Visualization of Vascular Inflammation in the Atherosclerotic Mouse by Ultrasmall Superparamagnetic Iron Oxide Vascular Cell Adhesion Molecule-1–Specific Nanoparticles

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Objective—Noninvasive imaging of atherosclerosis remains challenging in clinical applications. Here, we applied noninvasive molecular imaging to detect vascular cell adhesion molecule-1 in early and advanced atherosclerotic lesions of apolipoprotein E–deficient mice.

Methods and Results—Ultrasmall superparamagnetic iron oxide particles functionalized with (P03011) or without (P3007) vascular cell adhesion molecule-1–binding peptide were visualized by ultra high-field (17.6 T) magnetic resonance. Injection of P03011 resulted in a marked signal loss in the aortic root of apolipoprotein E–deficient mice fed a Western diet for 8 and 26 weeks in vivo and ex vivo, compared with preinjection measurements, P3007-injected mice, and P03011- or P3007-injected age-matched C57BL/6 controls. Histological analyses revealed iron accumulations in the intima, in colocalization with vascular cell adhesion molecule-1–expressing macrophages and endothelial cells. Coherent anti-Stokes Raman scattering microscopy demonstrated iron signals in the intima and media of the aortic root in the P03011-injected but not untreated apolipoprotein E–deficient mice, localized to macrophages, luminal endothelial-like cells, and medial regions containing smooth muscle cells. Electron microscopy confirmed iron particles enclosed in endothelial cells and in the vicinity of smooth muscle cells.

Conclusion—Using a combination of innovative imaging modalities, in this study, we demonstrate the feasibility of applying P03011 as a contrast agent for imaging of atherosclerosis. (Arterioscler Thromb Vase Biol. 2012;32:2350–2357.)

Key Words: iron oxide nanoparticles ■ noninvasive imaging ■ surface-enhanced coherent anti-Stokes Raman scattering ■ vascular cell adhesion molecule-1 ■ atherosclerosis

The field of clinical cardiovascular imaging is rapidly expanding, driven by the development of contrast agents and novel imaging modalities, which allow the visualization of molecular signatures down to the picomole level.1–4 The use of contrast agents in magnetic resonance imaging (MRI) greatly enhances the possibility to depict the vascular system and inflamed tissue, such as in arthritis, tumor angiogenesis, and atherosclerotic plaques.7 The field of molecular imaging has centered on magnetic nanoparticle–based contrast agents in preclinical and clinical research studies.5–11 Ultrasmall superparamagnetic iron oxide particles (USPIO) are extremely strong contrast enhancers for proton MRI,12,13 and because of their remarkable biocompatible and biodegradable properties, several USPIO particles with diverse size, coating, and targeting ability have been applied (eg, for imaging of the inflammatory process of atherosclerosis, myocarditis, lupus nephritis, and neurodegenerative disorders, such as Parkinson and Alzheimer diseases).14–18

Adhesion molecules are key mediators contributing to the onset and progression of atherosclerosis.19 Vascular cell adhesion molecule (VCAM)-1 is expressed and upregulated on endothelial cells, macrophages and smooth muscle cells (SMCs) within atherosclerotic plaques, and plays a key role in recruiting leukocytes to the vessel wall. Upregulation of VCAM-1 production in early and advanced lesions may be a promising imaging marker for the inflammatory process in atherosclerosis.19–23 Hence, P03011, developed by the conjugation of USPIO to a peptide specifically recognizing VCAM-1, holds potential for the visualization of atherosclerotic plaques by delivering nanoparticles specifically to VCAM-1–expressing cells within plaques.

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Contrast agents are also being tested for multimodal approaches; for example, the combination of MRI and optical imaging techniques such as surface-enhanced coherent anti-Stokes Raman scattering (SECARS) microscopy. Unlike other clinical imaging modalities, SECARS microscopy can be tuned to provide a variety of tissue contrasts with subcellular spatial and near real-time temporal resolution. Combining the imaging modalities of SECARS and MRI would, therefore, allow investigation of the exact localization of nanoparticle accumulations in areas of enhanced MRI signal.

The aim of our study was to evaluate the potential of the newly developed contrast agent P03011 to image early and advanced atherosclerosis in an established apolipoprotein E–deficient (ApoE−/−) mouse model of atherosclerosis. Furthermore, we aimed to scrutinize the cellular localization of P03011 in the vessel wall, by combining 2 innovative imaging modalities of SECARS and MRI, respectively. The probes were coupled with a VCAM-1 peptide specific for the recognition of VCAM-1. In contrast to P03011, the P3007 contrast agent was made by conjugating the USPIO particles to a VCAM-1 peptide specific for the recognition of VCAM-1.

Materials and Methods

For expanded Materials and Methods please refer to the online-only Data Supplement.

Animals

All studies were performed using ApoE−/− mice placed on a Western diet (TD88137) at the age of 4 weeks for 8 or 26 weeks (n=6 per group), or C57BL/6 mice kept on a normal chow until imaging at 12 or 30 weeks of age (n=6 per group). In vivo MRI measurements were conducted with and without injection of P03011 contrast agent or the nonfunctionalized control, P3007, in C57BL/6 and ApoE−/− mice. Experiments were approved by local authorities (Regierung von Unterfranken, Würzburg, Germany) to comply with German animal protection law.

Contrast Agent

Polyethylene glycol (PEG)-USPIO–VCAM-1 peptide (P03011) and nonfunctionalized control PEG-USPIO (P3007) nanoparticles were synthesized according to the methods described in patent WO2004/058275 (US 2004/253181) Guerbet Research (France) for the preparation of the colloidal magnetic particles and the complexation of the magnetic particle with the carboxylic function and the gem-bisphosphonate coating. The P03011 contrast agent was made by conjugation of the USPIO particles to a VCAM-1 peptide specific for the recognition of VCAM-1. In contrast to P03011, the P3007 contrast agent was made by coupling PEG compounds to USPIO particles with gem-bisphosphonate compounds. The diameter of P03011 is ≈26 nm with a 5-nm maghemit core, a PEG, the gem-bisphosphonate coating. The P03011 contrast agent was made by conjugation of the USPIO particles to a VCAM-1 peptide specific for the recognition of VCAM-1. In contrast to P03011, the P3007 contrast agent was made by coupling PEG compounds to USPIO particles with gem-bisphosphonate compounds. The diameter of P03011 is ≈26 nm with a 5-nm maghemit core, a PEG, the gem-bisphosphonate coating, and conjugated VCAM-1 cyclic peptide. The relaxivity r value of the nanoparticles measured at 17.6 T is 98 per mmol/L per second. The dissociation constant (Kd) value of the functionalized PEG-USPIO (P03011) particle is 61 mmol/L.

Pharmacokinetics and Biodistribution

Pharmacokinetics of P03011 and P3007 were validated by inductively coupled plasma emission spectrometry after intravenous injection of 600 μmol iron/kg in C57BL/6 (n=5 per time point) and ApoE−/− (n=5 per time point) mice. Blood samples were collected before injection and after 30 minutes and 2, 5, and 24 hours and the serum analyzed via inductively coupled plasma emission spectrometry, with the concentration of iron oxide contrast agent determined as mg/L. The half-life of the contrast agent was defined by the nonlinear least square fit to the equation: N(t)=N0e−ct, T1/2=log(2)/b, dT1/2=log(2)dN0/b2 with N0 referring to the initial amount of the contrast agent that decays; N(t) is the amount that still remains and has not yet decayed after a time t, T1/2 is the half-life time of the decaying contrast agent, fitted value b is connected to half-life time (T1/2), db is the error of the fitted value b, and dT1/2 is the error of the half-life time.

To determine the biodistribution of the USPIO contrast agents after 24 hours, C57BL/6 and ApoE−/− mice, which had been intravenously injected with P03011 or P3007 for MRI, were euthanized by cervical dislocation. The aorta, heart, lungs, kidneys, leg skeletal muscle, brain, paraaortic lymph nodes, spleen, and liver were removed and snap-frozen for further histological processing.

MRI

Validation of the contrast agent dose was determined through ex vivo measurements of agar phantoms of the aorta and aortic root of C57BL/6 and ApoE−/− mice fed a Western diet for 8 or 26 weeks and injected with P03011 or the control contrast agent P3007 (n=3 per group, 600 μmol iron/kg). Agar phantoms of the aorta of noninjected mice (n=3) were used in the ex vivo measurements and served as a reference. Hearts and aortae were isolated and embedded in 1.5% agarose, then ex vivo MRI imaging was performed using a 3D multipin echo sequence with a very high spatial resolution to obtain the T2 values. The dose of 600 μmol iron/kg was determined to give the best contrast to noise ratio and, hence, was used for all subsequent in vivo MRI measurements.

In vivo measurements were performed applying the same protocol using an ECG-triggered FLASH sequence before and after P03011 and P3007 contrast agent application in 12- and 30-week-old C57BL/6 (n=6 per group) and ApoE−/− mice (n=6 per group). The MRI image reconstruction was performed in Matlab (The Mathworks Inc, Natick). The quantification of signal to noise ratio (SNR) was performed by measuring the signal intensities (SI) in high resolution T2*-weighted FLASH in vivo images within the aortic plaque area pre- and postcontrast application. Regions of interest were adjusted manually to the aortic plaque area of aortic root to measure the mean signal intensity of the plaque (SIplaque) (Figure 2A). Regions of interest positioned outside of the mouse were used to determine the standard deviation of the background signal (SDbackground). SNR values were estimated using the following calculations:

\[\text{SNR} = \frac{\text{SI}_{\text{plaque}}}{\text{SD}_{\text{noise}}}\]

Contrast to noise ratio (CNR) was, in addition, calculated by measuring the SI of T2*-weighted FLASH images within the aortic blood and plaque area, pre- and postcontrast application. Regions of interest were adjusted to the aortic lumen to measure the mean blood signal (SIblood) and to the plaque area of aortic root to measure the mean signal intensity of the plaque (SIplaque) (Figure 2A). Regions of interest positioned outside of the mouse were used to determine the standard deviation of the background signal (SDbackground). CNR values were estimated using the following calculations:

\[\text{CNR}_{\text{pre}} = \frac{\text{SI}_{\text{blood pre}} - \text{SI}_{\text{plaque pre}}}{\text{SD}_{\text{noise pre}}}\]

\[\text{CNR}_{\text{post}} = \frac{\text{SI}_{\text{blood post}} - \text{SI}_{\text{plaque post}}}{\text{SD}_{\text{noise post}}}\]

SECARS Microscopy

Ex vivo SECARS experiments were performed on aortic root sections using near-infrared excitation of 1064 nm of mode-locked Nd: YVO4 (7 ps; 76 MHz), and Ti: sapphire laser (Coherent Mira HP; 3.5 W; 3 ps; 700–1000 nm) combined with a tunable optical parametric oscillator that covers the frequency range (200–3600 cm). The collinearly combined pump and stokes beams are sent into an inverted optical microscope. The beams were scanned over the sample and focused by water immersion objective lens with a numerical aperture of 1.2. The probe volume was reduced below the diffraction limit by tightly focusing the laser beams. The microscope is designed for the signal to be detected in both forward and epi-direction. The collected signal is filtered by bandpass filters and detected by a photomultiplier module. The images were recorded with a resolution of 5 seconds total acquisition time for 1 frame of 512×512 pixels.
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intravenous injection, based on the shortening of the relaxation times within the aortic root in ex vivo measurements and the almost complete clearance of iron from the blood at that time point. MRI measurements were performed in the aortic root region immediately before and 24 hours postinjection of P03011 (VCAM-USPIO) or control P3007 (USPIO particles alone) in C57BL/6 mice or ApoE−/− mice. Although application of P03011 and P3007 particles revealed no MR signal alterations in C57BL/6 mice, injection of P03011 but not P3007 resulted in a marked loss in signal intensity in ApoE−/− mice fed a Western diet for both 8 and 26 weeks in the aortic wall of the aortic root compared with preinjection measurements (Figure 2A–2D). Quantitation of the signal intensity alteration was performed by calculating the SNR as the mean signal intensity (SI) of the aortic wall (SI\text{plaque}) pre- and postcontrast application. A significant decrease in the SNR was revealed in ApoE−/− mice treated with P03011 particles and fed a Western diet for 8 and 26 weeks, when compared with signals before contrast injection.

As the change in SNR has no internal reference and, thus, may be prone to fluctuations because of measurements on different days or changes in the positioning of the mouse inside the coil, we have, in addition, calculated the CNR. The CNR is calculated based on the mean signal intensity (SI) differences between the blood (SI\text{blood}) and aortic root (SI\text{plaque}) and normalized to the noise of the image (SD\text{noise}). This signal difference increases after the application of the contrast agent, resulting in a positive ΔCNR. Calculation of the CNR revealed a significant increase in P03011-injected ApoE−/− mice fed a Western diet for 8 and 26 weeks, compared with signals before contrast injection (Figure 2E). In contrast, the SNR and CNR values in ApoE−/− mice fed a diet for 26 weeks and injected with P3007 showed only modest and nonsignificant changes when compared with the values before contrast agent injection (Figure 2E).

**P03011 Accumulation Within Aortic Root Atherosclerotic Plaques**

Prussian blue staining of sections taken from ApoE−/− mice fed a Western diet for both 8 and 26 weeks injected with P03011 revealed the accumulation of iron particles within early and advanced atherosclerotic plaques (Figure 3A–3D). The calculated signal to noise ratio (SNR) and contrast to noise ratio (CNR) value within aortic roots pre- and postcontrast agent application (n=6 per group). Data represent mean±SEM. ***P<0.001. NS indicates not significant.
advanced atherosclerotic plaques (Figure 3A–3D; Figure IIIA in the online-only Data Supplement). In contrast, the histo-
logical assessment of aortic root sections of P03011-injected C57BL/6 mice (mice without atherosclerotic plaque) or 
ApoE<sup>−/−</sup> mice injected with the control contrast agent P3007, revealed no iron depositions (data not shown and Figure 3E). These data indicate that the signal loss pattern seen during the 
MRI measurements of the aortic root correlated with the presence of iron particles within plaques.

Prussian blue–stained iron particles in plaques of P03011-injected ApoE<sup>−/−</sup> mice could be detected in colocalization with VCAM-1 staining, as demonstrated in adjacent sections (Figure 3B–3D and 3F; Figure IIIA in the online-only Data Supplement); iron staining could be detected in the fibrous cap, plaque shoulders, and luminal plaque areas, in regions which appear to be occupied by endothelial cells, SMCs, and newly recruited macrophages. Because P03011 binds to VCAM-1, we wanted to scrutinize the localization and cell type expressing VCAM-1 within lesions by performing double-immunofluorescence staining. VCAM-1 could be detected throughout the early and advanced plaque, and in colocalization with macrophages and endothelial cells (Figure 3F, and data not shown), in line with previous studies demonstrating the expression of VCAM-1 by these cell types in both human and mouse atherosclerosis. The same pattern of VCAM-1 staining was also observed in lesions from animals which had not been injected with any contrast agent (data not shown). However, although no Prussian blue staining could be observed in deeper plaque regions or medial cells, a strong VCAM-1 staining was observed in these regions, and double-immunofluorescence staining revealed its expression by SMCs (Figure 3F). Furthermore, we performed electron microscopy of sections adjacent to Prussian blue–stained sections (Figure 3C and 3D) and confirmed the presence of iron particles in the intima, within large endosome-like vesicles in the endothelium, in the extracellular matrix between endothelial and smooth muscle cells, and attached to the surface of smooth muscle cells, and in association with collagen fibrils in areas where smooth muscle cells are known to reside (Figure 3G).

**Ex Vivo Imaging Using SECARS Microscopy**

A major limitation of Prussian blue staining is low sensitivity. We therefore endeavored to localize iron depositions using an alternative method based on high-resolution imaging. In previous work, SECARS microscopy has been used to detect signal enhancement of USPIOs in specific pathological regions of the brain extracted from rat models with a neurodegenerative disorder. Based on the biodistribution of the contrast agent, we first attempted to perform SECARS experiments on unstained sections of the spleen and the liver from P03011-injected animals. Abundant SECARS signal enhancement was detected in regions that may represent iron accumulation in macrophages of the liver (Kupffer cells) and the red pulp of the spleen. The observed signal was consistent with the results from the Prussian blue staining (Figure IV in the online-only Data Supplement). The signal enhancement was further investigated by Raman imaging. Clear contrast enhancement was observed in regions highlighting accumulation of iron oxide.

**Figure 3.** Vascular cell adhesion molecule-1 (VCAM-1) expression and detection of iron particles within aortic root of atherosclerotic plaques of apolipoprotein E–deficient (ApoE<sup>−/−</sup>) mice injected with P03011. A and B, Prussian blue-stained sections of early plaques of P03011-injected ApoE<sup>−/−</sup> mice, revealing iron accumulations in the luminal lining, intima, and the media (magnification, ×20) and immunofluorescence staining of VCAM-1 (red) in adjacent sections (magnification B, ×40); arrow indicates iron accumulations. C and D, Prussian blue staining of advanced plaque sections P03011-injected ApoE<sup>−/−</sup> mice; arrows indicate iron accumulations. E, No iron particles were detected in sections from ApoE<sup>−/−</sup> mice injected with the control contrast agent, P3007 (magnification, ×20). F, Double-immunofluorescence staining of VCAM-1 (red) with macrophages (CD68), endothelial cells (von Willebrand factor [vWF]), and smooth muscle cells (SMCs; α-smooth muscle actin) in green; fluorescent images were taken from sections close to the Prussian blue–stained sections. Yellow staining indicates areas of colocalization in merged images; DAPI-stained nuclei are blue (magnification, ×40). Representative images of staining from n=6 mice are shown. G, Electron micrograph of an unstained ultrathin section through the aortic root of a P03011-treated ApoE<sup>−/−</sup> mouse. The endothelial cell lining (E) of the aortic root, an SMC, and the medial area (M) with collagen fibrils are indicated. Aggregates of nanoparticles are marked by arrows; higher magnification images of indicated aggregates are shown. Scale bar, 1 μm. Representative images from 2 mice. L indicates lumen; I, intima; M, media.
nanoparticles in sections of P03011-treated mice (Figure IV in the online-only Data Supplement).

In line with the results from the MRI of the atherosclerotic lesions, ex vivo SECARS measurements performed on unstained aortic sections from P03011–injected ApoE−/− mice adjacent to sections stained with Prussian blue (Figure 3C and 3D) demonstrated markedly increased signal enhancements within atherosclerotic lesions predominantly in luminal regions of the plaque (Figure 4A). Based on the histological pathology of the aortic specimens, these signals likely stem predominantly from endothelial cells, and macrophages within atherosclerotic plaques. However, upon closer investigation we could in addition identify iron particles in regions of spindle-shaped smooth muscle–like cells in the fibrous cap of the intima in areas where SMCs are known to reside (Figure III in the online-only Data Supplement) and in medial cells in the fibrous cap of the intima in areas where SMCs are known to reside (Figure III in the online-only Data Supplement) and in medial cells, as revealed by strong, localized signal enhancements (Figure 4B and 4C). Notably, no signal enhancements could be observed in uninjected control mice (Figure 4D). The bands that show significant intensity enhancement were ≈1435 and 1650 cm−1 in the fingerprint frequency region and 2850 and 2883 cm−1 corresponding to the CH2 unsaturated fat region. This indicates that the observed signal enhancement is correlated with the selective iron binding of surrounding lipid molecules adsorbed to the surface of iron oxide nanoparticles.

These results provide first evidence that the P03011 signal loss observed by MRI may reflect iron deposits not only within the plaque and VCAM-1–expressing macrophages and endothelial cells but also in the intimal and medial SMC, similarly displaying a strong upregulation of VCAM-1 in atherosclerosis.

Discussion

Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of inflammatory cells within the vessel wall.33–35 Early detection and diagnosis of atherosclerosis is crucial for the prevention of its sequelae, such as myocardial infarction and stroke. For this reason new imaging tools that specifically target atherosclerotic plaques and provide key information on plaque location and maturity are required.12,20,34–38 VCAM-1 adhesion molecules are upregulated in atherosclerotic plaques and play a key role in the pathogenesis of this disease.19,30,39 Previously, Nahrendorf et al20 have demonstrated the potential of imaging VCAM-1 expression by MRI and more recently positron emission tomography–computed tomography in murine models using specific molecular and multifunctional contrast agents.22 Hence, targeting VCAM-1 with contrast agents may be a useful tool for specific, noninvasive monitoring of vascular inflammation.

In the present study, we demonstrate the in vivo capability of the newly developed contrast agent P03011, an USPIO conjugated to a cyclic peptide specifically recognizing VCAM-1, to visualize early and advanced atherosclerotic plaques by MRI in vivo. ApoE−/− mice fed a high-fat diet for 26 weeks carry advanced lesions in the aortic root and aorta, characterized by the presence of VCAM-1–expressing endothelial cells, macrophages, and SMCs.41 ApoE−/− mice fed a Western diet for 8 weeks, although demonstrating much smaller, early lesions within the aortic root, displayed a very similar staining pattern for VCAM-1. Intravenous injection of P03011 into these atherosclerotic mice resulted in a significant increase in contrast enhancement within atherosclerotic plaques of the aortic root at 24 hours after administration, when virtually all P03011 had been cleared from the blood, unlike previous studies using a linear VCAM peptide conjugated to a cross-linked iron oxide nanoparticle, which demonstrated increased enhancement 48 hours after administration.22 Moreover, in the present study, lesions were detected with high spatial resolution achieved by using a high magnetic field 17.6-T vertical-bore MR scanner compared with the 9.4-T horizontal-bore system used by Nahrendorf et al.22 The short acquisition time triggered by ECG precluded imaging disturbances resulting from R-R intervals longer than the acquisition time.
from motion artefacts, and visualization of atherosclerotic lesions could similarly be confirmed by ex vivo 3D multipin echo measurement of the aortic root, hence excluding errors attributable to motion artefacts and the trigger procedure.

However, earlier studies have demonstrated uptake of unconjugated USPIOs by plaque macrophages and the subsequent detection of plaque-bound iron oxide particles using MRI in a hypercholesterolemic rabbit model of induced atherosclerosis; we could not detect any significant signals in atherosclerotic aortic root plaques of ApoE<sup>−/−</sup> mice injected with unconjugated USPIO particles using the same acquisition time and sensitivity as with mice injected with P03011. This corroborates that the VCAM-1–dependent accumulation of particles may be a prerequisite for the observed visualization of atherosclerosis. The only modest but nonsignificant accumulation of nontargeted USPIO particles may be related to differences in size and surface properties of the USPIO used in our study compared with previously used USPIO and also explain higher dosages and longer incubation times used in these previous studies compared with our study.

To examine the cellular distribution of USPIO–VCAM-1 contrast agent, we have chosen several independent approaches, namely histological staining, SECARS microscopy, and electron microscopy, which each have their advantages and provide additive information. The histological assessment of the aortic root of these mice enabled the cellular detection of P03011 within early and advanced atherosclerotic plaques. In line with previous findings using Prussian blue staining for iron, we detected USPIO particles in association with luminal plaque endothelial cells and macrophages. These cell types display a strong expression of VCAM-1, suggesting contrast agent accumulation in VCAM-1–expressing cells within the vessel wall. However, because of its low sensitivity, Prussian blue staining may only stain larger iron accumulations. Using SECARS microscopy, we could confirm that iron oxide particles localize to VCAM-1–expressing macrophages within the intima. However, despite the lack of any obvious Prussian blue positive particles, in this study, we also demonstrate for the first time that iron particles were present not only within the plaque itself but also accumulated in the media where predominantly SMCs reside. SECARS microscopic analysis is highly sensitive, and in addition can be performed on unstained material, keeping the molecular structure of the tissues unaltered.

Surface-enhanced signals detected by SECARS microscopy was previously shown to correlate with iron-lipid binding, as demonstrated for phosphatidic acid, phosphatidylserine, oleic, and stearic acids in filtration assays. The observed signal enhancement within plaques may, therefore, correlate with iron-binding enhancement from surrounding lipid molecules adsorbed to the surface of iron oxide nanoparticles either extracellularly or intracellularly after uptake. Importantly, no iron deposits were detectable by SECARS microscopy in P03011–injected C57BL/6 control or uninjected ApoE<sup>−/−</sup> mice, further corroborating the specificity of binding. Electron microscopy confirmed SECARS findings and further unravelled the exact cellular localization of iron particles enclosed in endosome-like vesicles within endothelium, in the extracellular matrix between endothelial and smooth muscle cells, and in association with collagen fibrils in areas where smooth muscle cells are known to reside. One downside of this technique, however, is that particles may get washed away during the staining preparation, if these are not bound to protein and cross-linked during tissue fixation.

VCAM-1 is upregulated on the endothelium under inflammatory conditions, including atherosclerosis and cardiac allograft rejection, and appears on the endothelial cell surface of atheroprone areas before the onset of visible disease. Its importance in the initiation of atherogenesis is demonstrated by delayed lesion development in mice carrying a mutation that hinders VCAM-1 function. Comparable with mouse atherosclerosis, VCAM-1 expression is induced early in human atheroma and is an important element in the inflammatory component of atherosclerosis, contributing to monocyte and lymphocyte recruitment from adventitial vessels and the arterial lumen.

Our data indicate that targeting VCAM-1 could have the potential to become a suitable molecular imaging target for the detection and diagnosis of acute and advanced inflammatory processes by in vivo MRI. USPIO–VCAM-1 particles may help to assess the spatial localization of the inflammatory responses by detecting increased VCAM-1 expression. The prompt clearance from the circulation make P03011 particles potentially interesting for its clinical application, where a high sensitivity and specificity is mandatory to detect inflamed regions.

In summary, this study demonstrates the capability of the VCAM-1 targeted contrast agent P03011 to visualize atherosclerotic plaques using a combination of 2 innovative imaging modalities: MRI with ultra high magnetic field (17.6 T) and SECARS microscopy. The surface-enhanced Raman scattering–based optical properties of iron oxide nanoparticles shows promise for the future of magnetic and optical probes for cell imaging applications and investigation of atherosclerotic plaques in vivo and ex vivo. Furthermore, the use of nanoparticles conjugated to targeted small peptides holds potential for nanoparticle-based drug delivery and prevention of atherosclerosis.

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Disclosures

None.

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Expanded Methods

Animals
At 4 weeks of age, ApoE<sup>-/-</sup> mice (backcrossed 6 times onto a C57Bl/6 background, Jackson Laboratory) were placed on a Western diet (TD88137, SSNIFF Experimental Fodder Rat/Mouse) for 8 and 26 weeks. C57Bl/6 mice (Charles River Laboratory) were used as controls and kept on a normal chow until imaging at 12 and 30 weeks of age. In vivo MRI measurements were conducted with and without injection of P03011 contrast agent or the non-functionalized control, P3007, in C57Bl/6 and ApoE<sup>-/-</sup> mice. Experiments were approved by local authorities (Regierung von Unterfranken, Würzburg, Germany) to comply with German animal protection law.

Contrast agent
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C57Bl/6 and ApoE⁻/⁻ mice, intravenously injected with 600 µmol iron/kg P03011 or P3007, were sacrificed by cervical dislocation after imaging. The aorta, heart, lungs, kidneys, leg skeletal muscle, brain, para-aortic lymph nodes, spleen, and liver were removed and snap-frozen for further histological processing.

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Validation of the contrast agent dose was determined through ex vivo measurements of agar phantoms of the aorta and aortic root of C57Bl/6 and ApoE⁻/⁻ mice fed a Western diet for 8 and 26 weeks, and injected with P03011 or the control contrast agent P3007 at doses ranging from 100 µmol up to 600 µmol iron/kg. Agar phantoms of the aorta of non-injected mice were also used in the ex vivo measurements and served as a reference. The heart and aortae were isolated and embedded in 1.5 % agarose, then ex vivo MRI imaging was performed using a 3D Multi Spin Echo (MSME) sequence with a very high spatial resolution to obtain the T2 values, using the following parameters: TR/TE = 1500/4.5 milliseconds, with an isotropic image resolution of 78 µm³ with a (5 x 5 x 20) mm³ transverse field-of-view, BW = 75 kHz, NA = 2, number of echo images 40. The experiment duration was 13 hours. Phantoms data were reconstructed and zerofilled inplane from 64 x 64 to 128 x 128 matrix. The quantification of the signal decay was calculated by fitting the T2 values pre and post-contrast via a non linear square fit to the function \( s(t) = \exp(-t/T2) \).
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\[
\text{SNR}_{\text{pre}} = \frac{\text{SI}_{\text{plaque pre}}}{\text{SD}_{\text{noise pre}}}
\]
\[
\text{SNR}_{\text{post}} = \frac{\text{SI}_{\text{plaque post}}}{\text{SD}_{\text{noise post}}}
\]

The quantification of contrast to noise ratio (CNR) was performed by measuring the signal intensities (SI) of in vivo high resolution T2*-weighted FLASH images within the aortic blood and plaque area to analyse pre- and post-contrast application. Regions of interest (ROI) were adjusted manually to the aortic lumen to measure the mean blood signal (SI\(_{\text{blood}}\)) and to the plaque area of aortic root to measure the mean signal intensity of the plaque (SI\(_{\text{plaque}}\)) (Figure 2A). ROI positioned outside of the mouse were used to determine the standard deviation of the background signal (SD\(_{\text{noise}}\)). CNR values were estimated using the following calculations:

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\text{CNR}_{\text{pre}} = \frac{(\text{SI}_{\text{blood pre}} - \text{SI}_{\text{plaque pre}})}{\text{SD}_{\text{noise pre}}}
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SECARS microscopy

SECARS microscopy is a vibrational nano-imaging technique based on the combination of a third-order nonlinear Raman optical effect with surface enhanced Raman scattering (SERS). Earlier published demonstrations of SECARS microscopy showed the feasibility of this method for enhanced detection sensitivity in biological systems\(^1,^2\). The method involves the input of two fields of frequencies \(\omega_P\) and \(\omega_S\) (\(\omega_P > \omega_S\)). Two photons of the pump field at frequency \(\omega_P\) interact with a single photon of the stokes field at frequency \(\omega_S\) to create an output field with a frequency of \(\omega_{AS} = 2\omega_P - \omega_S\). In this process, the two input frequencies are set so that \(\omega_P - \omega_S\) is near a Raman transition, which leads to large signal produced at the anti-Stokes frequency with the same average excitation power compared with spontaneous Raman signal. Consequently, the signal is enhanced by the aggregation of the nanoparticles in the presence of biological molecules, as molecules trapped in nanoparticle junctions. Optical imaging based on nonlinear microscopy has made a great contribution to life-science applications\(^3,^4\). The major advantage of SECARS microscopy compared with conventional methods is the drastic signal enhancement in the vicinity of nanoparticles that can increase the detection sensitivity reaching the single-molecule level, which make it a promising high resolution imaging technique in detecting the intracellular uptake of nanoparticles. In this study, the mechanism is discussed based on the vibrational resonance of the surrounding adsorbed molecules correlated with the SERS activity of iron nanoparticles\(^2,^5,^6\).

\textit{Ex vivo} SECARS experiments were performed on aortic root sections using NIR excitation of 1064-nm of mode-locked Nd: YVO\(_4\) (7 ps, 76 MHz), and Ti: sapphire laser (Coherent Mira HP, 3.5W 3ps, 700-1000nm) combined with a tunable optical parametric oscillator that covers the frequency range (200-3600 cm\(^{-1}\)). The collinearly combined pump and stokes beams are sent into an inverted optical microscope. The beams were scanned over the sample and focused by water immersion objective lens with 1.2 numerical aperture. The probe volume was reduced below the diffraction limit by tightly focusing the laser beams. The microscope is designed for the signal to be detected in both forward and epi-direction. The collected signal is filtered by bandpass filters and detected by a photomultiplier module. The images were recorded with a resolution of 5 seconds total acquisition time for one frame of 512 x 512 pixels.

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After imaging, the animals were sacrificed by cervical dislocation, the heart and ascending aorta removed, rinsed with PBS then snap-frozen in liquid nitrogen-cooled isopentane. Tissues were embedded in Tissue-Tek (Sakura, Netherlands) and sectioned at 8 \(\mu\)m for (immune-) histological staining and at 25 \(\mu\)m for iron staining, then acetone fixed. Tissues used for biodistribution analysis were stained for iron particle deposition using Prussian Blue\(^7\). In brief, slides were incubated in 1:1, 5% potassium ferrocyanide and 5% hydrochloric acid for 30 minutes, rinsed with distilled water, counter-stained with nuclear-fast red for 5 minutes, washed, dehydrated and cover slipped. Collagen
was stained using Picrosirius red and images of each section were recorded under brightfield and polarised light. Double immunofluorescent staining on aortic root sections was performed for VCAM-1 (rat anti-mouse CD106; Linaris Biologische Produkte GmbH, Wertheim-Bettingen), CD68 for macrophages (rat anti-mouse CD68, Clone FA-11, AbD Serotec, Germany), smooth muscle actin (α-SM actin, Abcam, Germany) and von Willebrand Factor (vWF) for endothelial cells (sheep anti-mouse vWF, Abcam, Cambridge, U.K.). Slides were blocked with 3% rabbit serum (Vector laboratories, Burlingame, U.S.A) then incubated with the first primary antibody (CD68, α-SM actin or vWF) overnight at 4°C. Secondary detection was performed using a biotinylated anti-rat antibody (Vector laboratories) followed by streptavidin-FITC (Vector Laboratories). Sections were then blocked for Avidin/Biotin (Avidin/Biotin Blocking Kit, Vector Laboratories) then incubated with the second primary antibody against VCAM-1 (biotinylated anti-rat) for 1 hour at room temperature. Secondary detection for VCAM-1 was performed using streptavidin-Alexa Fluor-546 (Invitrogen, Karlsruhe, Germany). Sections were counterstained with DAPI (1µg/mL; Merck, Darmstadt Germany) for visualisation of nuclei, then coverslipped using Vectorshield anti-fade mounting medium (Vector Laboratories). Images were taken using a Zeiss Axiovision fluorescent microscope (Carl Zeiss MicroImaging GmbH, Germany).

**Supplemental References**


Supplemental Figure I. Clearance of P03011 and P3007 nanoparticles from the blood stream after 30 min, 2 hours, 5 hours and 24 hours measured from serum samples of ApoE/− and C57Bl/6 mice aged 30 weeks (n=5). The pharmacokinetics were assessed via ICP-ES measurement and calculated as mg/L. The high concentration of the P03011 contrast agent measured after 30 minutes reverted mostly to the baseline after 24 hours. Data represent mean ± SD.
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Supplement Material.

Expanded Methods

Animals
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Supplemental References

1. Machtoub L, Bataveljic D, Andjus PR. Molecular imaging of brain lipid environment of lymphocytes in amyotrophic lateral sclerosis using magnetic resonance imaging and SECARS microscopy. Physiol Res. 2011;60 Suppl 1:S121-127
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