 according to the recommendations of the manufacturer. The cys2-M1234 variant, which does not bind PS³⁴, was coupled to maleimide-activated biotin similarly and served as a negative control.

Gd-DTPA and Gd-DTPA wedge: Biotin was coupled to Gd-DTPA as described by Langereis *et al.*³⁵; biotinylated Gd-wedge was synthesized similar to a method described previously³⁶.

Functionalized quantum dots: AnxA5-QDs were prepared in a 1:1 stoichiometry by mixing equimolar amounts of streptavidin-coated QDs (stock concentration 1 μ M, Invitrogen, Eugene, OR, with emission peaks at either 525 or 585 nm) with biotinylated AnxA5 in binding buffer (10 mM HEPES, pH 7.4, containing 150 mM NaCl, 5 mM KCl, 1 mM

MgCl₂, 2.5 mM CaCl₂ and 1 mg/mL Bovine Serum Albumin). The AnxA5-functionalized QDs were incubated with a minor excess of Gd-DTPA or Gd-wedge in order to saturate the unoccupied biotin binding sites of streptavidin. This procedure yields complexes with average stoichiometries of QD:AnxA5:Gd of 1:1:30 for AnxA5-QD-Gd and 1:1:240 for AnxA5-QD-Gd-wedge. These stoichiometries are derived from the information supplied by Invitrogen that each QD is coated with 8-12 streptavidin molecules.

Cryo-transmission electron microscopy: Specimen preparation for cryo-EM was done according to established procedures³⁷. Briefly, a thin film was prepared from the sample in the Vitrobot™, an automated vitrification robot with temperature and humidity control (patented by the University of Maastricht; patent licensed to FEI company, Hillsboro, OR/USA). The thin film was formed by applying 3 µl of the sample/suspension to a Quantifoil grid in the chamber of the Vitrobot at 22 °C and at a relative humidity >97%. Prior to sample application, the Quantifoil grid (R 2/2, Quantifoil GmbH, Jena/BRD) was glow-discharged to make the surface hydrophilic. Excess liquid was blotted away (one blot for one second) and the thin film thus formed was shot into melting ethane. The grids with vitrified thin films were transferred to the microscope using a Gatan 626 cryo-transfer/cryo-holder system (Gatan, Pleasanton, CA, USA). Micrographs were taken with the specimen at -170 °C, using low dose conditions in a CM12 transmission microscope (Philips, Eindhoven, The Netherlands) operating at 120 kV.

Cell culture, apoptosis stimulation, staining: Jurkat cells (ATCC, Middlesex, UK) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL), supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin and 10% heat-inactivated fetal bovine serum, and incubated in a humidified atmosphere at 37 °C and 5% CO₂. Cells were split every two or three days to 10⁵/mL. For experiments, cells were concentrated to 10⁶/mL. Apoptosis was induced by adding anti-Fas (anti-human antibody, clone 7C11, Immunotech, Marseille, France) to the culture medium at a final concentration of 200 ng/mL, and incubated in a humidified atmosphere at 37 °C and 5% CO₂ for three hours. Next, cells were washed with binding buffer, and stained, as indicated, with either of the two nanoparticles at a concentration of 40 nM for 10 minutes. After-

wards, cells were washed again with binding buffer and imaged with Two-Photon Laser Scanning Microscopy (TPLSM) or MRI.

For TPLSM experiments, cells were also incubated with a nucleic acid stain, either 2 μ M Syto41, a nucleic acid dye for viable and dead cells, (peak emission wavelength 450 nm, stock concentration 5 mM, Invitrogen, Eugene, OR/USA), or 2.5 μ g/mL Propidium Iodide (peak emission wavelength 620 nm, stock 250 μ g/mL, Molecular Probes, Leiden, the Netherlands) a nucleic acid dye for cells with a disrupted cellular membrane, i.e., dead cells. For internalization experiments, cells were incubated with 40 nM AnxA5-QD525-Gd, along with 200 ng/mL anti-Fas. After 5.5 hours cells were washed extensively in ethylene diamine tetra acetate (EDTA) to remove AnxA5 present on the outside of cells, and after washing with binding buffer, cells were stained with 40 nM AnxA5-QD585-Gd. Next cells were washed with binding buffer and imaged with TPLSM.

Whole blood collection, clot formation: Blood was collected with informed consent from healthy volunteers by venapuncture and anticoagulated in 0.1 volume of 129 mM trisodium citrate.

For TPLSM, clots were incubated with nanoparticles after clot-formation. Clot formation was induced by adding 15 mM CaCl_2 , and gentle mixing. Clots were allowed to form for one hour at 37 °C in coagulation tubes (BD Vacutainer SST II Advance, BD Diagnostics, Plymouth, UK). After coagulation, clots were washed in binding buffer, and next incubated with 40 nM AnxA5-QD-Gd-wedge, prepared as described above, or with 500 nM AnxA5-FITC (NeXins Research BV, Kattendijke, the Netherlands), for one hour at 37 °C. Clots were also stained with Acridine Red (AR), a cytoplasm stain, (Chroma-Gesellschaft, Köngen, Germany) or 2 μ M Syto41. In one instance, whole blood was also pre-incubated with FITC-labeled anti-fibrinogen (anti-human antibody, WAK FA AF FITC-2, WAK Chemie Medical, Steinbach, Germany). Afterwards, clots were again washed in binding buffer and enclosed in 2% agarose gel for imaging.

For MRI, whole blood was incubated with 40 nM or 200 nM AnxA5-QD525-Gd-wedge prior to clot formation to label as many platelets as possible, because incubation of the clots after formation did not result in sufficient labeling for detection. To compensate for the anticoagulant effect of AnxA5, whole-blood was incubated with thrombin and

collagen, triggering additional PS-exposure on platelets. Clot-formation was induced by adding 15 mM CaCl_2 , 1 nM thrombin, and 20 $\mu\text{g}/\text{mL}$ collagen in the presence of either 40 or 200 nM AnxA5-QD-Gd-wedge. Clots were allowed to form for one hour at 37 °C in coagulation tubes as described above. Clots were placed in 2% agarose gel without washing, to minimize the wash out of labeled platelets.

Wire-injured carotid artery model: An 18-week old male Swiss mouse was anesthetized by pentobarbital (110 mg/kg i.p.). The carotid artery was exposed while keeping it moist with saline and ligated at three sites: one proximal to the bifurcation of the artery and two distal to the bifurcation (one on each branch of the carotid artery). A flexible wire of 0.53 mm diameter was inserted in the common carotid artery via the external carotid artery and traversed three times to remove or damage the endothelium. Next, the blood flow was restored in the common carotid and internal branch; the external branch was tied off proximally of the incision with a fourth ligation³⁸. 45 minutes after injury the mouse was sacrificed by dissection of the diaphragm and cardiac perfusion with 5 mL of binding buffer in order to remove blood from the vasculature via liver-puncture. Then the carotid arteries were carefully excised, and connective and adipose tissue were removed. Next, the arteries were mounted into a home-built perfusion chamber³⁹ (IDEE BV, Maastricht, the Netherlands) in Hanks Balanced Salt Solution (HBSS, pH 7.4), containing 144 mM NaCl, 14.9 mM HEPES, 5.5 mM glucose, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM KH_2PO_4 , and 1.2 mM MgSO_4 . The artery was slowly perfused with 200 nM AnxA5-QD-Gd-wedge (500 μL in 30 minutes) using a microinjection pump (Predicor Infors, Basel, Switzerland). To stain cell nuclei and elastin in the vascular wall, the arteries were perfused with 500 μL HBSS containing syto41 (final concentration 2 μM) and eosin (final concentration 0.5 μM) in a similar way, while simultaneously removing unbound nanoparticles. A static transmural pressure of 40 mmHg was applied (using a modified Big Ben sphygmomanometer, Riester, Germany), and the arteries were imaged with TPLSM. Afterwards, the arteries were removed from the perfusion chamber and embedded in 2% agarose gel, and imaged with MRI.

Two-photon laser scanning microscopy: A standard Bio-Rad 2100MP (Hemel Hempstead, UK) was used in TPLSM mode. The excitation source was a Spectra Physics Tsunami Ti:Sapphire laser (Mountain View, CA/USA), tuned and mode-locked at 800 nm.

The Tsunami laser produces light pulses of about 140 fs width at a repetition rate of 82 MHz. Laser light reached the sample through the Nikon water immersion lenses (60x water dipping, numerical aperture (NA) 1.0, working distance (WD) 2 mm or 60x water immersion, NA 1.2 and WD 0.22 mm), incorporated in an upright Nikon E600FN microscope (Tokyo, Japan). Further magnification was achieved by optical zoom. Three photomultipliers detected the fluorescence. Each photomultiplier accepted a different, tunable wavelength region, which were color-coded: blue (for Syto41, 420-470 nm); green (for QD525 and FITC 510-540 nm); and red (for QD585 570-600 nm, for PI 580 nm and higher, and for acridine red 590 nm and higher). The three obtained images were combined into a single image. Only combined images are shown. No additional image processing was performed. An imaging speed of 0.1 Hz with a pixel dwell time of 39 μ s was used, while power at the sample was between 0.25 and 2.25 mW.

Cells were imaged by applying 20 μ L of buffer containing cells on a microscope slide, and covering it by a coverslip. Blood clots were imaged while enclosed in 2% agarose gel, covered with binding buffer.

Magnetic resonance imaging: MRI was performed at room temperature with a 1.5 T whole-body system (Intera, Philips Medical Systems, Best, the Netherlands) using an inversion recovery, turbo spin echo (IR-TSE) MR pulse sequence for visualization of Jurkat cells and whole-blood clots. Murine carotid arteries were imaged at room temperature with a Bruker Biospec 7.0T scanner (Bruker Biospin GmbH, Ettlingen, Germany) using a multi-slice spin echo sequence.

Jurkat cell samples were imaged using a 23 mm diameter surface radio-frequency coil (Philips Medical Systems, Best, the Netherlands). The imaging parameters for the single slice IR-TSE sequence were: repetition time (TR), 1580 ms; inversion time (TI), 546 ms; echo time (TE), 13 ms; field-of-view (FOV), 40x40 mm²; matrix size, 192x192; slice thickness, 1.5 mm; echo train length, 6; number of signal averages (NSA), 16. The obtained in-plane resolution was 208x208 μ m².

Imaging of whole-blood clots was performed using a 47 mm diameter surface radio-frequency coil (Philips Medical Systems, Best, the Netherlands) and slightly different parameters for the IR-TSE sequence: TR, 1500 ms; TI, 610 ms; TE, 13 ms; FOV, 40x40

mm²; matrix size, 192x192; slice thickness, 1.5 mm; echo train length, 6; NSA, 32. The obtained in-plane resolution was 208x208 μm^2 .

Carotid images were obtained using a 35 mm diameter quadrature coil (Bruker Biospin GmbH, Ettlingen, Germany) and a multi-slice spin echo sequence with the following parameters: TR, 800 ms; TE, 10.2 ms; FOV, 27.7x27.7 mm²; matrix size, 312x312; slice thickness, 0.5 mm; NSA, 125. The obtained in-plane resolution was 89x89 μm^2 .

Cells were imaged as a pellet in a PCR-eppendorf, while blood clots and mice carotid arteries were imaged enclosed in 2% agarose gel and covered by binding buffer to prevent dehydration.

In all images, regions of interest (ROIs) were drawn, and mean values and standard deviations were calculated using JiveX (version 4.1, VISUS Technology Transfer GmbH, Bochum, Germany) or Para-vision 4.0 (Bruker Biospin GmbH, Ettlingen, Germany). ROIs were placed in the center of the Jurkat cell samples and whole-blood clots. For the analysis of the carotid arteries ROIs were placed over the entire vessel wall of the carotid arteries, and divided into quadrants to allow analysis and comparison of different regions of the vascular wall.

Results

The first version of the nanoparticle consisted of biotinylated AnxA5 coupled to streptavidin coated QDs in a 1:1 stoichiometry. QDs had emission peaks of either 525 nm (QD525, green) or 585 nm (QD585, red). AnxA5 contained a single biotin, located apical to the PS binding sites. The remaining binding sites of streptavidin were saturated with biotinylated Gadolinium-DTPA ³⁵, an MRI contrast-agent (biotinylated Gd-DTPA, figure 1A; AnxA5-QD-Gd, figure 2A).

To increase the perceptibility of the nanoparticle in MRI, it was aimed to increase the load of Gd-DTPA. Therefore, a biotinylated construct was designed consisting of a lysine-wedge with eight Gd-DTPA complexes attached to the periphery ³⁶ (Gd-wedge, figure 1B). Addition of this wedge to the described QDs resulted in the nanoparticle represented in figure 2B (AnxA5-QD-Gd-wedge).

Figure 1. Molecular structure of the biotinylated Gd-DTPA (A) and biotinylated Gd-wedge (B) structures with biotin (red) and Gd-DTPA (blue). Color figure on page 181.

Figure 2. Schematic representation of the two nanoparticles. QDs contain approximately 1 AnxA5 and 10 streptavidin molecules. A: the nanoparticle with single biotinylated Gd-DTPA (AnxA5-QD-Gd); B: the nanoparticle with the biotinylated Gd-wedge, containing eight Gd-DTPA complexes each (AnxA5-QD-Gd-wedge). Green: QD, yellow: streptavidin, red dot: Gd-DTPA, red star: lysine-wedge, blue: AnxA5. Color figure on page 181.

Figure 3. Cryo transmission electron microscopy image of AnxA5-QD525-Gd. Bar = 10 nm.

Low temperature transmission electron microscopy (cryo-TEM, figure 3) showed that functionalized QDs in suspension were spherical, monodisperse, and non-aggregated. The spheric diameter was measured to be 6.7 ± 1.0 nm (mean $\pm$ standard deviation (SD)), $n = 124$. AnxA5 and Gd-DTPA are not visible on the EM image; however, a gap of at least 1 nm between any two adjacent QDs indicates the presence of molecules on the surface of QDs. These results are similar to results in literature⁴⁰.

In MRI, Gd-DTPA accelerates longitudinal relaxation (T_1 -relaxation) which can be quantified by its longitudinal relaxivity (r_1) expressed in $\text{mM}^{-1}\text{s}^{-1}$. The r_1 -values of AnxA5-QD-Gd and AnxA5-QD-Gd-wedge were estimated based on the r_1 per Gd-DTPA complex, which were subsequently multiplied by the number of Gd-DTPA complexes per nanoparticle. For biotinylated Gd-DTPA coupled to avidin r_1 was $17.5 \text{ mM}^{-1}\text{s}^{-1}$ per Gd-DTPA³⁵, and for biotinylated Gd-wedge coupled to avidin r_1 was $15.6 \text{ mM}^{-1}\text{s}^{-1}$ per Gd-DTPA³⁶. Based on information supplied by the manufacturer (≈ 10 streptavidin molecules per QD), it can be calculated that AnxA5-QD-Gd and AnxA5-QD-Gd-wedge have r_1 -values per nanoparticle of $420\text{--}630 \text{ mM}^{-1}\text{s}^{-1}$ and $3000\text{--}4500 \text{ mM}^{-1}\text{s}^{-1}$, respectively.

AnxA5-QD-Gd and AnxA5-QD-Gd-wedge nanoparticles were examined on their property to discriminate between living and dying cells using TPLSM. Jurkat cells were triggered to execute apoptosis by activation of Fas receptor using anti-Fas antibodies. Binding of AnxA5-QD-Gd and AnxA5-QD-Gd-wedge occurred to early and late apoptotic cells but not to viable cells (figure 4) in a manner comparable to Oregon Green labeled AnxA5 (not shown)⁴¹. During early apoptosis, small patches of green fluorescence were

Figure 4. TPLSM images showing cellular AnxA5-QD525 (green) distribution. All cells were counterstained with PI (red) to determine membrane leakage, i.e., determine the stage of apoptosis. Top-row: early apoptotic Jurkat cells labeled with A; AnxA5-QD-Gd and B; AnxA5-QD-Gd-wedge. Bottom-row: late apoptotic cells labeled with C; AnxA5-QD-Gd and D; AnxA5-QD-Gd-wedge. Bar = 10 μ m. Color figure on page 182.

Figure 5. TPLSM image of a Jurkat cell, incubated with AnxA5-QD525-Gd (green), while simultaneously inducing apoptosis by anti-Fas antibodies. After 5.5 hours cells were washed in EDTA buffer to remove AnxA5 present on the outside of the cells, and subsequently stained with AnxA5-QD585-Gd (red). Yellow indicates co-localization of QD525 and QD585 (probably caused by QD525 on the cell exterior that had not completely been removed). Cells were counterstained with Syto41 (blue), a nucleic acid stain. Bar = 10 μ m. Color figure on page 182

visible on the cellular membrane (figure 4A/B), whereas during late apoptosis the entire cellular membrane was brightly fluorescent (figure 4C/D). Late apoptotic phase is characterized by loss of plasma membrane integrity and concomitant nuclear uptake of propidium iodide (PI), showing a red, fragmented nucleus. In addition, AnxA5-QD-Gd was able to be internalized by Jurkat cells (figure 5), as was reported for AnxA5⁴¹. A movie, showing a z-stack of a cell, similar to the cell in figure 5, is also available at <http://pubs.acs.org>. Internalization of AnxA5-QD-Gd-wedge by Jurkat cells was not investigated in this study.

Binding of AnxA5-QD-Gd and AnxA5-QD-Gd-wedge to dying cells depends on cell surface exposure of PS, since QDs functionalized with M1234, a variant of AnxA5 that does not bind to PS³⁴, do not show binding to apoptotic cells (figure 6). All together, these findings clearly demonstrate that AnxA5 coupled to a QD retains its full biological property to bind to PS exposed on the surface of dying cells.

Figure 6. TPLSM images showing the cellular distributions of QD585 (red) coupled to AnxA5 or a mutant of AnxA5, unable to bind to PS (M1234), on apoptotic Jurkat cells. All cells were counterstained with Syto41 (blue), a nucleic acid stain. A: AnxA5-QD-Gd. B: AnxA5-QD-Gd-wedge. C: M1234-QD-Gd-wedge. Bar = 50 μm . Color figure on page 182.

Figure 7. Sections of a single MR image, showing pellets of apoptotic Jurkat cells incubated with AnxA5-QD-Gd or AnxA5-QD-Gd-wedge. A: T1-weighted MR-images of the four cell samples. B: Graph showing mean $\pm$ SD for signal intensities of the pixels in the regions of interest (ROIs), drawn within the samples shown in A. Cells incubated with: 1, buffer only (control 1); 2, 1.6 μM Gd-wedge only (control 2); 3, 40 nM AnxA5-QD-Gd; 4, 40 nM AnxA5-QD-Gd-wedge.

Having established the AnxA5-specific binding properties of AnxA5-QD-Gd and AnxA5-QD-Gd-wedge by fluorescence studies, the functional bimodality of the nanoparticle had to be confirmed. Apoptotic Jurkat cells were incubated with buffer (control 1), Gd-wedge (control 2), AnxA5-QD-Gd or AnxA5-QD-Gd-wedge and subsequently measured with MRI (figure 7). The amount of Gd-wedge in sample 2 (control) was equal to the amount used in sample 4.

Cells incubated with AnxA5-QD-Gd (figure 7, sample 3) exhibit a signal intensity that is a factor 5.9 and 6.9 higher as compared to control 1 and control 2, respectively. Cells incubated with AnxA5-QD-Gd-wedge (4) exhibit a signal intensity that is a factor 5.8 higher than 3, demonstrating that AnxA5-QD-Gd-wedge has a much better T_1 -lowering

effect than AnxA5-QD-Gd, as was expected from the differences in r_1 . This results in a higher MRI signal intensity in T_1 -weighted imaging using the same amount of nanoparticles.

Cultured cells are simple *in vitro* systems for testing molecular imaging agents. Results derived from such systems hardly give reliable predictions about behavior in the *in vivo* situation. Therefore, a more complex system was chosen to assess the robustness of this nanoparticle as a bimodal molecular imaging agent. Since activated platelets expose PS, a human whole-blood clot system was chosen. Human whole blood was incubated with AnxA5-QD-Gd-wedge; subsequently PS exposure by platelets was induced to trigger clot-formation. The concentration of nanoparticles within the clot was expected to be much lower as compared to cell pellets. Within the clot only platelets expose PS, and not, for example, captured erythrocytes. An additional complexity of this system comes from the facts that AnxA5 competes with coagulation factors for binding to PS and acts as an anticoagulant preventing platelets from being incorporated into the thrombus^{11, 42}. Hence, MRI signal intensities were not expected to achieve the levels of figure 7. Therefore, following experiments were performed with AnxA5-QD-Gd-wedge only.

In order to label as many platelets as possible, whole blood was incubated with AnxA5-QD-Gd-wedge prior to clot-formation. Coagulation in whole blood, mediated by thrombin generation and PS exposure on platelets, was triggered by adding Ca^{2+} to citrate-anticoagulated whole blood. Additional PS exposure was triggered by pre-activating the platelets in whole blood with thrombin and collagen¹¹, to counteract the anticoagulant effect of AnxA5. Whole-blood was incubated with buffer (control 1), Gd-wedge (control 2), 40 nM or 200 nM AnxA5-QD-Gd-wedge; subsequently clot-formation was induced and clots were measured with MRI (figure 8). The amount of Gd-wedge in sample 2 (control) was equal to the amount used in sample 4.

Signal intensities of the four clots are shown in figure 8B. Corresponding T_1 -values for clots 1, 2, 3, and 4 are 734 ± 85 , 740 ± 44 , 678 ± 89 , 628 ± 30 ms (mean $\pm$ SD), respectively. The signal intensity of clot 4 was approximately 47% and 36% higher than controls 1 and 2, respectively. These results demonstrate that MRI of PS exposure in a more complex system is feasible using the AnxA5-QD-Gd-wedge nanoparticle. A recent study

Figure 8. A: T₁-weighted sections of a single MR image, showing whole-blood clots, activated with Ca²⁺, thrombin and collagen, and incubated with AnxA5-QD-Gd-wedge enclosed in 2% agarose gel. B: graph showing mean $\pm$ SD for the signal intensities of the pixels in ROIs, drawn within the samples shown in A. Clots were incubated with: 1, buffer only (control 1); 2, 8 μ M Gd-wedge only (control 2); 3, 40 nM AnxA5-QD-Gd-wedge; 4, 200 nM AnxA5-QD-Gd-wedge. Clot 3 was slightly damaged, causing the black spot in the clot. Clot 4 was larger probably due to the fact that the higher concentration of AnxA5 inhibited clot retraction through its anticoagulant action.

showed that with SPECT imaging mural thrombus renewal in abdominal aortic aneurysms *in vivo* can be visualized using AnxA5 labeled with a radionuclide²⁵. This study proved that thrombus imaging *in vivo* is feasible using AnxA5.

Whole-blood clots were also analyzed with TPLSM. For TPLSM, clots were incubated with AnxA5-QD-Gd-wedge after clot-formation, because in contrast to MRI, a lower amount of AnxA5-QD-Gd-wedge was sufficient for visualization by TPLSM. These experiments could thus be performed without interfering with clot-formation.

Distribution of AnxA5-QD-Gd-wedge in the whole-blood clots was similar to the distribution of FITC-labeled AnxA5 (figure 9A). AnxA5-QD-Gd-wedge specifically labeled activated platelets (figure 9B/C), as can be deduced from the size of the labeled structures, measuring ca. 1-2 μ m. Larger cells incorporated in the clot and easily discernible with a cytoplasm-specific or nucleic acid-specific dye, were stained neither with AnxA5-FITC nor AnxA5-QD-Gd-wedge (figure 9A/B). Figure 9C shows whole blood that was pre-incubated with anti-fibrinogen-FITC and subsequently activated to form a clot. Staining the clot with AnxA5-QD-Gd-wedge reveals that PS exposing platelets are aligned along the fibrin network within the clot.

Figure 9: TPLSM images of whole-blood clots incubated with AnxA5 after clot-formation. A; green: AnxA5-FITC, blue: syto41, a nucleic acid stain; B; green: AnxA5-QD525-Gd, red: acridine red, a cytoplasmatic stain. C; red: AnxA5-QD585-Gd, green: anti-fibrinogen-FITC. Platelets show a bright cellular membrane, similar to apoptotic cells (compare to figures 4/5/6). Bar = 50 μm . Color figure on page 183.

To demonstrate the feasibility of this AnxA5-functionalized bimodal nanoparticle as a target-specific nanoparticle for cell death in the vascular wall, a murine carotid artery was mechanically injured *in vivo* by endothelial denudation using a metal wire as described elsewhere³⁸. This injury results in rapid PS exposure by cells of the tunica intima, media, and adventitia³⁸. Both damaged and undamaged carotid arteries were excised and mounted in a perfusion chamber. A static transmural pressure of 40 mmHg was applied, and the arteries were labeled intraluminally with AnxA5-QD-Gd-wedge by

Figure 10: A; TPLSM image of a wire-injured murine carotid artery, showing the various vascular wall layers. The tunica intima consists of endothelial cells (ECs); the tunica media mainly consists of smooth muscle cells (SMCs). Uptake of nanoparticles is visible in ECs and SMCs. Green: AnxAS-QD-Gd-wedge; red: eosin, labeling elastin laminae; blue: syto41, labeling cell nuclei. L: lumen; IEL: internal elastic lamina; EEL: external elastic lamina. Bar = 10 μ m. The imaging plane was slightly oblique, as opposed to a longitudinal direction of the artery. B; transversal MR image of two murine carotid arteries: undamaged (control, left) and damaged (right, same artery as shown in A). Bar = 0.5 mm. Color figure on page 184.

slow perfusion. The arteries were imaged by TPLSM as described in a recent article³⁹. Figure 10A shows uptake of AnxAS-QD-Gd-wedge in the endothelial cells (ECs) in the tunica intima and in smooth muscle cells (SMCs) in the tunica media. Apparently, nanoparticles are able to penetrate through elastic laminae deep into the tunica media. It is unclear why the first SMC layer hardly shows any uptake of the nanoparticle, while the layer of SMCs between the outer two elastic laminae does show abundant labeling. Furthermore, no labeled platelets were visible, as no blood was present at time of labeling. A movie of a 3-dimensional representation of the carotid artery in figure 10A is available at <http://pubs.acs.org>. The undamaged control artery hardly shows any labeling. Next, the arteries were removed from the perfusion chamber, and embedded in agarose gel for imaging with MRI. The amount of labeling of the damaged artery is sufficient to increase the MRI signal intensity compared to the control artery (figure 10B). To determine the increase in signal intensity, three transversal MRI slices of the carotid

arteries were divided into quadrants. In these quadrants, ROIs were drawn to select the vascular wall. This is because the uptake of nanoparticle was not homogeneous throughout the vascular wall, as it was unevenly damaged by the wire. A total of twelve signal intensities was acquired per artery. The mean signal intensity increased from 83.2 ± 3.6 for the control artery to 91.9 ± 13.5 for the damaged artery (arbitrary units, mean $\pm$ SD), and ranging from 73.5 to 86.9 for the control artery and 73.4 to 125 for the damaged artery. This indicates that the increase in signal intensity differs per location in the vascular wall and ranges from 0 to 50% compared to the control, undamaged artery.

Discussion

Reports about QD-labeled AnxA5 have been published recently. As mentioned before, Le Gac *et al.*²⁸ investigated photo-stability of streptavidin-coated, AnxA5-labeled QDs. In contrast to the biotinylated AnxA5 used in this study, the described AnxA5 contained three to four biotins, located at random sites on the AnxA5 polypeptide. This implies, as pointed out by the authors, that AnxA5 coupling to QDs may lead to masking of PS binding sites and aggregation of the QDs. The current study utilized cys2-AnxA5 which contains a single biotin attached to the N-terminal tail that is located opposite to the side of the AnxA5 molecule harboring the PS binding sites⁴³. Coupling of cys2-AnxA5 to streptavidin coated QDs neither induced aggregation nor caused a loss of biological activity. In addition, a recent study⁴⁴ showed that cells, labeled with AnxA5-QDs, can be imaged using an *in vivo* imaging system.

Another study investigated bimodal, paramagnetic micellular QDs that were functionalized with AnxA5⁴⁵. Table 1 summarizes the most important characteristics of AnxA5-functionalized bimodal nanoparticles published in literature. Two other T_1 -weighted nanoparticles, loaded with Gd-DTPA, are compared with the novel nanoparticle described in the current study. Furthermore, two T_2 -weighted nanoparticles, containing iron oxide, are listed. AnxA5-QD-Gd-wedge has several advantages. First, it is the smallest nanoparticle so far. This can be advantageous when the imaging target is outside the vessel lumen, as in, for example, atherosclerotic plaques or tumors. Furthermore, of the T_1 -weighted nanoparticles, AnxA5-QD-Gd-wedge is the nanoparticle with the highest relaxivity relative to the nanoparticle surface area. AnxA5-QD-Gd-wedge allows visi-

ble and near-infrared imaging and shows high fluorescence intensities. Additionally, QDs can be localized by electron microscopy⁴⁰. Moreover, the 1:1 stoichiometry of the AnxA5-QD complex provides the most optimal configuration for maximizing the amount of bimodal nanoparticle bound to the PS exposing surface. Other advantages of AnxA5-QD-Gd-wedge are straightforward preparation (add and mix), and variability of the nanoparticle's biological functionality. By replacing AnxA5 by a different biotinylated targeting function this nanoparticle can be used for molecular imaging of different biological processes⁴⁶.

The results in this paper demonstrate that the bimodal AnxA5-QD-Gd-wedge nanoparticle can be used to analyze biological samples as well as vascular structures with MRI at the anatomical level and with TPLSM at the cellular level. AnxA5-QD-Gd-wedge has high potential for *in vivo* imaging in the near future.

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Table 1. Comparison of five different bimodal AnxA5-conjugated nanoparticles. NIRF = near infrared fluorescence.

	nanoparticle	T ₁ or T ₂ -lowering	size (nm)	r ₁	r ₂	stoichiometry AnxA5: particle	optical
1	QD-AnxA5-Gd-wedge ^a	T ₁	~7	3000-4500 (mM ⁻¹ particle) ₁ ⁻	5600-8400 (mM ⁻¹ particle) ₁ ⁻	1:1	Visible, NIRF
2	Micellar QD-AnxA5 ^b	T ₁	< 10	~1860 (mM ⁻¹ particle) ₁ ⁻	~2700 (mM ⁻¹ particle) ₁ ⁻	>5:1 ^e	Visible, NIRF
3	AnxA5-fluorescein liposomes ^c	T ₁	100	~164,000 (mM ⁻¹ particle) ₁ ⁻	~272,000 (mM ⁻¹ particle) ₁ ⁻	>5:1 ^e	Visible
4	AnxA5-CLIO-Cy5.5 ^d	T ₂	50	19 (mM ⁻¹ Fe) ₁ ⁻	48 (mM ⁻¹ Fe) ₁ ⁻	3.5:1	NIRF
5	AnxA5-fluorescein micellar iron oxide ^c	T ₂	10	13.3 (mM ⁻¹ Fe) ₁ ⁻	159.6 (mM ⁻¹ Fe) ₁ ⁻	>5:1 ^e	Visible

a, this paper; b, van Tilborg *et al.*⁴⁵; c, van Tilborg *et al.*²⁰; d, Schellenberger *et al.*¹⁹; e, personal communication.

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Chapter 4

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Hot-spot labeling of apoptosis and
collagen in atherosclerotic plaques using
bimodal quantum dots

In preparation

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Abstract

Targeted bimodal quantum dots (Qdots) were utilized for imaging either collagen or cell death in atherosclerotic plaques of carotid arteries of ApoE^{-/-} mice. Two-photon laser scanning microscopy (TPLSM) revealed that both types of Qdots bound to hot-spots in intimal as well as deeper layers of the atherosclerotic plaque. Likely, Qdots get access to vascular targets only at sites of endothelial damage, reflecting plaque instability. MRI showed a minor increase in signal intensity for collagen-targeting Qdots.

Introduction

Molecular imaging is a fast growing research area. Specific molecular targeting of pathological sites by ligands coupled to contrast agents allows detection and diagnosis of disease using non-invasive imaging modalities such as MRI, PET, SPECT, ultrasound, CT, or fluorescence imaging. Molecular imaging of atherosclerosis would allow non-invasive and accurate visualization of atherosclerotic plaques, as well as discrimination between stable and vulnerable plaques in patients at risk. Insight into biology of developing atherosclerotic plaques has provided potential targets such as matrix metalloproteinases¹⁻³, macrophage markers⁴⁻⁶, and thrombus-specific molecular markers^{7,8}.

Two promising targets are phosphatidylserine (PS) and collagen. PS is a phospholipid exposed on dying cells⁹ and activated platelets¹⁰. As these are both present in ruptured plaques or plaques at risk of rupture¹¹, PS imaging may allow the detection of vulnerable plaques^{12,13}. Annexin A5 (Anx) is a ligand for PS with an affinity in the nanomolar range^{9,10}, and is successfully used in several pre-clinical^{12,14,15} and clinical¹⁶⁻¹⁸ imaging studies on the detection of cell death.

More recently, collagen was proposed as a suitable target for imaging atherosclerosis^{19,20}. Atherosclerotic plaques expose more collagen to the lumen compared to non-plaque areas of the vascular wall for two reasons: first, the intimal layer of the plaque is activated and possibly disrupted, which means that it is no longer a seamless cover of the collagen-containing basal lamina²⁰. Second, as plaques become more advanced, a fibrous cap develops, consisting for a large part of collagen types I, III, IV, and V²¹. CNA35 (CNA) is a recently developed polypeptide that binds collagen types I, III, and IV with an affinity of 0.5 μM ²², and has shown to be a suitable probe for collagen targeting in atherosclerotic plaques in ApoE^{-/-} mice¹⁹.

Recently, we reported a bimodal nanoparticle for molecular imaging. The nanoparticle consists of a streptavidin-coated quantum dot (Qdot, for fluorescence imaging) to which biotinylated Gadolinium-DTPA (for MR imaging) was coupled²³. Bimodal imaging has the advantage of allowing non-invasive imaging by MRI and high-resolution imaging at subcellular level by fluorescence imaging of the same specimen. Qdots are fluores-

Figure 1 (left). Schematic representation of Anx- or CNA-Qdot. Red: Qdot; yellow: streptavidin; grey: Gd-DTPA8 wedge; blue: Anx or CNA. Color figure on page 184.

cent semiconductor nanocrystals, having several advantages over organic fluorophores: i) they have high quantum yields (i.e., they are brightly fluorescent)²⁴, ii) low photobleaching²⁵, allowing improved non-invasive²⁶⁻²⁸ and repetitive, long-term fluorescence imaging²⁹, iii) they have broad excitation and narrow emission spectra, allowing simultaneous imaging of multiple targets using Qdots with different emission peaks³⁰. These advantages render Qdots into attractive nanoparticles for *in vivo* molecular imaging, as demonstrated in imaging studies of tumor tissue³¹⁻³³, sentinel lymph nodes³⁴, and reticulo endothelial system (RES)³⁵.

Qdots have previously been functionalized with additional contrast agents allowing magnetic resonance imaging (MRI)^{23, 33, 36} or positron emission tomography³⁷. In the present study, we attempted to visualize vulnerable atherosclerotic plaques by both MRI and two-photon laser scanning microscopy (TPLSM). In our approach, we equipped streptavidin-conjugated Qdots with biotinylated Gadolinium-DTPA lysine-wedges, each containing 8 Gd-DTPA moieties for increased MRI sensitivity, as previously described²³. This Qdot was further equipped with either biotinylated Anx or biotinylated CNA (both are polypeptides of approximately 35 kDa) for atherosclerotic plaque imaging (see figure 1).

First, biodistribution and pharmacokinetics of these bimodal CNA- and Anx-Qdots were determined in mice. Next, bimodal nanoparticles were investigated for their capability to detect atherosclerotic plaques in ApoE ^{-/-} mice. Atherosclerosis was induced by a high-fat diet and silicon perivascular collars around both carotid arteries³⁸. Three or six weeks after collar placement, mice were scanned *in vivo* with MRI and injected with CNA-, Anx-, or control-Qdots. Afterwards, mice were sacrificed; carotid arteries were excised and imaged with TPLSM at sub-cellular resolution.

Materials and methods

Annexin A5: A cysteine residue was engineered in the N-terminal tail, which is located apical to the PS binding sites (cys2-Anx). Maleimide-activated biotin (Pierce, Rockford, IL) was coupled to cys2-Anx according to the recommendations of the manufacturer.

CNA: CNA was produced as described previously²², and was biotinylated by coupling a C-terminal thioester on CNA to an N-terminal cysteine residue on biotin using native chemical ligation.

Gd-DTPA wedge: Biotinylated Gd-wedge was synthesized similar to a method described previously^{23, 39}.

Bimodal Qdots: Anx-Qdots, CNA-Qdots, and control-Qdots were prepared as described previously²³, by mixing streptavidin-coated QDots (stock concentration 1 μ M and emission peak at 525 or 585 nm, Invitrogen, Eugene, OR/USA) with biotinylated Anx or CNA, and biotinylated Gd-wedge in Hanks Balanced Salt Solution (HBSS, pH 7.4), containing 144 mM NaCl, 14.9 mM HEPES, 5.5 mM glucose, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM KH_2PO_4 , and 1.2 mM MgSO_4 . This procedure yields complexes with average stoichiometries of QD:Anx/CNA:Gd-wedge of 1:6:24. These stoichiometries were derived from information supplied by Invitrogen that each Qdot is coated with 8-12 streptavidin molecules. Control-Qdots lack both Anx and CNA.

Clearance and biodistribution study: Animal experiments were approved by the local ethics committee. Male Swiss mice 40.1 ± 4.4 gr (mean $\pm$ SD, n=1 or 2 per group) were anesthetized by 3% isoflurane applied through a mask, anesthesia was maintained by 1.5% isoflurane. Mice were then cannulated in the femoral vein for intravenous injection of Qdot585 solution (100 pmol Qdots per animal) and on the opposite side in the femoral artery for blood sample withdrawal. A blank blood sample was drawn prior to Qdot injection, and after injection samples were drawn 1', 2', 5', 10', 15', 20', 30', 45', 60' and 90' after injection. Samples of 50 μ l were drawn using a syringe, and kept at 4 °C until analysis. After 90' of circulation, mice were sacrificed by cervical dislocation, and liver, spleen, kidney, heart, lung, and intestine tissue were collected. To determine the fluorescence intensity in blood, samples were centrifuged for 3' at 278 g and serum was collected. Whole-blood samples could not be measured due to the high absorbance of

erythrocytes. The residue did not contain Qdots, as was checked with TPLSM. Fluorescence intensities of blood samples were measured using a fluorospectrometer, the NanoDrop 3300 (NanoDrop Technologies, Inc, Wilmington, DE/USA). Two- μ l serum samples were applied to the NanoDrop pedestal and the fluorescence intensities were measured (arbitrary units) at the peak of the emission spectrum. In order to determine mono-exponential fits of the data and corresponding circulation half-lives, GraphPad Prism 4 (GraphPad Software, Inc.) was used. Figure 4 shows the clearance of Qdots normalized to the fluorescence intensity at $t = 2'$; intensity at $t = 1'$ was too variable (actual time of sample withdrawal could be between $0'$ and $2'$) due to too many handling steps in a short time.

Atherosclerotic mice: Experiments were approved by the local ethics committee. Fifteen-week old male ApoE $-/-$ mice (Charles River Laboratories, Maastricht, the Netherlands), $n = 4$ per group, were fed a high-fat diet (Hope Farms diet 4021.06, Woerden, the Netherlands) for two weeks. After subcutaneous injection of buprenorphine (0.4 mg/kg), and while under isoflurane anesthesia applied through a mask, perivascular silicon collars (Standard Silicon Tubing, Helix Medical, Carpinteria, CA/USA; inner diameter 0.31 mm, outer diameter 0.64 mm, length 3 mm) were placed bilaterally around the common carotid arteries and ligated using three ligatures, as described by Von der Thüsen *et al.*³⁸, inducing atherosclerotic plaque growth proximal to the collar. Mice remained on high-fat diet. Either 3 or 6 weeks after collar placement, mice were again anesthetized by isoflurane, and received a cannula in the femoral vein. Three-week mice weighed 27.8 ± 3.1 gr (mean $\pm$ SD), six-week mice weighed 29.2 ± 1.5 gr (mean $\pm$ SD) at time of cannulation. This cannula was approximately 40 cm in length, and already contained the Qdot525 solution. This length is necessary to allow injection from outside the MRI scanner without having to remove the mice from the scanner. This way, pre- and post-injection scans could be made of the carotid arteries at exactly the same position. The injected dose was 200 pmol Qdots per animal, which equals ≈ 1.3 μ mol/kg Gd-DTPA. Mice were sacrificed after the scan-procedures were completed (after approximately 2 hours of circulation) by dissection of the diaphragm and cardiac perfusion with 5 mL of HBSS in order to remove blood from the vasculature via atrial-

puncture. Then the carotid arteries were carefully excised; collars, connective tissue, and adipose tissue were removed.

MRI: Mice were placed in an animal holder and scanned in a 7T Bruker Biospec (Bruker Biospin, GmbH, Ettlingen, Germany), while under isoflurane anesthesia and warmed by a heating pad. Respiration and temperature were continuously monitored. Carotid images were obtained using a 35 mm diameter quadrature coil (Bruker Biospin GmbH, Ettlingen, Germany). First, scout images were acquired in order to plan the transverse slices perpendicular to the carotids. Next, RARE T_1 -weighted pulse sequences were performed with the following parameters: TR, 1014 ms; TE, 8.2 ms; field-of-view, 30x30 mm²; matrix size, 312x312; slice thickness, 0.5 mm; number of averages, 32; rare partitions, 4. The obtained in-plane resolution was 96x96 μm^2 . Pre- and post contrast scans were made with the same gain settings. Post-injection scans were started 10 minutes after injection. Regions of interest (ROIs) were drawn on the vascular wall on the first transverse slice proximal to the collar independently by two observers. Absolute enhancement (SI_{enh}) was calculated by dividing mean MR signal intensities of the ROI on the post-injection image by the mean MR signal intensities of the ROI on the pre-injection image. Measurements of two independent observers were averaged. SPSS 15.0 (SPSS Inc) was used to determine statistical signal enhancement by testing if $SI_{\text{enh}} \neq 1$ with a two-way, one-sample t-test. $P < 0.05$ was considered statistically significant.

Tissue preparation for TPLSM imaging: In the biodistribution study, samples of non-fixed, fresh tissue were embedded in 2% agarose gel, and covered by HBSS. For the imaging of Qdot uptake in plaques, fresh carotid arteries were imaged in either of three different ways (because plaque tissue can only be partially imaged due to high light scattering by fat tissue): 1) mounted into a home-built perfusion chamber⁴⁰ (IDEE BV, Maastricht, the Netherlands) in HBSS, while under a physiological transmural pressure of 80 mmHg (using a modified Big Ben sphygmomanometer, Riester, Germany) and viewed from the adventitial side; 2) dissected longitudinally, and viewed from the luminal side ('en face'); 3) dissected transversally so that the cross-section of the artery could be imaged.

To stain cell nuclei in the vascular wall, arteries were incubated in 2 μM syto44 (Invitrogen, Eugene, OR/USA). Nile Red (Invitrogen, Eugene, OR/USA) was used to stain lipids.

Nile Red was dissolved in HBSS at 500 µg/mL, containing 1% DMSO and Pleuronic, and filtered using a 0.45 µm filter. For proper labeling, arteries were incubated with either dye for at least 30'. For *ex vivo* labeling with CNA-streptavidin-Alexa568, one artery was incubated for 30' in 500 µl of 320 nM streptavidin-Alexa568 (Invitrogen, Eugene, OR/USA) with bound biotinylated CNA in HBSS.

Two-photon laser scanning microscopy: A standard Bio-Rad 2100MP (Hemel Hempstead, UK) was used in TPLSM mode. The excitation source was a Spectra Physics Tsunami Ti:Sapphire laser (Mountain View, CA/USA), tuned and mode-locked at 800 nm. The Tsunami laser produces light pulses of about 100 fs width at a repetition rate of 82 MHz. Laser light reached the sample through a Nikon water immersion lens (60x water dipping, numerical aperture (NA) 1.0, working distance 2.8 mm), incorporated in an upright Nikon E600FN microscope (Tokyo, Japan). Behind the objective, laser pulse width is estimated to be around 300 fs, based on optical properties as given by the microscope manufacturer. Three photomultipliers detected the fluorescence. Each photomultiplier accepted a different, tunable wavelength region. Initially, these filter settings were used: blue (for autofluorescence up to 500 nm; for syto44, 420-470 nm); green (for autofluorescence 500-560 nm; for Qdot525 510-540 nm); and red (for autofluorescence and Nile Red 560 nm and higher; for Qdot585, 570-600 nm). All three channels were optimized individually, such that they showed maximal intensities while avoiding saturation and leak-through to other channels. The three obtained images were combined into a single image. Only combined images are shown. No additional image processing was performed. An imaging speed of 0.1 Hz with a pixel dwell time of 37.8 µs was used, and a Kalman filter over 1 frame was applied; or an imaging speed of 0.14 Hz, pixel dwell time 10.5 µs in combination of a Kalman filter over 2 frames.

Results

The biodistribution of Qdots at 90min after intravenous injection was examined by *ex vivo* TPLSM. Anx- and CNA-Qdots localized mainly around liver and spleen vasculature, presumably inside vesicles in macrophages in the vicinity of the vasculature (figure 2). No kidney clearance was found, presumably due to the relatively large size of the Qdot (diameter ≈ 10 nm²³). These results confirmed previous reports^{25, 26, 32, 35}. Though the

Figure 2 (below). Distribution of CNA-Qdots in A) spleen, inset is magnification of box; B) liver, inset is magnification of box; C) kidney glomerulus. Distribution of Anx-Qdots in D) spleen and E) liver. Qdots (red), autofluorescence (blue). Arrows in A) and B) indicate collagen-binding Qdots. Bar = 10 μ m. Color figure on page 185.

biodistributions of CNA- and Anx-Qdots were largely similar, some subtle differences became apparent. Anx-Qdots were only observed within spots; CNA-Qdots also appeared to bind collagen in vascular walls in spleen and liver (indicated by the arrows in figure 2). This is more clearly visible in figure 3, showing spleen and liver of a mouse injected with both CNA-Qdots (red) and bare streptavidin-Qdots (green). Though Qdots are not cleared by the kidneys, CNA-Qdots were observed within the glomerulus (figure 2C). CNA-, Anx-, and control-Qdots were never observed in kidney tubuli, confirming that Qdots were not renally excreted. Lung tissue showed some aggregates of Qdots, while intestine and heart tissue only sporadically showed small aggregates within microvessels (not shown).

Circulation half-lives reported in literature are highly variable, depending on, among others, size and surface chemistry⁴¹. Half-life values varying between 4.6min and 19.8h^{26, 28, 32, 42-44} have been reported. The clearance of Anx- and CNA-Qdots followed a mono-exponential clearance curve with circulation half-lives ($t_{1/2}$) of 3.5min for CNA-Qdots and 5.3min for Anx-Qdots (figure 4). To determine the component of the bimodal Qdot that causes fast clearance, also circulation half-lives of bare streptavidin-Qdots (without ligand or Gd-wedge) and Qtracker (Qdot without streptavidin) were determined. Bare

Figure 3. Difference in distribution of bare Qdots (green) and CNA-Qdots (red). A) spleen, B) liver, C) magnification of boxed part in A), D) magnification of boxed part in B). Arrows indicate collagen-specific binding of CNA-Qdots. Bar = 10 μ m. Color figure on page 185.

Figure 4. Clearance of CNA-Qdots, Anx-Qdots, bare Qdots (without ligand and Gd-wedge, but with streptavidin), and Qtracker (Qdot without streptavidin). Data points are normalized to fluorescence intensity at $t = 2$ min (see the Materials and Methods section). Mono-exponential curves are fitted through the data. The fit for Qtracker is indicative due to scattering of data points.

streptavidin-Qdots have a half-life of 6.6min, while the half-life of Qtracker could not be determined due to high variation in data points. However, it is clear from figure 4 that this half-life is much greater (> 90 min) than for any of the streptavidin-coated Qdots. Consequently, the presence of streptavidin is most likely responsible for the fast clearance of the bimodal Qdots.

Figure 5. Plaque types in carotid arteries for three-week and six-week collar-bearing ApoE^{-/-} mice. Plaque types of both carotid arteries of all mice are combined. Types were binned as follows: 0, no plaque; 1, small, not occlusive; 2, moderate, up to 50% occlusive in lumen diameter; 3, large, 50-70% occlusive in lumen diameter; 4, severe, 70-100% occlusive in lumen diameter.

The size of the collar-induced plaques in ApoE^{-/-} mice was determined based on light-microscopic evaluation after excision of the carotids. Plaques were assessed as one of the following types: 0, no plaque; 1, small, non-occlusive plaque; 2, moderate plaque, up to 50% occlusion of lumen diameter; 3, large plaque, 50-70% occlusion of lumen diameter; and 4, severe plaque, more than 70% occlusion of lumen diameter. The median for three-week collar plaques was type 1, while for six-week collar plaques the median was of type 2 (figure 5). Type 1 plaques consisted mainly of a collection of small lipid cores. With increasing plaque size, the size of the lipid cores increased while the number of lipid cores decreased. Type 4 plaques consist generally of few but very large lipid cores.

Arteries were imaged *ex vivo* with TPLSM. Plaque tissue is difficult to visualize due to limited penetration depth of light in plaque (fat) tissue. With the set-up used, plaques could be imaged up to a thickness of approximately 100 μm ²⁰. Depending on the view-angle ('en face', transversal, or mounted arteries, see Materials and Methods), various aspects of the plaque could be visualized. When imaging mounted arteries, generally less Qdots were observed. This can be explained by the fact that Qdots were localized

Table 1. Overview of CNA-, Anx-, and control-Qdot labeling in plaque-containing carotid arteries.

ligand	plaque type	no. of arteries	Qdot labeling of plaque
Control	0	2	In one artery minor endothelial labeling, in one artery no labeling.
	1	3	In one artery minor endothelial labeling, two arteries showed no labeling.
	2	8	In seven arteries minor to moderate endothelial labeling (figure 6A/B/C), in one artery no labeling.
	3	1	Minor endothelial labeling in one artery.
	4	1	Moderate endothelial labeling in one artery (figure 6D).
CNA	0	3	Minor Qdot labeling of fibrous caps in three arteries.
	1	5	Minor to moderate Qdot labeling of fibrous cap around lipid pools in all five arteries (figure 7A).
	2	1	The artery shows weak labeling of fibrous cap.
	3	3	In one carotid artery strong labeling of fibrous cap (figure 8A/B), a second artery shows CNA-Qdots on fibrous cap covering lipid pools (figure 8C); the third artery shows weak labeling. Figures 8A/B/D/F show the same artery.
	4	2	In one artery strong labeling inside lipid pool (figure 7B), in other artery strong labeling of basal lamina around lipid pools (figure 8E)
Anx	0	3	Minor endothelial labeling in three arteries.
	1	3	Some endothelial labeling in two arteries that also show minor uptake of Qdots in lipid pools (figure 10C/D). One artery shows no labeling.
	2	5	Minor to moderate endothelial labeling in four arteries. Minor labeling inside lipid pool in another artery (figure 11C)
	3	1	This artery showed endothelial labeling, but also labeling inside lipid pools (figure 10A/B, same artery). Stronger labeling than in types 1-2.
	4	3	Two arteries were completely occluded (collaterals had formed), no Qdot labeling. One artery showed strong labeling inside lipid pool (figure 11B/D), and also endothelial labeling (figure 11A, 11A/B/D are the same artery).

on or just underneath the endothelium, and excitation as well as emission light had to go through plaque tissue in order to reach the Qdots. In general, this means that only plaque shoulders could be imaged properly. However, an advantage of mounted arteries is that they are in a near-physiological situation as they are pressurized at normal transmural pressures. For an overview of the entire plaque, the artery was best imaged

Figure 6. A and B) Control-Qdots (green) in three-week collar plaques. Both images show carotid arteries in a flow chamber and under 80 mmHg transmural pressure. A) Control-Qdots are bound to endothelium covering lipid pools (type 2). B) Control-Qdots are seen in the grooves of elastic laminae in an area adjacent to plaque (type 2). C and D) Control-Qdots (green) in six-week collar plaques. C) Mounted carotid artery under 80 mmHg (type 2). Endothelial cells on plaque are labeled with control-Qdots, indicated by arrow. D) 'En face' view of carotid artery (type 4). White lines indicate border between media and intima layers. Arrows indicate control-Qdots bound to endothelium. Blue in A) and C) are nucleic acids, and in B) and D) autofluorescence. Red is lipid. 'L' is lipid pool, 'VW' is vascular wall. Bar = 10 μ m. Color figure on page 186.

'en face'; the luminal surface of lipid pools could then be visualized. Finally, cross-sections were best suitable to visualize the inside of a plaque or lipid pool.

Each of three views revealed different parts of the plaque, and they were used in this study alternately to gather as much information on the Qdot intra-plaque distribution as possible. A quantitative analysis of plaque labeling is impossible using TPLSM images, as the plaque cannot be imaged entirely with either of the view angles. Therefore, a semi-quantitative and qualitative description of our findings is presented here. An overview of the results of 3 and 6 week old collar-bearing ApoE $-/-$ mice is given in table 1 and is further discussed in the following paragraphs.

Figure 7. CNA-Qdots (green) in three-week collar plaques. A) 'En face' view (type 1). B) Transverse view (type 4). Dashed line indicates border between plaque and lumen. In A) blue are nucleic acids, in B) autofluorescence. B) shows the sum of a small stack of images. 'L' is lipid core, 'VW' is vascular wall. Bar = 10 μ m. Color figure on page 186.

Three- and six-week plaques showed minor staining with control-Qdots. If labeling was observed, it localized to endothelium, and mostly plaque-endothelium (figure 6A/C/D). In smaller plaques, the grooves of the elastic laminae are visible due to the endothelial staining (figure 6B).

In three-week plaques, CNA-Qdots were mainly found directly underneath the endothelial layer, i.e. on the fibrous caps surrounding lipid cores for all plaque types. When visualized 'en face', lipid cores were visible by their fibrous caps containing collagen-bound CNA-Qdots, indicating that the lipids were embedded in fibrous caps (figure 7A). On one occasion, CNA-Qdots were also clearly visible inside a type 4 plaque (figure 7B). On another occasion, CNA-Qdots also bound to the basal lamina of a non-plaque containing carotid artery (not shown).

In six-week collar-carotids, plaques were generally larger in size as compared to plaques of three-week collar carotids (see figure 5). Larger plaques contained more CNA-Qdots. Collagen-networks were visualized at the shoulder of plaques (fibrous caps, figure 8A/B). When viewed 'en face', collagen-networks were visualized just underneath the plaque-endothelium around (figure 8C) and in between lipid-cores (figure 8D/E).

It appeared that only specific spots in the plaque were labeled with CNA-Qdots (hot-spots, figures 8D/8F/7B). This is in contrast to the labeling behavior of the much smaller CNA-OG488, which labels collagen in the entire plaque¹⁹. In order to verify that CNA-Qdot indeed labels specific sites of collagen and to exclude that the cause of this difference is a different collagen content, an atherosclerotic artery labeled *in vivo* with CNA-Qdots (green) was subsequently labeled *ex vivo* with CNA-streptavidin-Alexa568 (red, figure 9). *Ex vivo* labeling generally results in more labeling than *in vivo* labeling. Fur-

Figure 8. CNA-Qdots (green) in six-week collar plaques. A) and B) Mounted artery under 80 mmHg transmurial pressure, showing fibrous caps labeled with CNA-Qdots (same artery, type 3). C) 'En face' view, showing a lipid pool with CNA-Qdots just underneath the endothelium (type 3). D) 'En face' view, collagen network visible in between two lipid pools (type 3). E) 'En face' view, collagen network visible in between two lipid pools (type 4). F) 'En face' view showing multiple lipid pools, but only one (same as in D) shows bound CNA-Qdots. Blue are nucleic acids, red is lipid. White lines indicate border between media and intima, dotted lines in E) indicate border of lipid pools. 'L' is lipid pool, 'VW' is vascular wall. Bar = 10 μ m, except in F), bar = 50 μ m. Color figure on page 187.

thermore, since CNA-streptavidin-Alexa568 is much smaller than CNA-Qdot, CNA-streptavidin-Alexa568 labels more collagen present. High amounts of broadly distributed collagen are present in these lesions (figure 9). This suggests that CNA-Qdots indeed preferentially label accessible hot-spots of collagen in a plaque.

Anx-Qdots localized to endothelium (figure 10A/B, white arrows) and inside lipid cores of three-week plaques. Inside lipid cores, Anx was visible in vesicle-shaped structures (figure 10C/D, orange arrows), or seemed attached to cells inside the cores, possibly macrophages (figure 10A/D, yellow arrows).

Anx-Qdots bound to endothelium of six-week plaques (figure 11A). In addition, Anx-Qdots were found inside lipid cores, on one occasion showing very strong labeling (figure 11B). Anx-Qdots also bound to (foam) cells (figure 11C/D) inside lipid pools. As with

Figure 9. Two-photon image showing 'en face' view of a plaque, *in vivo* injected with CNA-Qdot (green) and *ex vivo* labeled with CNA-streptavidin-Alexa568 (red). *Ex vivo* labeling resulted in abundant collagen labeling, revealing that CNA-Qdots indeed only label accessible hot-spots of collagen within the plaque. Blue is syto44. Bar = 50 μm . Color figure on page 187.

CNA-Qdots, Anx-Qdot penetration into the plaque is restricted and occurs only at hot-spots.

In vivo MRI was also performed. To quantify the signal intensity in the plaque, regions of interest (ROIs) were drawn independently by two observers on a transverse slice of a T_1 -weighted pulse sequence, on the plaque-portion of the carotid artery. Three- and six-week collar mice combined, CNA-Qdots lead on average to a minor, but significant signal intensity increase (post-scan compared to pre-scan) of $6.2\% \pm 2.1\%$ (mean $\pm$ SEM), while Anx-Qdots as well as control-Qdots lead to a non-significant average decrease of $2.3\% \pm 1.4\%$ and $0.23\% \pm 2.2\%$, respectively. This coincides with TPLSM results, as there appears to be more labeling in the plaques labeled with CNA-Qdots than those labeled with Anx-Qdots (see table 1 and figures 7/8/10/11). The fact that these changes in signal intensity are small, despite the high longitudinal relaxivity ($3000\text{-}4500 \text{ mM}^{-1}\text{s}^{-1}$ per Qdot) reported previously²³, can be explained by the low total injected dose of Gd-DTPA ($1.3 \mu\text{mol/kg}$). Furthermore, as can be seen in the TPLSM images, CNA- and Anx-Qdots were present only in hot-spots in the plaque, and not in the entire vascular wall. This is unfavorable for visualization with MRI as this imaging technique is relatively insensitive to contrast agents. This means that a high pay-load of contrast agents within the plaque is required for MRI visualization. In the used experimental set-up, higher dosing is limited by low stock concentration of Qdots. Similar bimodal Qdots, functionalized with angiogenic ligand cNGR, have been used successfully in the MRI visualization of tumor tissue using a low dose³³.

Figure 10. Anx-Qdots (green) in three-week collar plaques. A) Transverse view of plaque (type 3). B) Transverse view of plaque (type 3). C) 'En face' view of plaque (type 1). Vascular wall is visible because the cutting surface curled up. D) 'En face' view of plaque (type 1). White lines indicate border between media and intima. White arrows indicate endothelial labeling by Anx-Qdots, yellow arrows indicate cellular Anx-Qdots, and orange arrows indicate vesicular Anx-Qdots. 'L' indicates lipid pool, 'VW' indicates vascular wall. Red/orange is lipid; blue are nucleic acids. Bar = 10 μ m. Color figure on page 188.

Figure 11. Anx-Qdots (green) in six-week collar plaques. A) Transverse slice of carotid artery. Anx-Qdots are visible on the endothelium covering the lipid pool (type 4). B) Transverse slice of carotid artery, showing a high load of Anx-Qdots inside a lipid pool (type 4). C) 'En face' view of carotid artery (type 2). Anx-Qdots are visible in a foam cell within a lipid pool. D) Transverse view of plaque in carotid artery (type 4). Arrows indicate (foam) cell that had taken up Anx-Qdots. White lines indicate border between media and intima. 'L' is lipid pool; 'VW' is vascular wall. Blue are nucleic acids, red is lipid. Bar = 10 μ m. Color figure on page 188.

Figure 12. Pre- and post-MR images of first transverse slice proximal to collar. This is a 6-week collar mouse, intravenously injected with CNA-Qdots. Bottom row shows magnification of boxed parts on top row. 'A' indicates anterior, 'L' indicates left. White (non-dashed) arrows indicate signal intensity increases in vascular walls of carotid arteries. Dashed arrow indicates trachea. Left carotid artery is the same artery as in figure 8C.

Discussion

Qdots have to cross several barriers in order to reach the inside of a plaque. The glycocalyx is the first barrier, a matrix consisting of proteoglycans and glycoproteins attached to and covering the endothelium. It is hypothesized that this layer is perturbed at sites of atherosclerosis⁴⁵. The next barrier is the endothelium. Endothelial permeability is based on two processes: transcytosis through the endothelial cells (active vesicle transport), and paracellular transport through gaps between the endothelial cells (passive diffusion)⁴⁶. Healthy endothelium allows solutes up to 2 nm in diameter⁴⁷ to pass via these intercellular gaps. Larger structures may pass the endothelium by transcytosis. As a result of endothelial activation, both transcytosis is increased and endothelial gaps become larger, which is known to occur at sites of inflammation, such as in atheroscle-

rotic plaques⁴⁸. Increased endothelial permeability is induced by, e.g., reactive oxygen species⁴⁹ and cytokines⁵⁰ released by inflammatory cells. The third barrier is the fibrous cap, an extracellular matrix consisting of collagen and elastin. Although the synthesis of extracellular matrix components is upregulated in plaques, also matrix-degrading matrix metalloproteinases that are released by, among others, macrophages are active¹, presumably resulting in less organized extracellular matrix. Qdots are indeed able to pass through the intimal layer, but only at specific sites in the plaque, as shown in figures 7, 10, and 11, most likely through perturbed endothelium.

Hot-spot labeling by CNA-Qdot (figures 7/8) contrasts a previous study using CNA-OG488 to visualize atherosclerotic plaques in old ApoE -/- mice¹⁹. CNA-OG488, a fluorescent polypeptide, is substantially smaller than CNA-Qdot (≈ 0.5 nm and 10 nm in diameter, respectively). CNA-OG488 labeled fibrous caps in the entire plaque and even the tunica intima adjacent to plaques¹⁹. Even though in the CNA-OG488 study ten-fold more CNA was injected and a different animal model was used, hot-spots of CNA-Qdots in plaques strongly suggest that CNA-Qdots do bind at specific sites within the plaque. Based on the assumption that CNA-Qdots (≈ 10 nm) enter the plaque through sufficiently large gaps in endothelium, this may also be assumed for Anx-Qdots. Indeed, Anx-Qdots were only seen inside few lipid pools, though this may also result from PS-expressing cells or debris present only in these lipid pools. On the other hand, ^{99m}Tc-Annexin, of similar size as CNA-OG488, has been used to image atherosclerotic plaques in ApoE -/- aortas. ^{99m}Tc-Annexin was indeed able to label large areas of aortic plaques, suggesting that ^{99m}Tc-Annexin does not label plaque tissue size-selectively¹².

Hot-spot labeling by Qdots indicates increased intimal permeability and/ or disruption, and increased inflammation at these sites. It is intriguing to speculate that sites where Qdots penetrate the plaque, also platelets and plasma proteins, such as coagulation factors, extravasate into the plaque. Hence, the presence of Qdots at sites inside a plaque may indicate where inflammation is increased and rupture-prone sites are located. Whether these sites indeed indicate rupture-prone sites remains to be elucidated. CNA- and Anx-Qdots provide a promising novel approach of vulnerable plaque imaging, as it is based on size-selection, complementary to molecular targeting.

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Molecular MRI of early thrombus formation using a bimodal α_2 -antiplasmin-based contrast-agent

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Abstract

Objectives: We aimed to investigate whether early thrombus formation can be visualized with *in vivo* MRI using a novel bimodal α_2 -antiplasmin-based contrast agent (CA).

Background: Thrombus formation plays a central role in several vascular diseases and non-invasive detection of early thrombus formation is a major challenge in clinical practice. During the early phases of thrombus formation, activated factor XIII (FXIIIa) covalently cross-links α_2 -antiplasmin to fibrin, indicating the potential of α_2 -antiplasmin-based CAs in detecting early thrombus formation.

Methods: A bimodal CA was synthesized by coupling gadolinium-DTPA and rhodamine to an α_2 -antiplasmin-based peptide. For the control CA, a glutamine residue essential for cross-linking was replaced by alanine. *In vitro* generated thrombi were exposed to both CAs and imaged by MRI and two-photon laser-scanning microscopy (TPLSM). Immunohistochemistry was performed on human pulmonary emboli sections to determine α_2 -antiplasmin and FXIII presence in different thrombus remodeling phases. *In vivo* feasibility of the CA in detecting early thrombus formation specifically, was investigated with MRI.

Results: *In vitro* generated thrombi exposed to the α_2 -antiplasmin-based CA showed hyperintense MR signal intensities at the thrombus edge. No hyperintense signal was observed using the α_2 -antiplasmin-based CA in the presence of FXIII inhibitor dansylcaldaverine, nor when using the control CA. TPLSM demonstrated that the α_2 -antiplasmin-based CA bound to fibrin. Immunohistochemistry demonstrated substantial α_2 -antiplasmin staining in fresh compared to lytic and organized thrombi. *In vivo* CA administration within seconds after inducing thrombus formation increased contrast-to-noise ratios (CNR: 2.28 ± 0.39 , $N=6$) at the site of thrombus formation compared to the control CA (CNR: -0.14 ± 0.55 , $P=0.003$, $N=6$) and α_2 -antiplasmin-based CA administration 24-48 hours after thrombus formation (CNR: 0.11 ± 0.23 , $P=0.006$, $N=6$).

Conclusions: A novel bimodal CA was developed, characterized, and validated. Our results showed that this bimodal CA enabled non-invasive *in vivo* MR visualization of early thrombus formation.

Introduction

Thrombotic complications such as myocardial infarction, stroke, deep venous thrombosis, and pulmonary embolism, are major causes of morbidity and mortality^{1,2}. The resistance of thrombi to fibrinolytic therapy increases with thrombus age³, and fibrinolytic agents can induce severe complications such as bleeding^{4,5}. This indicates the necessity to accurately diagnose early thrombus formation as it may improve selection of patients that will benefit from fibrinolytic therapy. Currently, non-invasive detection of early thrombus formation is a major problem in clinical practice. Several specific contrast agents (CAs) have been developed to visualize thrombi using molecular imaging techniques⁶⁻⁸. Fibrin has frequently been used as a target for *in vivo* thrombus visualization using various magnetic resonance imaging (MRI) CAs, such as fibrin-targeted perfluorocarbon nanoparticles⁹, and fibrin-specific peptide-based CAs¹⁰⁻¹⁷. Recently, initial results of a human study using a fibrin-specific peptide-based CA (EP-2104R) were published, suggesting selective molecular MR imaging of thrombi¹⁸. Nonetheless, these CAs have low sensitivity for the estimation of thrombus age.

In this study, we searched for other coagulation factors that could be used as a target for thrombus imaging. We focused on activated factor XIII (FXIIIa) as it cross-links fibrin α - and γ -chains of neighboring fibrin molecules and covalently cross-links α_2 -antiplasmin (α_2 -AP) to fibrin¹⁹. In this latter process, the glutamine (Gln2) substrate in the N-terminal domain of α_2 -AP is the primary substrate site for FXIIIa²⁰. Robinson *et al.*²¹ have shown that the catalytic ability of FXIIIa in cross-linking α_2 -AP into formed thrombi declines with a short half-life. Therefore, a CA based on α_2 -AP might enable specific visualization of early thrombus formation.

Recently, a near-infrared (NIR) fluorescent probe (A15) and an MRI CA (A14), both based on α_2 -AP, enabled visualization of *in vitro* formed thrombi with NIR fluorescence and *ex vivo* MRI²². Subsequently, *in vivo* detection of FXIII activity was achieved in ferric chloride treated murine femoral vessels²³ and in the superior sagittal sinus²⁴ by intravital fluorescence microscopy using the A15 probe. However, translation of this probe to human studies in the near future is unlikely due to the limited penetration depth of NIR

fluorescence microscopy. Non-invasive thrombus visualization using imaging modalities such as MRI, may overcome this limitation.

In the present study, we first explored the feasibility of a bimodal α_2 -AP-based CA for visualization of *in vitro* formed thrombi with MRI and two-photon laser-scanning microscopy (TPLSM). Secondly, we analyzed the presence of FXIII and α_2 -AP in fresh, lytic and organized human pulmonary emboli using immunohistochemistry. Subsequently, we investigated whether early thrombus formation can be visualized *in vivo* with MRI using a bimodal α_2 -AP-based CA. Additionally, TPLSM was used to investigate whether the α_2 -AP based CA co-localizes with the fibrin polymers.

Materials and methods

Synthesis of the CAs

An α_2 -AP based peptide sequence (GNQEQVSPLTLL) that binds covalently to fibrin^{21, 23} was synthesized using tertbutyloxycarbonyl (tBoc) solid-phase peptide synthesis (SPPS)²⁵. The peptide was bimodally labeled with rhodamine and a DTPA-chelate (Bi- α_2 AP-CA) by cross-linking maleimide-DTPA²⁶ and succinimidyl-rhodamine (Invitrogen, Molecular Probes, Breda, The Netherlands) to an additional C-terminal KW dipeptide, branched at the lysine ϵ -amino group with a cysteine. A control CA (Bi-con-CA) was obtained by replacing a glutamine residue essential for cross-linking by alanine (Q3→A3) to prevent binding to fibrin (figure 1). Bi- α_2 AP-CA and Bi-con-CA were characterized by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Gadolinium chloride (GdCl₃) was added in a 0.9:1 molar ratio after which no MALDI spectrum was observed due to poor ionization.

Contrast agent validation

For r_1 relaxivity measurements, the gadolinium-labeled peptides were solved (concentrations ranging from 25-150 μ M) in phosphate buffered saline solution (PBS). Solutions were imaged at room temperature with an 1.5 Tesla MR-scanner (MR Intera, Philips Healthcare, Best, The Netherlands) using a commercially available head coil and a 7.0 Tesla Bruker Biospec scanner (Bruker Biospin GmbH, Ettlingen, Germany) using a 154

Figure 1: Schematic representation of the bimodal α_2 -AP-based peptide labeled with rhodamine and DTPA (Bi- α_2 AP-CA) and the corresponding mass spectra. MALDI-MS shows a molecular mass of 2832.92 g/mol for Bi- α_2 AP-CA. Substitution of one amino acid (Q3→A3) results in a bimodal control CA (Bi-con-CA). Color figure on page 189.

mm diameter quadrature transmit-receive radio-frequency coil. T_1 relaxation times were obtained using inversion recovery sequences with different inversion times (TI), ranging from 50-5000 ms. Additional parameters: repetition time (TR), 7500 ms; echo time (TE), 14 ms (1.5T) or 8.4 (7.0T) ms; slice thickness, 3 mm; number of signal averages (NSA), 1; in-plane resolution, $0.55 \times 0.55 \text{ mm}^2$. Gadolinium content was determined using inductively coupled plasma mass spectrometry (ICP-MS).

The effect of Bi- α_2 AP-CA on thrombus formation was determined with a thrombin generation assay using platelet poor plasma with different concentrations (0, 10, 20, and 40 μM) of Bi- α_2 AP-CA.

In vitro thrombus imaging

Human blood was obtained by venous puncture from a healthy volunteer and collected in vacutainer tubes containing trisodium citrate (BD Vacutainer Systems, Preanalytical solutions, Plymouth, UK). Murine blood was obtained by right ventricle puncture. Human and murine thrombi were allowed to form during 90 minutes at 37 °C in the presence of 30 μl of 1.5 mg/ml Oregon Green 488-labeled fibrinogen (Invitrogen, Molecular Probes, Breda, The Netherlands). Thereafter, thrombi were incubated with 150 μM Bi- α_2 AP-CA or Bi-con-CA during 90 minutes at 37 °C, followed by extensive washing with

PBS. To determine the effect of FXIIIa on thrombus visualization, a human thrombus was formed and exposed to Bi- α_2 AP-CA in the presence of dansylcadaverine (final concentration 2 mM) (Fluka Chemie AG, Buchs, Switzerland), since dansylcadaverine is a competitive substrate for transglutaminases such as FXIII²⁷.

Visualization with TPLSM was performed as previously described by Megens *et al.*²⁸. Briefly, a BioRad 2100MP (Hemel Hempstead, UK) was used in TPLSM mode. Fluorescence was detected by 3 photomultipliers. Filter settings: 420-470nm (blue), 510-540nm (green, fibrin network) and 570-590nm (red, rhodamine detection of the CAs).

After TPLSM and approximately 4 hours after CA incubation, thrombi were embedded in 2% agarose gel and imaged at 1.5 Tesla using a commercially available 47 mm diameter surface coil (Philips Healthcare, Best, The Netherlands). Images were acquired using a T₁-weighted Inversion Recovery Turbo Spin Echo (IR-TSE) sequence with the following scan-parameters: TR/TE/TI, 1580/13/546 ms; echo train length, 6; NSA, 16; slice thickness, 1.5 mm; field of view (FOV), 40x40 mm²; matrix size, 192x192; in-plane resolution, 0.21x0.21 mm².

Thrombus age classification

To assess whether FXIII and α_2 -AP can be seen as markers for early thrombus formation, tissue sections with pulmonary emboli were evaluated by immunohistochemistry (IHC). Twenty-two paraffin-embedded blocks (15 from 8 autopsy patients and 7 from 3 lung biopsy tissues) containing pulmonary emboli were used to analyze FXIII and α_2 -AP presence. All tissues were obtained from the Maastricht Pathology Tissue Collection (MPTC). Collection, storage, and use of tissue and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (<http://www.fmwv.nl>).

Immunohistochemistry (IHC) was performed using conventional methods. Briefly, mouse monoclonal anti-human FXIII alpha subunit (1:300; clone AC-1A1, Lab Vision, Fremont, CA), rabbit-anti human α_2 -AP polyclonal (1:200; Biogenesis-MorphoSys AG, Martinsried/Munich, Germany), and mouse monoclonal anti-human fibrin II beta chain (1:100; clone T2G1, Accurate Chemical, Westbury, NY) were used as primary antibodies. For the negative controls, no primary antibody was used. Visualization was achieved

with vectastain red (alkaline phosphatase substrate kit I, Vector Laboratories, Burlingame, CA).

Thrombi were classified into fresh (<1 day; layered patterns of platelets, fibrin, erythrocytes, and intact granulocytes), lytic (1-5 days; areas of colliquation necrosis and karyorrhexis of granulocytes), organized (>5 days; ingrowth of smooth muscle cells with or without deposition of connective tissue and capillary vessel ingrowth), or in thrombi containing more than one of these age classifications²⁹. Percentages of thrombi positive for FXIII, α_2 -AP, and fibrin staining were determined for the various thrombus age classifications. Additionally, the degree of staining was scored for each thrombus age classification separately in no staining (score 0), slightly stained (score 1), positively stained (score 2), and strongly stained (score 3) (by VH, intraobserver variability <10%).

Clearance and biodistribution

All animal experiments were approved by the local ethical review committee. Mice were anesthetized by inhalation of air with 2% isoflurane inhalation gas. Half-life of Bi- α_2 AP-CA (n=3) and Bi-con-CA (n=3) was determined by collecting blood samples before and at different time-points (1, 10, 15, 30, 60, and 90 minutes) after contrast administration (dose: 5.0 μ mol/kg body weight). Using TPLSM, mean fluorescence of the red channel was determined in all blood samples. Exponential fitting was used for half-life determination (GraphPad Prism 4.0, La Jolla, USA). After blood collection, mice were sacrificed using an excess of pentobarbital. Biodistribution of both CAs was determined with TPLSM by studying CA uptake in excised liver, spleen, kidney, heart, lung, and intestine. Filter settings were: $\leq$ 500nm (blue), 500-560nm (green) and 560-610nm (red, rhodamine detection of the CAs).

In vivo thrombus imaging

The right carotid artery was exposed by separating the sternocleidomastoid muscle from the trachea. Thrombus formation was induced by applying a strip of filter paper soaked in 10% ferric chloride (FeCl_3) on the carotid artery. After 5 minutes the filter paper was removed and the carotid artery was washed with PBS. Within seconds after FeCl_3 -induced thrombus formation, 5.0 μ mol/kg body weight Bi- α_2 AP-CA (n=6) or Bi-

con-CA (n=6) was administered intravenously. In 6 other mice, Bi- α_2 AP-CA was administered 24-48 hours after FeCl₃-induced thrombus formation.

MR imaging was performed at 7.0 Tesla using a 35 mm diameter quadrature transmit-receive radio-frequency coil (Bruker Biospin GmbH, Ettlingen, Germany). After scout scans, sagittal carotid MR images were obtained approximately 90 minutes after CA administration using a rapid acquisition with relaxation enhancement (RARE) inversion recovery pulse sequence. Parameters were: TR/TE/TI, 7500/8.9/1654 ms; 21-25 slices; slice thickness, 0.25 mm; interslice distance, 0.3-0.35 mm; NSA, 2; rare partitions, 4; FOV, 30x30 mm²; matrix size, 312x312; in-plane resolution, 96x96 μ m². Additionally, after Bi- α_2 AP-CA administration within seconds after thrombus formation, repeated MR measurements were performed (50, 65, 85, 100, 115, and 130 minutes, n=2).

For TPLSM, carotid arteries (n=4 for each group) were excised and mounted into a home-built perfusion chamber (IDEE BV, Maastricht, The Netherlands), immersed in Hanks Balanced Salt Solution, maintaining 60-80 mmHg transmural pressure²⁸. Syto 13 (Invitrogen, Eugene, OR) was added (final concentration: 2.5 μ M) to visualize nucleic acids. Filter settings: 420-470 nm (blue), 510-530 nm (green, nucleic acid staining) and 560-610 nm (red, rhodamine detection of the CAs).

MR contrast-to-noise ratios (CNRs) were calculated by dividing the difference between the mean MR SIs from a ROI positioned in the thrombus and that in neighboring muscle tissue by the noise measured in air. Noise values were corrected for magnitude effects by the Rayleigh factor. ROIs were drawn in ParaVision version 4.0 (Bruker Biospin GmbH, Ettlingen, Germany).

Statistics

Data are presented as mean $\pm$ standard error. Statistical analyses were performed on the histology scores using the Mann-Whitney U test. For *in vivo* measurements the one-way ANOVA test was used. Due to Bonferroni correction for multiple group comparison, differences with a P-value less than 0.025 were considered significant.

Results

Synthesis of the CAs

MALDI-MS showed a molecular mass for Bi- α_2 AP-CA of 2832.9 g/mol, in agreement with the theoretical average mass of 2893.1 g/mol without Gd³⁺ bound (figure 1). The Bi-con-CA with a Q3 to A mutation had a molecular mass of 2775.4 g/mol, corresponding to the theoretical average mass of 2776.1 g/mol without Gd³⁺ attached (data not shown).

Contrast agent validation

The r_1 relaxation times of the Bi- α_2 AP-CA at 1.5 and 7.0 Tesla were 5.6 ± 0.4 and 3.0 ± 0.3 mM⁻¹s⁻¹, respectively. The r_1 relaxation times obtained for Bi-con-CA were 6.5 ± 0.5 mM⁻¹s⁻¹ (1.5T) and 3.3 ± 0.2 mM⁻¹s⁻¹ (7.0T).

For all Bi- α_2 AP-CA concentrations (0, 10, 20, and 40 μ M) the endogenous thrombin potentials were comparable (708, 742, 733, and 717 nM·min, respectively). In addition, no effects of Bi- α_2 AP-CA on thrombin peak heights or lag times of thrombin generation were observed. These results indicate that Bi- α_2 AP-CA did not interfere with thrombin generation and subsequent thrombus formation.

In vitro thrombus imaging

TPLSM showed co-staining of the rhodamine-labeled Bi- α_2 AP-CA (red) and the OG488-labeled fibrin network (green) resulting in yellow areas of co-localization, indicating binding of Bi- α_2 AP-CA to fibrin in human and murine thrombi (figures 2B and H). In contrast, the control agent Bi-con-CA showed limited labeling and co-localization with the fibrin network (figures 2D and J). Hyperintense MR signals were found at the edge of thrombi incubated with Bi- α_2 AP-CA (figures 2A and G), which were absent using the Bi-con-CA (figures 2C and I). FXIIIa inhibition by dansylcadaverine decreased binding and co-localization of Bi- α_2 AP-CA with fibrin, thereby lowering the MR signal intensity at the edge of a human thrombus exposed to Bi- α_2 AP-CA (figures 2E-F). This finding confirms that FXIII presence is needed for Bi- α_2 AP-CA binding to fibrin.

Figure 2: Two-photon laser-scanning microscopy (TPLSM) and magnetic resonance imaging (MRI) results for human (A-F) and murine (G-J) thrombi incubated with the bimodal α_2 -AP-based contrast agent (Bi- α_2 AP-CA) (A, B, E-H) and the bimodal control CA (Bi-con-CA) (C, D, I, and J). For TPLSM, red color originated from the rhodamine-labeled CAs. Green color indicated the fibrin network due to use of fibrinogen-Oregon green 488. Yellow color illustrated co-localization of red and green. Hyperintense MR signals were found at the edge of thrombi incubated with Bi- α_2 AP-CA (A, G). Lower MR signal intensities were found at the edge of a thrombus exposed to dansylcadaverine and Bi- α_2 AP-CA (E). Color figure on page 189.

IHC staining for FXIII and α_2 -AP on a human thrombus formed during three hours showed that FXIII was present throughout the thrombus (figure 3A), while α_2 -AP was strongly expressed at the edge (figure 3B). This is in line with the increased MR signal intensities at the edge of thrombi exposed to Bi- α_2 AP-CA.

Thrombus age classification

We evaluated 44 thrombi for FXIII, 38 for α_2 -AP, and 47 for fibrin and categorized them in fresh, lytic, or organized. High percentages of positive fibrin staining were found in all categories, whereas positive α_2 -AP and FXIII staining was mainly present in fresh thrombi followed by lytic and organized thrombi. Additionally, mean α_2 -AP score was significantly higher in fresh thrombi compared to lytic and organized thrombi, and mean FXIII score was significantly higher in fresh compared to organized thrombi while no significant difference was found between fresh and lytic thrombi. Mean fibrin score was relatively high in all thrombus age classifications (table 1). Additionally, FXIII was expressed in inflammatory cells (mainly macrophages), representing the cellular component of FXIII. A typical example of a thrombus containing fresh and organized components is shown in figure 4.

Figure 3: Immunohistochemical stainings of thrombi. A) factor XIII (FXIII) staining, B) α_2 -antiplasmin (α_2 -AP) staining. Positive staining is depicted in red/pink color. FXIII staining (scanty spots) was present throughout the whole thrombus while α_2 -AP staining was mainly depicted at the edge of the thrombus (arrows). Bars: 50 μ m. Color figure on page 190.

Table 1: Percentages and scores of α_2 -antiplasmin, factor XIII, and fibrin stainings in lung emboli tissue sections.

α_2 -antiplasmin			factor XIII		fibrin	
Thrombus age	Positive staining	Mean score $\pm$ s.e.	Positive staining	Mean score $\pm$ s.e.	Positive staining	Mean score $\pm$ s.e.
Fresh (<1 day)	95%	1.4 $\pm$ 0.1	92%	1.5 $\pm$ 0.1	100%	2.1 $\pm$ 0.1
Lytic (1-5 days)	29%	0.3 $\pm$ 0.2	75%	0.8 $\pm$ 0.3	83%	2.0 $\pm$ 0.4
Organized (>5 days)	20%	0.2 $\pm$ 0.1	31%	0.3 $\pm$ 0.1	92%	1.9 $\pm$ 0.2

*Significantly different, s.e. indicates standard error

Clearance and biodistribution

Both CAs were rapidly cleared from the blood. Circulation half-lives of Bi- α_2 AP-CA and Bi-con-CA were 14.1 $\pm$ 8.4 and 16.3 $\pm$ 4.0 minutes, respectively. TPLSM on excised organs showed that both CAs were mainly cleared through the kidneys, while limited CA was

Figure 4: Lung emboli sections. Middle panels: overview of lung emboli, from top to bottom: Hematoxyline/Eosin stained, immunohistochemically stained for fibrin, factor XIII, and α_2 -antiplasmin. Bars: 300 μ m. Left panels: enlargements of boxed areas indicating an organized part of the lung emboli. In the enlarged FXIII stained area clear cellular FXIII staining is visible. Bars: 50 μ m. Right panels: enlargements of boxed areas indicating a fresh segment (arrows). Bars: 50 μ m. Color figure on page 190.

found in other organs (figure 5), indicating that there is no or limited accumulation of the CAs on organ level.

In vivo thrombus imaging

Clear hyperintense signals from early thrombus formation were observed with *in vivo* MRI (CNR: 2.29 ± 0.39) when Bi- α_2 AP-CA was administered immediately after FeCl_3 -induced thrombus formation. No hyperintense MR signals were found using the control agent (Bi-con-CA) for fresh thrombus visualization (CNR: -0.14 ± 0.55 , $P=0.003$) nor when using Bi- α_2 AP-CA for the visualization of 24-48 hours old thrombi (CNR: 0.11 ± 0.23 , $P=0.006$) (figures 6A-D). Additionally, CNR-ratios as a function of time after Bi- α_2 AP-CA administration (50, 65, 85, 100, 115, and 130 minutes) were comparable (2.65 ± 0.11 , 2.43 ± 0.06 , 2.51 ± 0.15 , 2.43 ± 0.37 , 2.24 ± 0.21 , 2.01 ± 0.44 , respectively).

TPLSM of excised carotid arteries confirmed Bi- α_2 AP-CA presence in the thrombi formed in the lumen of the FeCl_3 -treated artery when Bi- α_2 AP-CA was administered within seconds after thrombus formation (figure 6E). Limited fluorescence was found using Bi-con-CA (figure 6F) or when Bi- α_2 AP-CA was administered 24-48 hours after

Figure 5: Clearance and kidney uptake of Bi- α_2 AP-CA and Bi-con-CA. Relative fluorescence measured in blood samples decreases with time after contrast administration (A). Half-life of the CAs is 14.1 ± 8.4 and 16.3 ± 4.0 minutes for Bi- α_2 AP-CA and Bi-con-CA, respectively. Both CAs are mainly cleared through the kidney (B-C). Panels B-C: Red: Bimodal CA present in kidney tubuli. Color figure on page 191.

FeCl₃-induced thrombus formation (figure 6G). Occasionally, fluorescence was found in the medial layer (predominantly smooth muscle cells) of vessels that were treated with FeCl₃ 24-48 hours prior to imaging. No fluorescence was detected in the non-treated contralateral carotid arteries. Histology confirmed presence of thrombi in all FeCl₃-treated carotid arteries.

Discussion

Since α_2 -AP covalently crosslinks to fibrin during the early phases of thrombus formation, α_2 -AP-based CAs might enable non-invasive detection of early thrombus formation. We demonstrated that the α_2 -AP-based CA enabled MR imaging and TPLSM of *in vitro* generated thrombi and that FXIII presence is required for binding of the α_2 -AP-based CA to fibrin. Additionally, we showed that α_2 -AP and FXIII are predominantly present in fresh compared to lytic and organized human pulmonary emboli, indicating the potential of α_2 -AP-based CAs in detecting early thrombus formation. Subsequently, we demonstrated that the α_2 -AP-based CA enabled *in vivo* visualization of early thrombus formation with MRI. Two-photon laser-scanning microscopy confirmed specific binding of the α_2 -AP-based CA to fibrin.

Previously, α_2 -AP-based peptides labeled with either Alexa680 (A15) or gadolinium (A14) were used *in vitro* for visualization with NIR fluorescence microscopy and MRI. Both probes were tested for their ability to visualize murine²² and human thrombi²³ by adding these probes during thrombus formation. Additionally, it was shown that α_2 -AP-based peptides bind covalently to fibrin^{21,23}, indicating that the K_d -value approaches 0.

Figure 6: *In vivo* magnetic resonance images (MRI) (A-C) and *ex vivo* two-photon laser-scanning microscopy (TPLSM) results (E-G) of early thrombus formation and 24-48 hour old thrombi in murine carotid arteries after injection of the bimodal α_2 -antiplasmin-based CA (Bi- α_2 AP-CA) (A, C, E, and G) and the bimodal control CA (Bi-con-CA) (B and F). Contrast-to-noise ratios (CNR) are elevated in the early phases of thrombus formation after Bi- α_2 AP-CA administration (D). Panels E-G: Red: Bimodal CA. Green: Nuclear stain. Lumen is located between dotted lines. Blue arrows indicate leukocytes trapped in the thrombus. Elongated cells are smooth muscle cells (white arrows) and endothelial cells (red arrow). Color figure on page 191.

These findings were confirmed by the present study since contrast addition 90 minutes after the initiation of thrombus formation resulted in hyperintense MR signal at the edge of human as well as murine blood-derived thrombi.

In previous murine studies, intravital fluorescence microscopy enabled visualization of carotid²³ and cerebral²⁴ thrombi using the A15 probe. This probe proved to be specific

for the early phases of thrombus formation²³. Nevertheless, due to the limited penetration depth of NIR fluorescent microscopy it is unlikely that this probe can be used for human applications. Our α_2 -AP-based CA enabled *in vivo* visualization of early thrombus formation with non-invasive MRI. In our MRI study a higher CA dose was used compared to the NIR studies^{23, 24}. However, the present dose (5 μ mol/kg) is still low for MRI. Biological amplification due to the abundance of fibrin and r_1 relaxivity increase of the CA upon binding may explain the observed signal enhancement achieved with this low dose. Other fibrin-targeted molecular imaging studies used comparable CA concentrations¹⁰⁻¹⁸. CAs like fibrin-targeted perfluorocarbon nanoparticles⁹, EP-1242¹⁷, EP-1873¹⁶, and EP-2104R^{10-15, 18} proved their ability to visualize thrombi in different animal models with MRI. Recently, initial results showed that EP-2104R allows *in vivo* MR imaging of thrombi in humans¹⁸. Additionally, EP-2104R demonstrated a time-dependent thrombus enhancement, showing a slow decrease in contrast-to-noise ratio (CNR) over a 8 weeks time period¹². However, since increased CNRs were observed in all thrombi compared with pre-contrast imaging, it is unlikely that this CA could effectively discriminate between the early stages of thrombus formation and organized thrombi. In the present study, we demonstrated that the α_2 -AP-based CA significantly increased CNRs during early thrombus formation compared with 24-48 hours old thrombi. Therefore, our bimodal α_2 -AP-based CA is promising for the differentiation between the early phases of thrombus formation and organized thrombi.

Immunohistochemistry showed presence of FXIII in pulmonary thrombi, but cellular FXIII staining was also observed. The cellular form of FXIII consists of a homodimer of A-subunits¹⁹. We used a primary antibody addressed to the α -chain of FXIII, explaining the cellular FXIII staining. The importance of FXIII beyond its function in coagulation is still not well known. An *in vivo* study, in which an¹¹¹In-labeled α_2 -AP-based CA was used to visualize FXIII activity in myocardial healing, suggested that FXIII plays a role in wound healing and tissue repair³⁰. Therefore, the fluorescence observed in the medial layer of some carotid arteries might be explained by tissue repair 24-48 hours after FeCl_3 -treatment. Abdalla *et al.*³¹ suggested that intracellular FXIIIa mediated monocyte adhesion during the atherosclerotic process.

Non-invasive assessment of early thrombus formation versus organized thrombi could be of major clinical relevance since specific detection of early thrombus formation may improve selection of patients eligible for fibrinolytic therapy, thereby reducing serious side-effects. Whether our CA is able to detect thrombi that are sensitive to fibrinolytic therapy remains to be determined. We have demonstrated that the α_2 -AP-based CA did not interfere with the thrombin generation, but it remains to be established whether this α_2 -AP-based CA inhibits thrombolysis. However, this CA only contains the part that is responsible for binding of α_2 -AP, which on its own is insufficient to inhibit plasmin activity. Finally, determination of the optimal dosage is still needed.

In conclusion, our bimodal α_2 -AP-based CA enabled specific, non-invasive visualization of the early phases of thrombus formation, while no hyperintense signal was observed in 24-48 hours old thrombi. Therefore, this bimodal α_2 -AP-based CA might be able to specifically select patients who are eligible for fibrinolytic therapy.

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Chapter 6

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Seven

General discussion

In this thesis, the feasibility of bimodal imaging of vulnerable plaques in murine carotid arteries was investigated. Three different molecular target/ligand combinations based on two different nanoparticles were explored (Anx-Qdot, CNA-Qdot, and Bi- α_2 AP-CA) for their use in imaging with fluorescence microscopy and magnetic resonance imaging (MRI). The fluorescence microscopy imaging technique used in this thesis was two-photon laser scanning microscopy (TPLSM); a technique with several advantages, such as a larger penetration depth, less bleaching, and less tissue damage due to reduced laser-induced heat development. Dying cells, collagen, and fresh thrombi were used as targets of vulnerable plaques. The bimodal nanoparticles were applied to mouse models of atherosclerosis and thrombosis and imaged both *in vivo* with MRI and *ex vivo* with TPLSM. TPLSM allowed accurate cellular localization of the nanoparticles within the plaque or thrombus. MRI allowed minor visualization of CNA-Qdot and good visualization of Bi- α_2 AP-CA within the carotid arteries at tissue level. Anx-Qdot could not be visualized with MRI.

Qdots are nanoparticles with excellent fluorescent properties. They are very bright, hardly bleach, and have favorable excitation and emission spectra. Disadvantages are their potential toxicity, low stock concentration as provided by the manufacturer, and their high costs. In chapters 4 and 5, Qdots were utilized as bimodal nanoparticles by adding Gd-DTPA wedges, first in an *in vitro/ex vivo* proof-of-principle study and next in an *in vivo* atherosclerosis study. Two different ligands were explored. CNA targets collagen present in atherosclerotic plaques as was already shown previously in two TPLSM studies^{1, 2}, while Anx targets phosphatidylserine (PS) exposed by dying cells and activated platelets^{3, 4}. TPLSM results showed Anx-Qdots labeling plaque endothelium as well as distinct regions and foam cells within lipid pools. CNA-Qdots were visualized mainly on fibrous caps just underneath the endothelium in plaque areas and were also observed inside plaque on one occasion. These target-specific results strongly indicate that Qdots are able to enter plaques only at sites with increased permeability, presumably related to plaque vulnerability. Visualization with MRI should be further optimized. This is discussed below.

In chapter 6 TPLSM results showed that peptide-based Bi- α_2 AP-CA specifically targeted activated factor XIII and co-localizes with fibrin. This was shown both in *in vitro* whole

blood clots and in carotid arteries containing thrombi after FeCl_3 treatment and intra-venous injection with the bimodal contrast agent. Furthermore, $\text{Bi-}\alpha_2\text{AP-CA}$ was proven to be specific for fresh thrombi; thrombi of 24-48h old were not targeted by $\text{Bi-}\alpha_2\text{AP-CA}$. Information about thrombus age is important in fibrinolytic treatment after infarctions. MRI results showed a contrast-to-noise ratio (CNR) of ≈ 2.5 for $\text{Bi-}\alpha_2\text{AP-CA}$ in fresh thrombi, which is sufficient for visualization.

For MRI, visualization of nanoparticles is more difficult than for TPLSM as shown in chapters 4 and 5, because sensitivity of MRI is relatively low. Many factors play a role in the potential of functionalized nanoparticles to visualize vulnerable plaques, and should be optimized for MRI visualization. First, the bioavailability of the target (both the number of targets present and the location of the target) is important. Fibrin and activated factor XIII are targets readily available in the vascular lumen in high amounts in case of active thrombus formation, in contrast to collagen and PS that are localized underneath the endothelium and presumably present to a lower extent than fibrin.

Second, the affinity of the ligand for the target plays an important role. Binding of Anx and CNA on one hand and $\alpha_2\text{-AP}$ on the other to their respective targets are based on different biochemical processes. Anx and CNA (chapter 5) are ligands that bind their targets (PS and collagen, respectively) through dis/dissociation equilibria with high affinities. Affinity of a ligand for its target is described by its dissociation constant (K_d), which is defined as the concentration of ligand at which half of the number of target sites is occupied with the ligand; the lower this concentration the higher its affinity. For both CNA and Anx (chapter 5) these affinities are high, in the nanomolar range ³⁻⁵. In the case of thrombus formation (chapter 6), 20-30% of target sites on fibrin are occupied with $\alpha_2\text{-AP}$ ⁶ through covalent coupling, catalyzed by activated factor XIII ⁷. The overall reaction rate in enzyme kinetics is described by its Michaelis constant, K_m , defined as the concentration of substrate at which the reaction rate is half maximum; or, the lower this concentration the faster this reaction may occur. For $\alpha_2\text{-AP}$ coupling to fibrin catalyzed by activated factor XIII, K_m is $\approx 5 \mu\text{M}$ ⁸, which is reasonably low for enzymes in general ⁹. All three target/ligand combinations are therefore biochemically suitable for utilization in molecular imaging.

Third, the availability (dose and circulation half-life) of the nanoparticle, and in case of MRI the Gd-content of this nanoparticle, is crucial. The total injected dose of Gd in the α_2 -AP-study (chapter 6) was approximately 4 times higher than in the bimodal Qdot study (chapter 5), despite the use of Gd-DTPA wedges that amplified the number of Gd-DTPA moieties by a factor eight. Furthermore, in both studies the circulation half-lives were short, reducing the chance of the nanoparticle to reach its target before being cleared from the circulation; ≈ 5 min for CNA- and Anx-Qdots and ≈ 15 min for Bi- α_2 AP-CA. Altogether, availability of Gd-DTPA in the thrombus study was approximately 12 times higher than in the atherosclerosis study.

Fourth, the size of the nanoparticle contributes to the amount of labeling. Bi- α_2 AP-CA (≈ 3 kD, or < 1 nm) is much smaller than the bimodal Qdots (≈ 10 nm). The size of the nanoparticle mainly plays a role when the target is subendothelial, such as in the atherosclerosis study (chapter 5), because then the nanoparticle has to pass through the endothelium or intimal layer. Endothelial cells are, in healthy condition, neatly arranged. This means that nanoparticles up to 2 nm^{10} are able to passively diffuse through the endothelium into the subendothelial space. Most nanoparticles are larger (see chapter 2), such as the bimodal Qdots used in chapter 5, and are thus unable to label subendothelial targets when the vascular wall is intact. However, endothelial permeability increases when tissue underlying the endothelium becomes inflamed, such as in atherosclerosis. As the plaque progresses into an advanced lesion and cells within the plaque release more cytokines and enzymes such as matrix degrading enzymes (matrix metalloproteinases), pathologic disruption of endothelium and fibrous cap occurs, leading to even larger gaps. The ultimate event of atherosclerotic plaque development may be plaque rupture, a process in which endothelium and underlying fibrous cap are completely destructed. Targeting subendothelial objects by size-selection is, in addition to molecular targeting, a tool in development of an appropriate nanoparticle for the visualization of vulnerable atherosclerotic plaques, especially in subendothelial targets, as was shown in chapter 5. In other words, the size of the nanoparticle may be adjusted to allow specific labeling of sites with a certain amount of endothelial or fibrous cap damage. Future experiments may utilize Qdots of various

sizes ($\approx 5\text{-}20\text{ nm}^{11}$), and hence, different emission spectra, to show size-specific labeling of atherosclerotic plaques.

These four factors combined, it may be concluded that due to the higher bioavailability of factor XIII/fibrin, and due to the higher availability of Gd-DTPA, the *in vivo* thrombus study described in chapter 6 lead to better results with MRI than in the atherosclerosis model described in chapter 5. An additional cause of the disappointing MRI results of the Qdot study is that CNA- and Anx-Qdots were found only locally within plaques. This hot-spot labeling of CNA- and Anx-Qdots hinders MRI visualization, as resolution is $\approx 100\text{ }\mu\text{m}$, about the size of these labeled spots.

In vivo atherosclerosis imaging studies with MRI have been performed previously, either non-targeted¹²⁻¹⁴, or targeting angiogenesis^{15, 16}, macrophages¹⁷⁻¹⁹, dying cells²⁰, matrix metalloproteinases²¹, activated platelets²², or adhesion molecules²³. Most of these studies used Gd-DTPA or Gd-DOTA as MRI contrast agent. In these studies a higher dose of Gd was used compared to the dose used in chapters 5 or 6, and/or had slower clearance rates. Often lipidic structures such as micelles or liposomes were used, that can contain up to tens of thousands of Gd-moieties. A common disadvantage of lipidic structures is the relatively high unspecific binding^{16, 17, 19} that is presumably related to merging of lipids with cellular membranes. Unspecific binding is proven by the fact that untargeted liposomes also label atherosclerotic plaques¹⁴. This hinders specific molecular targeting and accurate discrimination between stable and vulnerable plaques. Other studies used iron-containing particles, inducing negative contrast (reduction of signal intensity). Bimodal Qdots used in chapter 5 have a high r_1 relaxivity of $3000\text{-}4500\text{ mM}^{-1}\text{s}^{-1}$ per particle; higher than other Anx-functionalized nanoparticles with respect to size (see chapter 4). Furthermore, aspecific binding to atherosclerotic plaques is negligible, as can be seen on TPLSM images in chapter 5. However, due to the low injected dose MRI visualization was not optimal. Of course, with increasing dose aspecific binding may also increase. Future experiments will show whether custom-made, high-dose bimodal Qdots are able to improve visualization of vulnerable plaques with both TPLSM and MRI.

It is difficult to say which target/ligand combination would be optimal for the imaging of vulnerable plaques. On one hand, target should be readily available to allow sufficient

labeling. On the other hand, the target should be specific for vulnerable plaques; in the ideal case stable and vulnerable plaques are perfectly distinguishable. So far, a single target that predicts plaque vulnerability has not yet been discovered. The presence of thrombus is obvious for the presence of vulnerable plaque, though not necessarily at the same location. Whether Anx or CNA are ligands specific for vulnerable plaques still remains to be elucidated; there is one study concerning Anx in patients that points towards its potency to discriminate between plaque vulnerability and stability²⁴. As discussed above and in chapter 5, combining specific molecular targets with size-selective targeting may improve the specific visualization of targets inside vulnerable plaques.

As discussed in chapter 2, not many *in vivo* studies have been performed using optical techniques. Non-optical and non-invasive techniques such as MRI, PET, and SPECT are more common in the clinical setting and more likely to implement molecular imaging. On the other hand, endoscope-based techniques such as OCT or TPLSM may provide complementary information due to the higher resolution. In the future, optical techniques may very well enter the clinical setting as well. As non-invasive imaging techniques are concerned, MRI is a promising technique to allow clinical molecular imaging, due to the relatively good resolution (compared to PET/SPECT/ultrasound), the lack of radiation (as opposed to PET/SPECT/CT), and anatomical information (as opposed to PET/SPECT). A disadvantage is the low sensitivity to contrast agents. Nanoparticles should be optimized for visualization with MRI, as discussed above. An alternative to MRI may be PET imaging in the case of low-signal enhancement. Gd-DTPA used in this thesis may easily be replaced by Ga⁶⁸-DTPA, a PET contrast agent. Future experiments may thus include combined PET/TPLSM experiments.

Concluding, this thesis provides novel bimodal nanoparticles for the specific visualization of vulnerable atherosclerotic plaques with TPLSM and MRI. Future research will show if the described bimodal Qdots will allow improved MRI visualization and if molecular imaging of vulnerable plaques is clinically applicable.

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Eight

Summary

Samenvatting

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Summary

Atherosclerosis is a systemic disease, developing in most humans during aging in which, among others, cholesterol-esters and monocytes are deposited at specific sites in the vascular wall. This causes an increase in vascular wall thickness, which may be remodeled either into the lumen (narrowing the lumen) or outward. Furthermore, inflammation occurs at these sites due to the presence of monocytes and macrophages. Eventually, plaque rupture may occur, causing downstream infarctions. Development of atherosclerosis can be more progressive due to, for example, genetic predisposition, high-fat diet, or smoking. During development of atherosclerotic plaques, not only plaque size increases, but also the molecular and cellular composition changes. Furthermore, there are molecular and cellular differences between stable plaques and plaques at risk of rupture. Visualization of specific pathologic characteristics would allow accurate distinction between non-symptomatic plaques and plaques that are potentially dangerous. In this thesis, the potential of nanoparticles in the molecular imaging of vulnerable plaques is examined.

In **chapter 2** basic principles of imaging of murine carotid arteries and atherosclerosis are discussed. First, the cellular composition of vascular walls is explained. Though the development of the disease is similar, there are differences between human and murine atherosclerosis. This is discussed next. Morphological features of vulnerable atherosclerotic plaques are discussed. The physical principles of two-photon laser scanning microscopy (TPLSM) are explained. Finally, practical information on the imaging of atherosclerotic plaques in murine carotid arteries using TPLSM is provided.

Chapter 3 reviews various novel optical nanoparticles for the labeling of vulnerable atherosclerotic plaques. Advantages and disadvantages of quantum dots, and gold, diamond, carbon, and silica nanoparticles are discussed with respect to their optical characteristics and potential toxicity. Furthermore, endoscope-based optical imaging techniques that are in development for *in vivo* or clinical use are discussed. In the future these techniques may in the future contribute to personalized, specifically targeted treatment of cardiovascular disease.

Chapter 4 describes a quantum dot-based nanoparticle that allows imaging of apoptosis and activated platelets. Bimodality of the nanoparticle allows visualization by both fluorescence microscopy (by the quantum dot) and magnetic resonance imaging (MRI) imaging (by the attached Gd-DTPA moieties). This nanoparticle is functionalized by Annexin A5, a ligand that specifically targets phosphatidylserine (PS). PS is exposed on the cellular membrane of apoptotic cells and activated platelets. The functionality of this nanoparticle was shown with both TPLSM and MRI. TPLSM results demonstrated that the nanoparticle specifically bound to PS on the outer cellular membrane of apoptotic Jurkats cells and activated platelets. The nanoparticle also bound to damaged cells in the wire-injured carotid artery, as shown with TPLSM. Longitudinal relaxivity of the bimodal nanoparticle was substantially increased by the use of lysine-backbone Gd-DTPA wedges instead of single Gd-DTPA. This improves the ability of the nanoparticle to be visualized by MRI, as the use of Gd-DTPA wedges amplifies the number of MRI-contrast agents by a factor eight. Indeed, MRI results showed that the same nanoparticle allowed visualization of the three *in vitro* samples with MRI.

The bimodal quantum dot, described in chapter 4, was utilized for imaging either collagen or cell death in atherosclerotic plaques of carotid arteries of ApoE^{-/-} mice in **chapter 5**. TPLSM revealed that both types of Qdots bound to hot-spots in intimal as well as deeper layers of the atherosclerotic plaque. This was shown to be specific binding as the control particle, without any targeting ligand, hardly exhibited any labeling of plaques. Likely, quantum dots access vascular targets only at sites showing a certain permeability, or endothelial damage. MRI showed a minor increase in signal intensity for collagen-targeting quantum dots only. Future experiments may reveal whether MRI visualization can be improved and if the relation between the amount of plaque labeling by quantum dots and plaque vulnerability can be determined.

Chapter 6 describes a study in which a novel bimodal α_2 -antiplasmin-based contrast agent was directed against fresh thrombi. During the early phases of thrombus formation, activated factor XIII (FXIIIa) covalently cross-links α_2 -antiplasmin to fibrin, indicating the potential of α_2 -antiplasmin-based contrast agents (CAs) in detecting early thrombus formation. A bimodal CA was synthesized by coupling gadolinium-DTPA and rhodamine to an α_2 -antiplasmin-based peptide. *In vitro* results showed that the CA

binds specifically to fibrin in whole-blood clots mediated by FXIIIa, as was shown both with MRI and TPLSM. In an *in vivo* mouse model of thrombus formation the combination of MRI and TPLSM was used to show the applicability of the CA to specifically enhance the signal intensity of fresh thrombi *in vivo*.

The General Discussion in **chapter 7** discusses the requirements for optimized visualization of novel nanoparticles. Three different nanoparticles were used in this thesis. Because of their different composition they exhibit different optical and magnetic resonant properties and they showed different *in vivo* behavior. For optimized MRI visualization, which has lower sensitivity than TPLSM, four properties should be kept in mind. These are the bioavailability of the target, the affinity of the ligand for the target, the availability of the nanoparticle, and the size of the nanoparticle in case of subendothelial targets. The various characteristics of these nanoparticles are compared and their influence on the outcome of the experiments is discussed.

Samenvatting

Aderverkalking is een ziekte die bij bijna iedereen voorkomt, in bepaalde grote bloedvaten door het hele lichaam. Het verloop van de ziekte kan versneld worden door bijvoorbeeld genetische eigenschappen, slechte voeding, of roken. Het is een proces waarbij cholesterol op specifieke plekken in de vaatwand worden gedeponeerd. Deze plekken worden plaques genoemd. Witte bloedcellen (ontstekingscellen) gaan daar actief naar toe om cholesterol te verwijderen. Doordat cholesterol in de vaatwand gedeponeerd blijft worden, kunnen de witte bloedcellen het 'opruimen' van cholesterol niet meer bijhouden en kunnen ze doodgaan. Vervolgens gaat een plaque ontsteken, waarbij er nog meer witte bloedcellen worden aangetrokken vanuit de bloedbaan. Uiteindelijk leidt dit tot de vorming van de 'necrotic core', oftewel een dode kern in de plaque. Door verschillende processen verandert de moleculaire en cellulaire samenstelling van de plaque in de loop van de tijd. Dit kan op den duur leiden tot scheuren van de plaque (plaque-ruptuur). Zo wordt bijvoorbeeld het fibreuse kapsel, een laag bestaande uit voornamelijk collageen die vermoedelijk zorgt voor de stabiliteit van de plaque, dunner door aanwezige enzymen (afkomstig uit onder andere macrofagen). Vele moleculaire en cellulaire processen spelen een rol in plaque-ruptuur; het is nog niet duidelijk welke de belangrijkste is. Bij plaque-ruptuur komt de necrotic core van de plaque in aanraking met de bloedstroom, waardoor bloedstolsels ontstaan en verstoppingen (infarcten) kunnen optreden.

De groei van plaques vindt niet alleen plaats naar binnen toe (bloedvat-vernauwend), maar kan ook naar buiten toe plaats vinden, waarbij het bloedvat niet vernauwd wordt. Dit heeft tot gevolg dat niet alle plaques zichtbaar zijn bij angiografie, waarbij de bloedstroom in de bloedvaten zichtbaar gemaakt wordt. Bovendien zijn er moleculaire en cellulaire verschillen tussen plaques die stabiel zijn en instabiele plaques die mogelijk wel scheuren en daardoor infarcten kunnen veroorzaken. Het is dan ook van belang dat de mogelijk gevaarlijke plaques op een eenduidige manier zichtbaar gemaakt kunnen worden. Wanneer de specifieke moleculaire kenmerken van een instabiele plaque zichtbaar gemaakt kunnen worden zou het dus mogelijk moeten zijn om onderscheid te

maken tussen de stabiele en instabiele plaques. Het zichtbaar maken van moleculaire kenmerken door nanodeeltjes (deeltjes in de grootte orde van een miljoenste van een millimeter) heet 'Molecular Imaging'. In dit proefschrift is het vermogen van verschillende nanodeeltjes onderzocht om, op basis van molecular imaging, instabiele plaques zichtbaar te maken. Er is gekeken naar drie verschillende plaque-componenten: dode cellen, collageen en geactiveerde bloedplaatjes (die in bloedstolsels voorkomen). Hiervoor zijn, naast *in vitro* systemen (zoals celculturen) ook *in vivo* (levende) muismodellen van atherosclerose en bloedstolselvorming gebruikt. Verder zijn twee verschillende beeldvormingstechnieken gebruikt: fluorescentie microscopie vanwege de mogelijkheid om details van cellen te bekijken, en magnetic resonance imaging (MRI) vanwege de klinische toepasbaarheid en de mogelijkheid om van buitenaf bloedvaten te bekijken.

In **hoofdstuk 2** wordt eerst de algemene cellulaire samenstelling van de vaatwand behandeld, waarbij aandacht wordt besteed aan een aantal eigenschappen van instabiele plaques. De vorming van aderverkalking in muizen wordt apart besproken, omdat deze nogal verschilt van aderverkalking bij mensen. Vervolgens worden de essentiële elementen van de gebruikte microscopische techniek (twee-foton laser scanning microscopie; TPLSM) besproken. Als laatste worden de praktische kanten met betrekking tot het met TPLSM waarnemen van plaques in muizen-halsslagaders behandeld.

Hoofdstuk 3 is een literatuuroverzicht van nieuwe nanodeeltjes die gebruikt kunnen worden bij optische beeldvormingstechnieken, toegepast op instabiele atherosclerotische plaques. De optische kenmerken en mogelijke toxiciteit van quantum dots (dit zijn sterk fluorescerende nanodeeltjes gemaakt van halfgeleider kristallen), en goud-, diamant-, koolstof- en silicadeeltjes worden één voor één besproken. Aangezien microscopie technieken niet eenvoudig klinisch toepasbaar zijn, worden ook mogelijkheden voor klinisch gebruik van optische beeldvormingstechnieken besproken. Endoscopietechnieken, waarbij microscopie mogelijk gemaakt wordt met behulp van optische fibers die een bloedvat ingeschoven kunnen worden, zouden het mogelijk kunnen maken om van binnen uit het bloedvat de vaatwand optisch te bekijken met een hogere resolutie dan met bijvoorbeeld MRI of computed tomography (CT). Deze

technieken kunnen in de toekomst mogelijk bijdragen aan individuele en specifieke behandeling van hart- en vaatziekten.

In **hoofdstuk 4** wordt een nanodeeltje beschreven dat als basis uit een quantum dot bestaat. Dit nanodeeltje is specifiek gericht tegen cellen, die apoptose, ofwel geprogrammeerde celdood, ondergaan. Tijdens apoptose is een specifiek molecuul, fosfatidylserine (PS), aan de buitenkant van de cel aanwezig. PS is niet aanwezig aan de buitenkant van gezonde cellen. PS is verder ook zichtbaar aan de buitenkant van geactiveerde bloedplaatjes, die in bloedstolsels voorkomen. Dit nanodeeltje hecht zich aan PS door middel van Annexine A5 dat aan het nanodeeltje gekoppeld is. Dit molecuul zorgt ervoor dat het nanodeeltje specifiek aan apoptotische cellen en geactiveerde bloedplaatjes bindt. Dit nanodeeltje kan zichtbaar gemaakt worden met twee verschillende beeldvormingstechnieken: fluorescentie microscopie en MRI; dit wordt bimodaliteit genoemd. Het nanodeeltje is fluorescerend dankzij de quantum dot. Aan deze quantum dot is gadolinium gekoppeld, dat ervoor zorgt dat het zichtbaar is met MRI. Voor een verdere versterking van de zichtbaarheid met MRI is gebruik gemaakt van een gadolinium-wedge, een acht-armig molecuul dat het aantal gadolinium-ionen verachtvoudigt. Het resulterende nanodeeltje is getest op een celcultuur in apoptose en op bloedstolsels buiten het dier en bekeken met zowel TPLSM als MRI. Om de fysiologische relevantie beter aan te kunnen tonen is ook een beschadigde muizenhalsslagader na operatieve verwijdering gelabeld met dit nanodeeltje. Ook in dit bloedvat was het nanodeeltje zichtbaar met zowel TPLSM als MRI.

Hoofdstuk 5 beschrijft een *in vivo* studie waarin het nanodeeltje uit hoofdstuk 4 is gebruikt in muizen met aderverkalking. Er zijn twee versies van het nanodeeltje gebruikt: in het eerste geval is het nanodeeltje gekoppeld aan Annexine A5 en bindt het dus aan apoptotische cellen en geactiveerde bloedplaatjes (die beide aanwezig kunnen zijn in instabiele atherosclerotische plaques); in het tweede geval bindt het nanodeeltje aan collageen dankzij het molecuul CNA35. Collageen is aanwezig in zowel stabiele als instabiele plaques. Vermoedelijk is echter collageen in instabiele plaques meer toegankelijk voor het nanodeeltje omdat het endotheel dat erboven ligt meer beschadigd is. TPLSM opnames lieten zien dat beide versies van het nanodeeltje zowel oppervlakkig als in diepere lagen van de plaque binden. Aangezien de nanodeeltjes

alleen in specifieke ‘hot-spots’ zichtbaar zijn lijkt het erop dat ze alleen de plaque labelen wanneer het endotheel toegankelijk, ofwel beschadigd genoeg is. Deze mate van labeling door quantum dots zou een maat kunnen zijn voor plaque instabiliteit. MRI resultaten lieten alleen voor het CNA35-nanodeeltje een zwak signaal zien; het Annexine A5-nanodeeltje was niet zichtbaar met MRI. Aanvullend onderzoek moet gedaan worden om zichtbaarheid met MRI te optimaliseren. Ook de relatie tussen de mate van plaque labeling door quantum dots en plaque instabiliteit moet nog verder worden uitgezocht.

In **hoofdstuk 6** wordt een ander soort bimodaal nanodeeltje beschreven dat specifiek gericht is op verse bloedstolsels of trombi. Dit is een veel kleiner deeltje, dat α_2 -anti-plasmine gebruikt als bindingsfactor. α_2 -antiplasmine is een molecuul dat op een speciale manier aan het bloedstolsel wordt gekoppeld onder invloed van geactiveerd stollingsfactor XIII. Dit gebeurt alleen in de vroege fase van trombusvorming. Het nanodeeltje was bimodaal vanwege de combinatie van het fluorescerende rhodamine en het MRI-zichtbare gadolinium. Tests met bloedstolsels buiten het lichaam laten zien dat het nanodeeltje specifiek bindt aan een onderdeel van bloedstolsels, fibrine. Bovendien laten deze tests zien dat het nanodeeltje zichtbaar is met zowel MRI als met TPLSM. Dit nanodeeltje is ook *in vivo* getest in een muismodel van acute trombusvorming. Ook hier is aangetoond dat het nanodeeltje gevisualiseerd kan worden specifiek in verse trombi met zowel MRI als TPLSM.

In de algemene discussie in **hoofdstuk 7** worden de vereisten voor het optimaal zichtbaar maken van nieuwe nanodeeltjes besproken. Drie verschillende nanodeeltjes zijn gebruikt in dit proefschrift. Omdat deze verschillend zijn samengesteld hebben ze ook verschillende optische en magnetische resonantie eigenschappen. Bovendien vertonen ze ook verschillend gedrag in levende dieren. Omdat MRI visualisatie van gadolinium moeilijker is dan de weergave van fluorescentie met TPLSM, moet visualisatie met MRI geoptimaliseerd worden. Hiervoor zijn in de algemene discussie vier verschillende eigenschappen genoemd. Dit zijn de beschikbaarheid van het doelmolecuul, de sterkte van binding van het ligand aan het doelmolecuul, de beschikbaarheid van het nanodeeltje, en de grootte van het nanodeeltje wanneer het doelmolecuul onder het endotheel ligt. De verschillende eigenschappen van deze

Chapter 8

nanodeeltjes worden vergeleken en de invloed van deze eigenschappen op het resultaat van de experimenten wordt besproken.

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Curriculum Vitae

Lenneke Prinzen was born on April 1, 1980 in Maastricht, the Netherlands. From 1992 until 1999 she attended secondary education (VWO) at the Trichter College, Maastricht, intermitted by a year (1995-1996), during which she attended Towson High School, Towson, MD, USA. Next, she studied Biomedical Engineering at the Eindhoven University of Technology (TU/e) from 1999-2004. During this time, she performed an internship at the Biochemistry department at Maastricht University, and an externship at the Physiology department at the Ludwig Maximilians Universität, München, Germany; her graduation thesis was done at the department of Molecular Cell Biology, Maastricht University. She received her Master's degree in 2004 with the designation 'cum laude'. Furthermore, she was nominated for the TU/e 2005 Mignot Graduation Award. In 2005 she started as a PhD-student at the department of Biophysics (now Biomedical Engineering) at Maastricht University on the subject of 'Quantum dots in ischemic diseases', which was part of the SenterNovem/BSIK project on 'Molecular Imaging of Ischemic Heart Disease'. During this time she attended several CARIM courses as well as general education courses. She also attended a number of international conferences, such as the Joint Molecular Imaging Conference and the conference organized by the International Society on Magnetic Resonance in Medicine. She received three travel awards for these conferences. As of May 1, 2009, she is working as a Post-Doc at the department of Biomedical Engineering at the Maastricht University.

List of publications

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Nine

Full color figures

Chapter 2 - figure 2. Schematic representation of transverse view of arterial wall of a murine carotid artery. '1' indicates tunica intima, '2' tunica media, and '3' tunica adventitia.

Chapter 2 - figure 2. Simplified Jablonski diagram showing single (blue) and two (red) -photon excitation.

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Chapter 3 - figure 1. Schematic overview of the composition of A) quantum dot, B) gold nanoparticle, C) diamond nanoparticle, D1) carbon nanotube, D2) carbon dot (C-Dot), E1) silica nanoparticle with encapsulated organic fluorophores, E2) silica nanoparticle with fluorescent core. Several chemical methods for surface binding of bio-active compounds are mentioned in the text. See Table 2 for spatial dimensions.

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Chapter 4 - figure 5. TPLSM image of a Jurkat cell, incubated with AnxA5-QD525-Gd (green), while simultaneously inducing apoptosis by anti-Fas antibodies. After 5.5 hours cells were washed in EDTA buffer to remove AnxA5 present on the outside of the cells, and subsequently stained with AnxA5-QD585-Gd (red). Yellow indicates co-localization of QD525 and QD585 (probably caused by QD525 on the cell exterior that had not completely been removed). Cells were counterstained with Syto41 (blue), a nucleic acid stain. Bar = 10 μ m.

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Chapter 4 - figure 9: TPLSM images of whole-blood clots incubated with AnxA5 after clot-formation. A; green: AnxA5-FITC, blue: syto41, a nucleic acid stain; B; green: AnxA5-QD525-Gd, red: acridine red, a cytoplasmic stain. C; red: AnxA5-QD585-Gd, green: anti-fibrinogen-FITC. Platelets show a bright cellular membrane, similar to apoptotic cells (compare to figures 4/5/6). Bar = 50 μ m.

Chapter 4 - figure 10: A; TPLSM image of a wire-injured murine carotid artery, showing the various vascular wall layers. The tunica intima consists of endothelial cells (ECs); the tunica media mainly consists of smooth muscle cells (SMCs). Uptake of nanoparticles is visible in ECs and SMCs. Green: AnxA5-QD-Gd-wedge; red: eosin, labeling elastin laminae; blue, syto41, labeling cell nuclei. L: lumen; IEL: internal elastic lamina; EEL: external elastic lamina. Bar = 10 μm. The imaging plane was slightly oblique, as opposed to a longitudinal direction of the artery. B; transversal MR image of two murine carotid arteries: undamaged (control, left) and damaged (right, same artery as shown in A). Bar = 0.5 mm.

Chapter 5 - figure 1. Schematic representation of Anx- or CNA-Qdot. Red: Qdot; yellow: streptavidin; grey: Gd-DTPA8 wedge; blue: Anx or CNA.

Chapter 5 - figure 2. Distribution of CNA-Qdots in A) spleen, inset is magnification of box; B) liver, inset is magnification of box; C) kidney glomerulus. Distribution of Anx-Qdots in D) spleen and E) liver. Qdots (red), autofluorescence (blue). Arrows in A) and B) indicate collagen-binding Qdots. Bar = 10 μ m.

Chapter 5 - figure 3. Difference in distribution of bare Qdots (green) and CNA-Qdots (red). A) spleen, B) liver, C) magnification of boxed part in A), D) magnification of boxed part in B). Arrows indicate collagen-specific binding of CNA-Qdots. Bar = 10 μ m.

Chapter 5 - figure 6. A and B) Control-Qdots (green) in three-week collar plaques. Both images show carotid arteries in a flow chamber and under 80 mmHg transmural pressure. A) Control-Qdots are bound to endothelium covering lipid pools (type 2). B) Control-Qdots are seen in the grooves of elastic laminae in an area adjacent to plaque (type 2). C and D) Control-Qdots (green) in six-week collar plaques. C) Mounted carotid artery under 80 mmHg (type 2). Endothelial cells on plaque are labeled with control-Qdots, indicated by arrow. D) 'En face' view of carotid artery (type 4). White lines indicate border between media and intima layers. Arrows indicate control-Qdots bound to endothelium. Blue in A) and C) are nucleic acids, and in B) and D) autofluorescence. Red is lipid. 'L' is lipid pool, 'VW' is vascular wall. Bar = 10 μ m.

Chapter 5 - figure 7. CNA-Qdots (green) in three-week collar plaques. A) 'En face' view (type 1). B) Transverse view (type 4). Dashed line indicates border between plaque and lumen. In A) blue are nucleic acids, in B) autofluorescence. B) shows the sum of a small stack of images. 'L' is lipid core, 'VW' is vascular wall. Bar = 10 μ m.

Chapter 5 - figure 8. CNA-Qdots (green) in six-week collar plaques. A) and B) Mounted artery under 80 mmHg transmural pressure, showing fibrous caps labeled with CNA-Qdots (same artery, type 3). C) 'En face' view, showing a lipid pool with CNA-Qdots just underneath the endothelium (type 3). D) 'En face' view, collagen network visible in between two lipid pools (type 3). E) 'En face' view, collagen network visible in between two lipid pools (type 4). F) 'En face' view showing multiple lipid pools, but only one (same as in D) shows bound CNA-Qdots. Blue are nucleic acids, red is lipid. White lines indicate border between media and intima, dotted lines in E) indicate border of lipid pools. 'L' is lipid pool, 'VW' is vascular wall. Bar = 10 μ m, except in F), bar = 50 μ m.

Chapter 5 - figure 9. Two-photon image showing 'en face' view of a plaque, *in vivo* injected with CNA-Qdot (green) and *ex vivo* labeled with CNA-streptavidin-Alexa568 (red). *Ex vivo* labeling resulted in abundant collagen labeling, revealing that CNA-Qdots indeed only label accessible hot-spots of collagen within the plaque. Blue is syto44. Bar = 50 μ m.

Chapter 5 - figure 10. Anx-Qdots (green) in three-week collar plaques. A) Transverse view of plaque (type 3). B) Transverse view of plaque (type 3). C) 'En face' view of plaque (type 1). Vascular wall is visible because the cutting surface curled up. D) 'En face' view of plaque (type 1). White lines indicate border between media and intima. White arrows indicate endothelial labeling by Anx-Qdots, yellow arrows indicate cellular Anx-Qdots, and orange arrows indicate vesicular Anx-Qdots. 'L' indicates lipid pool, 'VW' indicates vascular wall. Red/orange is lipid; blue are nucleic acids. Bar = 10 μ m.

Chapter 5 - figure 11. Anx-Qdots (green) in six-week collar plaques. A) Transverse slice of carotid artery. Anx-Qdots are visible on the endothelium covering the lipid pool (type 4). B) Transverse slice of carotid artery, showing a high load of Anx-Qdots inside a lipid pool (type 4). C) 'En face' view of carotid artery (type 2). Anx-Qdots are visible in a foam cell within a lipid pool. D) Transverse view of plaque in carotid artery (type 4). Arrows indicate (foam) cell that had taken up Anx-Qdots. White lines indicate border between media and intima. 'L' is lipid pool; 'VW' is vascular wall. Blue are nucleic acids, red is lipid. Bar = 10 μ m.

Chapter 6 - figure 1: Schematic representation of the bimodal α_2 -AP-based peptide labeled with rhodamine and DTPA (Bi- α_2 AP-CA) and the corresponding mass spectra. MALDI-MS shows a molecular mass of 2832.92 g/mol for Bi- α_2 AP-CA. Substitution of one amino acid (Q3→A3) results in a bimodal control CA (Bi-con-CA).

Chapter 6 - figure 2: Two-photon laser-scanning microscopy (TPLSM) and magnetic resonance imaging (MRI) results for human (A-F) and murine (G-J) thrombi incubated with the bimodal α_2 -AP-based contrast agent (Bi- α_2 AP-CA) (A, B, E-H) and the bimodal control CA (Bi-con-CA) (C, D, I, and J). For TPLSM, red color originated from the rhodamine-labeled CAs. Green color indicated the fibrin network due to use of fibrinogen-Oregon green 488. Yellow color illustrated co-localization of red and green. Hyperintense MR signals were found at the edge of thrombi incubated with Bi- α_2 AP-CA (A, G). Lower MR signal intensities were found at the edge of a thrombus exposed to dansylcadaverine and Bi- α_2 AP-CA (E).

Chapter 6 - figure 3: Immunohistochemical stainings of thrombi. A) factor XIII (FXIII) staining, B) α_2 -antiplasmin (α_2 -AP) staining. Positive staining is depicted in red/pink color. FXIII staining (scanty spots) was present throughout the whole thrombus while α_2 -AP staining was mainly depicted at the edge of the thrombus (arrows). Bars: 50 μ m.

Chapter 6 - figure 4: Lung emboli sections. Middle panels: overview of lung emboli, from top to bottom: Hematoxylin/Eosin stained, immunohistochemically stained for fibrin, factor XIII, and α_2 -antiplasmin. Bars: 300 μ m. Left panels: enlargements of boxed areas indicating an organized part of the lung emboli. In the enlarged FXIII stained area clear cellular FXIII staining is visible. Bars: 50 μ m. Right panels: enlargements of boxed areas indicating a fresh segment (arrows). Bars: 50 μ m.

Chapter 6 - figure 6: *In vivo* magnetic resonance images (MRI) (A-C) and *ex vivo* two-photon laser-scanning microscopy (TPLSM) results (E-G) of early thrombus formation and 24-48 hour old thrombi in murine carotid arteries after injection of the bimodal α_2 -antiplasmin-based CA ($\text{Bi-}\alpha_2\text{AP-CA}$) (A, C, E, and G) and the bimodal control CA (Bi-con-CA) (B and F). Contrast-to-noise ratios (CNR) are elevated in the early phases of thrombus formation after $\text{Bi-}\alpha_2\text{AP-CA}$ administration (D). Panels E-G: Red: Bimodal CA. Green: Nuclear stain. Lumen is located between dotted lines. Blue arrows indicate leukocytes trapped in the thrombus. Elongated cells are smooth muscle cells (white arrows) and endothelial cells (red arrow).