

1 Article

# 2 Contrast-Enhanced, Molecular Imaging of Vascular 3 Inflammation in the Mouse Model by Simultaneous 4 PET/MRI

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22 **Abstract:** Despite advances in diagnosis and management, cardiovascular diseases (CVDs) remain  
23 the leading cause of death in the US. Atherosclerosis is the most common form of CVD and the  
24 vulnerability of atherosclerotic plaques to rupture is a primary determinant for risk of catastrophic  
25 ischemic events. Current imaging of atherosclerotic disease focuses on assessing plaque size and  
26 the degree of luminal stenosis, which are not good predictors of plaque stability. Functional  
27 methods to identify biomarkers of inflammation in plaques could facilitate assessment of plaque  
28 instability to allow early intervention. In this study, we validate the use of a purpose-built,  
29 magnetic resonance imaging (MRI)-compatible positron emission tomography (PET) insert for  
30 multimodal, molecular imaging of vulnerable plaques in mice. We illustrate the application of PET  
31 to screen for inflamed regions to guide the application of MRI. Molecular MRI visualizes regions  
32 of vascular inflammation and is coupled with anatomical MRI to generate detailed maps of the  
33 inflammatory marker within the context of an individual vessel. As a testbed for this imaging  
34 methodology, we developed a multimodal, iron oxide nanoparticle (NP) targeting vascular cell  
35 adhesion molecule-1 (VCAM-1) for simultaneous PET/MRI of vascular inflammation performed  
36 on a mouse carotid ligation model. *In vitro* cell studies confirmed that the NPs are not cytotoxic to  
37 liver cells. *In vivo* simultaneous PET/MRI imaging identified regions of inflammation.  
38 Three-dimensional rendering of the MRI data facilitated high-resolution visualization of patterns  
39 of inflammation along the injured vessel. Histology validated the co-localization of the NPs with  
40 VCAM-1 expression at sites of induced inflammation. The results of this work validate the utility  
41 of the simultaneous PET/MR insert as a research tool for small animals and lays groundwork to  
42 further advance the potential clinical utility of integrated imaging systems.

43 **Keywords:** Multimodality imaging; MRI/PET; dual-mode imaging; nanoparticle; atherosclerosis;  
44 vulnerable plaque; cardiovascular imaging; vessel wall; VCAM.  
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## 46 1. Introduction

47 Cardiovascular disease is the leading cause of death for both males and females in the United  
48 States.[1] In particular, atherosclerosis is responsible for catastrophic manifestations of heart disease  
49 such as myocardial infarction and stroke.[2] Recent understanding of the pathophysiology of  
50 plaque formation has identified chronic inflammation as a hallmark of plaque development;  
51 localized inflammatory response can lead to the development of “vulnerable” plaques that are  
52 prone to rupture and cause downstream vascular occlusion.[3] Imaging can play a role in  
53 identifying patients with vascular lesions susceptible to acute cardiovascular events, who may be  
54 amenable to treatment with anti-inflammatories or interventional procedures. Recent literature has  
55 shown that plaque lesion composition, particularly the presence of inflammatory markers and  
56 immune cells, as opposed to the degree of vessel stenosis, is a better predictor of patient mortality  
57 and morbidity; and assessment of plaque inflammation is an excellent target for noninvasive  
58 imaging.[4] However, current clinical imaging techniques seldom provide specific information  
59 about inflammation.

60 Current clinical imaging techniques such as coronary angiography, vascular ultrasound and  
61 computed tomography focus on identifying stenotic disease and can miss vulnerable plaques that  
62 do not cause significant structural stenosis.[5] Anatomical features identified by coronary computed  
63 tomography (CT) and magnetic resonance imaging (MRI) that could be used to classify plaques,  
64 have not been fully validated for prediction of vulnerability.[6] Targeted molecular imaging has  
65 potential for greater predictive value. For example, imaging of plaque inflammation has been  
66 actively pursued using  $^{18}\text{F}$ -labeled fluorodeoxyglucose (FDG) positron emission tomography (PET)  
67 and has shown promise for identifying inflammation, generally in large vessels such as the carotid  
68 and aorta.[7-10] However, it is challenging to accurately assess inflammatory burden in small  
69 vessels with the limited resolution of PET.[11] Moreover, FDG is a non-specific marker of  
70 inflammation that measures only glucose uptake, which presents challenges for coronary artery  
71 imaging against the high metabolic background of the myocardium.[12] Enthusiasm for the clinical  
72 use of  $^{18}\text{F}$ FDG to identify plaques has diminished over recent years with new tracers such as  $^{18}\text{NaF}$   
73 receiving greater attention; but specific imaging of plaque inflammation is still not available.[13]  
74 New, targeted imaging strategies that can identify inflamed plaques in smaller vessels, such as the  
75 coronary arteries, with higher specificity are needed.

76 We have previously shown that multimodal agents combining positron emission tomography  
77 (PET) and magnetic resonance imaging (MRI) can detect macrophage density in plaques, using the  
78 quantitative ability of PET for high sensitivity mapping of the cells, and MRI for high spatial  
79 resolution molecular imaging and soft tissue mapping.[14] Our previous work utilized separate  
80 scanners to visualize the synergistic PET and MRI information. This required laborious spatial  
81 co-registration (prone to misalignment since they are acquired sequentially), more involved  
82 handling of subjects with longer sedation, and time delay due to the need for transport between  
83 PET and MR scanners. The latter factor also prevents temporal co-registration of the PET and MRI  
84 signals. Not only does this hinder throughput of preclinical research pursuits, but also may limit  
85 the translatability of such agents to the clinic. The use of hybrid modality instruments for  
86 simultaneous signal acquisition is, thus, of increasing interest in the imaging field and clinical  
87 hybrid instruments are now available; but the ideal cardiovascular applications for these hybrid  
88 systems are still under investigation.[15]

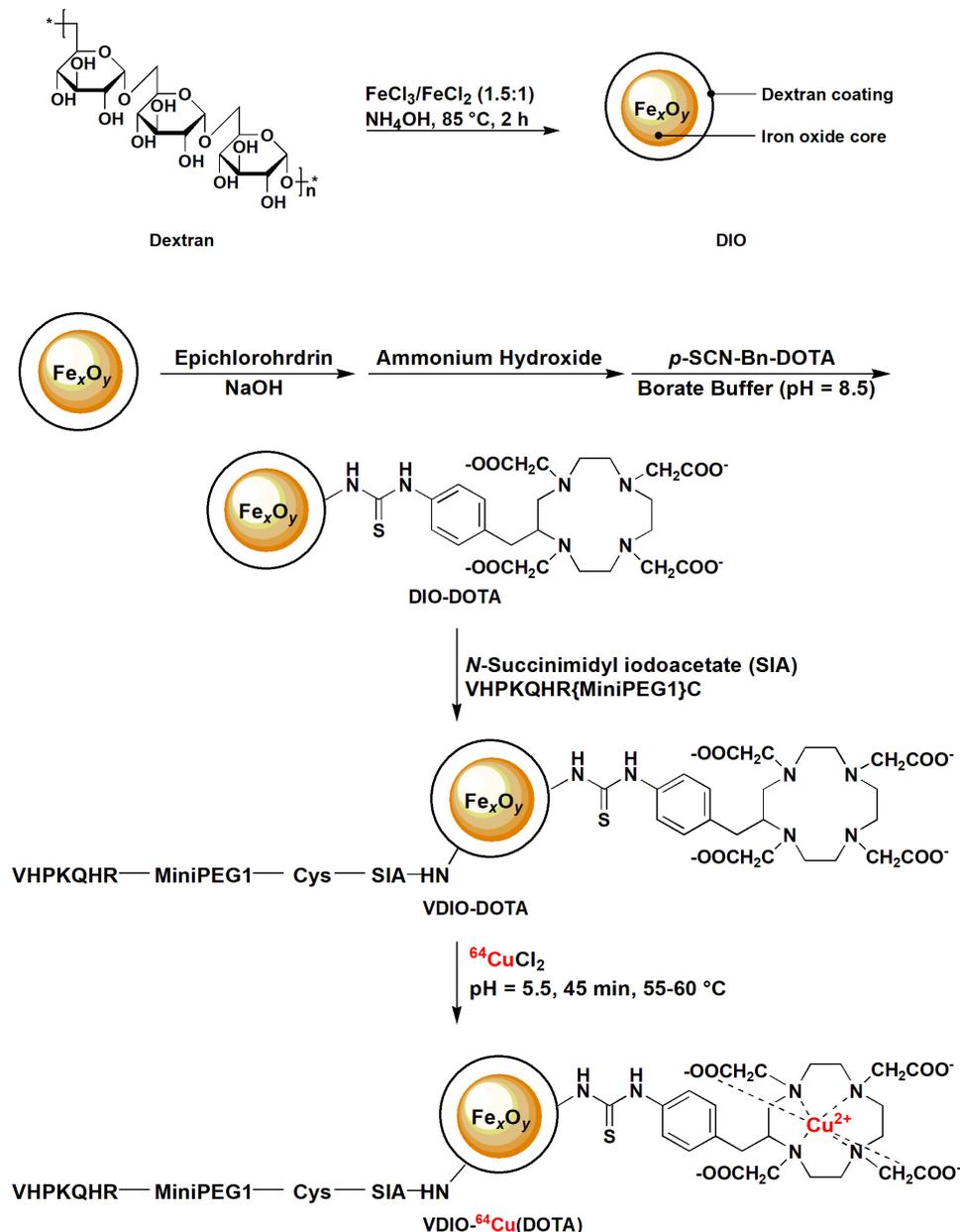
89 In this proof of concept study, we demonstrate the utility of simultaneous PET and MRI to  
90 facilitate PET-guided MRI mapping of localized inflammation in small vessels using an integrated  
91 small animal simultaneous PET/MRI imaging system developed at UC Davis and a dual-mode  
92 contrast agent targeted to Vascular Cell Adhesion Molecule 1 (VCAM-1).[16, 17] Vascular Cell  
93 Adhesion Molecule VCAM -1 has received attention for imaging of atherosclerotic plaques due to  
94 its overexpression in the pathogenesis of vulnerable plaques. It has been studied for single modality  
95 imaging by MRI[18], SPECT[19, 20], and ultrasound[21]. Each of these modalities holds inherent  
96 limitations for targeted vascular imaging. MRI and ultrasound can provide excellent local, spatial  
97 information at the lesion site, but are ill-suited to whole-body screening. PET is superior for  
98 screening, but lacks the spatial resolution to identify individual vessels. While computed

99 tomography (CT), can provide structural information, but does not provide physiological  
100 information at the lesion site. MRI/PET has the potential to overcome these difficulties by  
101 combining the screening power of PET with the resolution power of MRI, enabling concurrent  
102 assessment of cardiovascular structural and physiological abnormalities.[22] We evaluated the  
103 ability of the integrated PET/MRI instrument to visualize sites of localized inflammation induced by  
104 carotid injury in a mouse model; the mouse carotid is of similar size to human coronary arteries.  
105 PET provided an overview of inflamed regions, which was used to focus MRI interrogation and  
106 obtain higher resolution, 3-dimensional details of VCAM-1 expression in single vessels.

## 107 2. Results and Discussion

108 Nanoparticles (NP) were successfully synthesized (outlined in Figure 1 and described in  
109 Methods).

110 **Figure 1.** Synthesis of VDIO-DOTA nanoparticles. (a) Synthesis of dextran coated iron oxide  
111 nanoparticle (b) Conjugation of DOTA and VCAM-1 targeting peptides to nanoparticles.



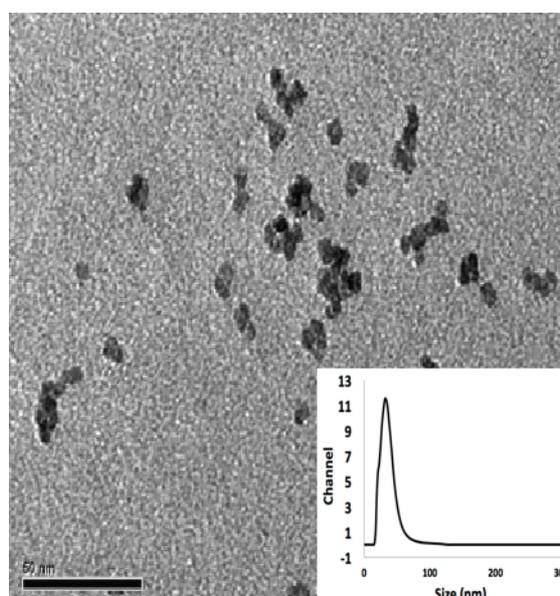
113 2.1 Characterization of VDIO-DOTA and DIO (control)

114 DIO (dextran-coated iron oxide) was synthesized as a matched control to the prepared  
 115 VDIO-DOTA (DOTA= 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid; VDIO=  
 116 VCAM-conjugated DIO). The physical properties of VDIO-DOTA and its precursor DIO are  
 117 summarized in Figure 2a. Following the syntheses described, the DIO and VDIO-DOTA iron oxide  
 118 core sizes were measured to be  $7.3 \pm 2.9$  nm by averaging 500 particle measurements from TEM  
 119 images as shown in representative images in Figure 2b and 2c. Inset plots show that the  
 120 hydrodynamic diameters of DIO and VDIO-DOTA were found to be  $39.7 \pm 15.0$  nm and  $48.1 \pm$   
 121  $19.5$ nm, respectively, using dynamic light scattering (DLS). The increased hydrodynamic diameter  
 122 could be explained by the addition of peptide polymers on the VDIO surface holding the dextran  
 123 polymers apart via steric hindrance. However, the core sizes remained the same.

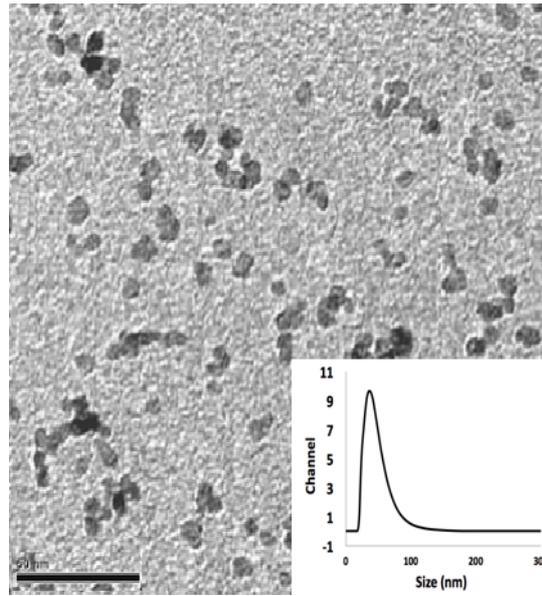
124 **Figure 2.** (a) Summary of nanoparticle properties. TEM images of (b) DIO (core size:  $7.30 \pm 2.92$ nm)  
 125 (C) VDIO-DOTA (core size:  $7.30 \pm 2.92$ nm). Scale bars = 50 nm, insets are DLS data.

Nanoparticles	Core size (nm)	Average hydrodynamic diameter (nm)	Relaxivity ( $\text{mM}^{-1}\text{s}^{-1}$ ) (1.4 T, 37°C)		% Iron
			r <sub>1</sub>	r <sub>2</sub>	
VDIO	$7.30 \pm 2.92$	48.10	7.38	67.85	21
DIO	$7.30 \pm 2.92$	39.7	10.05	68.83	16

(a)



(b)

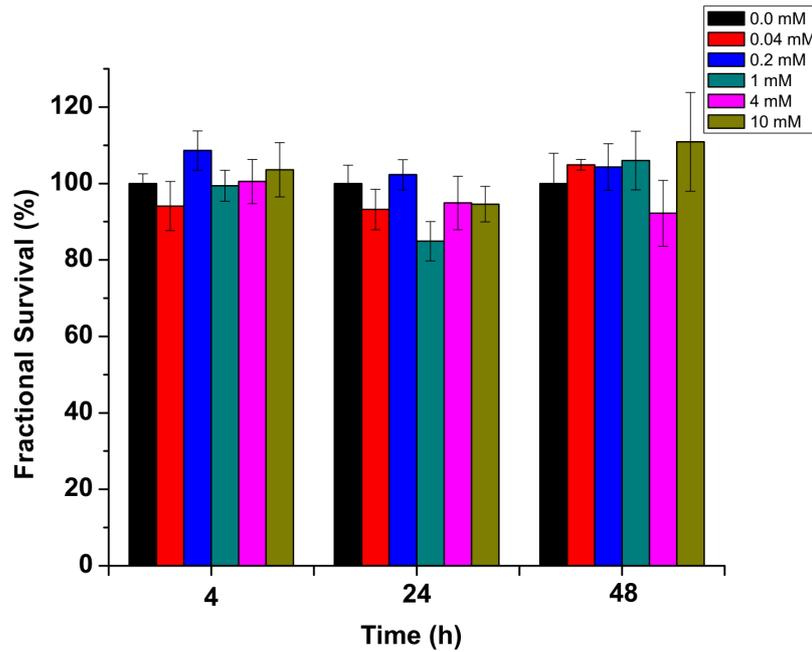


(c)

126 The iron concentration (mg per unit mass) in DIO and VDIO-DOTA was measured by atomic  
127 absorption (AA) to be 0.161 and 0.037 mg Fe/mg of nanoparticles, respectively. The longitudinal ( $r_1$ )  
128 relaxivity of VDIO-DOTA at 60 MHz (1.4 T, 37 °C, pH = 7) was  $7.4 \text{ mM}^{-1}\text{s}^{-1}$  and transverse relaxivity  
129 ( $r_2$ ) was measured as  $67.9 \text{ mM}^{-1}\text{s}^{-1}$ . DIO had  $r_1$  and  $r_2$  values of  $10.1 \text{ mM}^{-1}\text{s}^{-1}$  and  $68.8 \text{ mM}^{-1}\text{s}^{-1}$ ,  
130 respectively. The  $r_2$  to  $r_1$  ratio for both reflects their suitability as  $T_2$ -weighted MRI contrast  
131 agents.[17] VDIO-DOTA relaxivity and size remained stable after 1 year of dry storage at room  
132 temperature.

### 133 2.2 Contrast Agent is Not Cytotoxic to Liver Cells *in vitro*

134 Given that NPs are expected to clear through the liver, liver cells may be exposed to the  
135 highest off-target concentration of injected contrast agent. Thus,  $C_{12}$  – Resazurin viability assays  
136 were performed on HepG2 liver cells to evaluate any toxicity exhibited by VDIO-DOTA.[23] In this  
137 assay resazurin is reduced to resorufin in proliferating cells.[23] Cell survival was evaluated after  
138 incubation for 4, 24, and 48 hours with different concentrations of VDIO-DOTA, ranging from 0.04  
139 mM iron (red), 0.2 mM iron (blue), 1 mM iron (teal), 4 mM iron (pink) to 10 mM iron (khaki). With  
140 90% confidence interval by t-test there were no differences between the untreated control (0.0 mM  
141 iron, black) and cells treated at all concentrations of VDIO-DOTA tested from 0.04 mM iron up to 10  
142 mM iron. These results support that the nanoparticles are nontoxic to liver cells at relatively high  
143 concentrations.

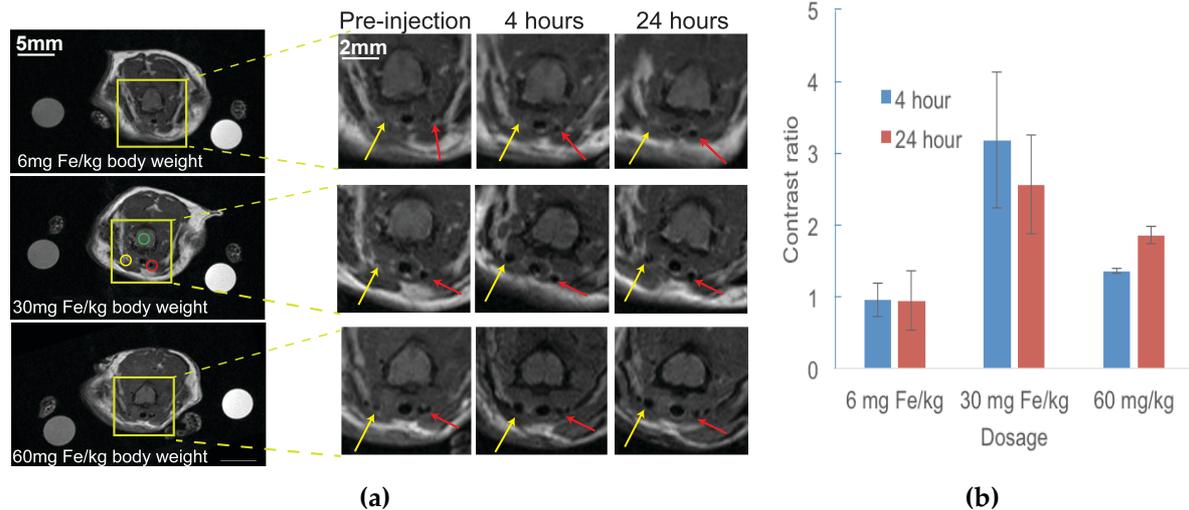


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145 **Figure 3.** The fractional survival of HepG2 after 4, 24 and 48 hours of incubation with VDIO  
146 solutions of varying concentrations. At each time point, fluorescent intensities reflecting survival  
147 fractions (y axis) were normalized against the signal from the untreated control group (black bar at  
148 each time point).

### 149 2.3 VDIO-DOTA can to detect inflamed vessels in vivo

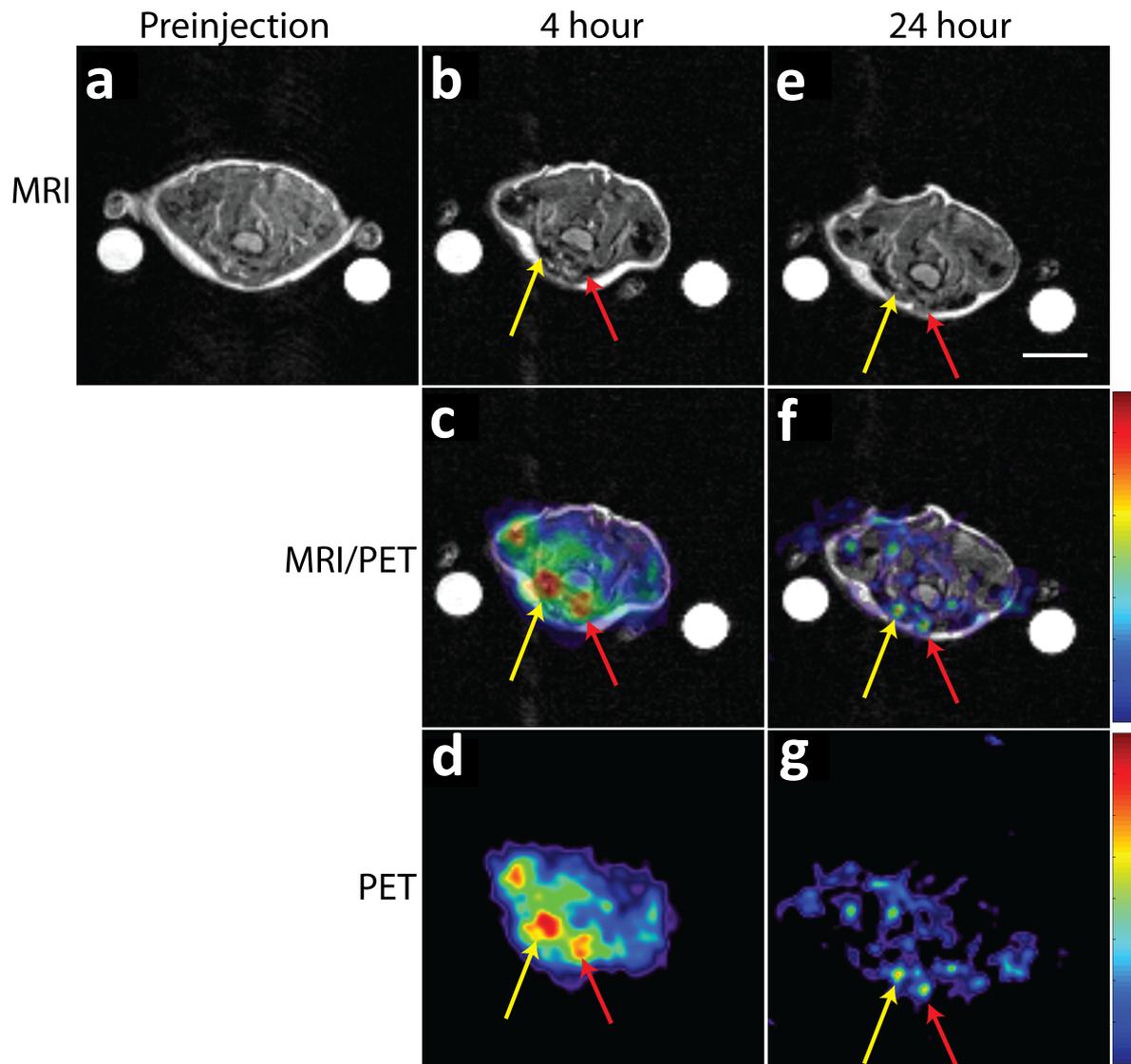
150 MRI only studies were performed to determine appropriate dosing prior to PET/MRI studies.  
151 Representative MRI scans showing VDIO-DOTA uptake at the three injection concentrations are  
152 shown in Figure 4a (n = 4 mice per concentration). The leftmost column shows cross sections  
153 through the entire animal for, from top to bottom 6mg/ml, 30mg/ml and 60 mg/ml injection of  
154 nanoparticles. These are labeled with colored circles to indicate the injured vessel (yellow circle),  
155 uninjured contralateral vessel (red circle) and spinal cord (green circle). The co-registration  
156 standards are also visible to left and right of the animal. The regions outlined by the yellow boxes  
157 are presented in zoomed views on the matrix of images on the right, which show, from left to right,  
158 images from this region taken pre-injection, and images 4 and 24 hours after injection of contrast.  
159 For all concentrations, the inflamed vessel (yellow arrows) showed expected increased dephasing  
160 (seen as signal dropout) as the concentration of VDIO-DOTA injected is increased resulting in  
161 greater accumulation at the ligated site compared to the nonligated contralateral vessel (red  
162 arrows). This darkening in the inflamed arteries persists through the 24h timepoint for all  
163 concentrations, showing sustained retention of the particles at the site of inflammation. Robust  
164 signal for 6ml/ml suggested that even lower injection concentrations could be employed. The CR  
165 was calculated as a function of time after injection and dosage and shown in the accompanying  
166 graph in Figure 4b. Although the 60 mg Fe/kg dose showed the greatest signal decrease on the  
167 images, maximal CR was achieved with the 30 mg Fe/kg dosage at both 4h and 24h after injection of  
168 contrast. This highlights the need to achieve a balance between local accumulation of the NP,  
169 retention over time, and its systemic circulation for optimal visualization of the region of interest.  
170 Using these results as a guide, 30mg Fe/kg was used for the remainder of the studies.



171 **Figure 4.**(a) MRI-only study showing VDIO-DOTA uptake at different injection concentrations over  
172 24 hours. The yellow arrows point to the inflamed vessel, while the red arrows indicate the  
173 non-inflamed control vessel. There was signal decrease at the site of injury at all dosages; but the  
174 decrease was most prominent at the 30 and 60 mg Fe/kg. The regions of interest for Contrast Ratio  
175 (CR) calculation are shown by the circles. (Scale bar = 5 mm). (b) Contrast ratio as function of time  
176 and dose for MRI-only studies. Compared to baseline, the 6 mg Fe/kg dose level did not  
177 demonstrate significant local accumulation of NP at the site of inflammation. Both the 30 mg Fe/kg  
178 and 60 mg Fe/kg showed increased accumulation of NP at the site of inflammation; the 30 mg Fe/kg  
179 dose demonstrated the highest CR, likely due to the reduction of signal differentiation between the  
180 localized accumulation and systemic distribution of NP at the 60 mg Fe/kg dosage. Thus the 30 mg  
181 Fe/kg dose was used for subsequent studies. (Error bars denote the SEM).

182 Following these MRI only studies, we evaluated VDIO-<sup>64</sup>Cu-DOTA nanoparticle accumulation  
183 *in vivo* by hybrid PET/MRI. Figure 5 shows image slices by MRI (top row), by PET (middle row) and  
184 the overlay (bottom row) from the same cross section in a representative mouse (n = 4 mice total).  
185 Prior to injection of VDIO-<sup>64</sup>Cu-DOTA, no MR or PET signal beyond background levels was seen  
186 within the animal. At 4 h post injection, both carotids demonstrated PET signal, which was higher  
187 in the injured vessel, suggestive of radiolabeled NP accumulation at the site of injury along with  
188 continued systemic circulation of unbound NP. Another focus of PET uptake within the field of  
189 view appear to correspond with a vein, supporting that systemic circulation of the NP remains at  
190 this time point, but which could represent another site of inflammation in the animal. In the slice  
191 shown, at 4 h there is a signal increase in the ligated vessel (yellow arrow) at a ratio of 1.19  
192 compared to the uninjured contralateral vessel; at the 24 h time point the ratio of the ligated site  
193 compared to the contralateral artery was 1.07; partial volume effects limits the accuracy of these  
194 measurement. We also calculated the MRI contrast ratio (CR; see equation in methods 4.6.3) from  
195 the concurrent MRI dataset. Note that CR also is a comparison against the contralateral control,  
196 thus taking into account contributions from signal in the blood. For this particular animal, the MRI  
197 CR was 1.41 at the 4 h time point and 0.62 by 24 h, indicating elevated NP accumulation at the  
198 ligated site at the 4 h timepoint with decreased MRI signal by 24 h, commensurate with the PET  
199 results. This slice was selected to illustrate the point that these trends are for this slice, in this  
200 particular animal and based on this slice only one may conclude there was no inflammation in this  
201 vessel. For a more accurate view of inflammation the entire affected volume should be considered,  
202 as well as the full set of experimental subjects. As shown later, mapping inflammation throughout  
203 the vessel provides a clearer picture of inflammatory burden. The MRI CRs for the entire PET/MRI  
204 cohort were  $1.65 \pm 0.26$  at 4 h and  $1.66 \pm 0.39$  at 24 hours respectively. In general, this is in agreement  
205 with the data for the MRI only cohort, which also demonstrated an increased MRI CR over the 24 h  
206 period (Figure 5b). Note that these results support that ligation does not prevent contrast agent

207 access to the injured carotid—if this had been the case there would be less signal drop out due to  
208 reduced perfusion to the region.

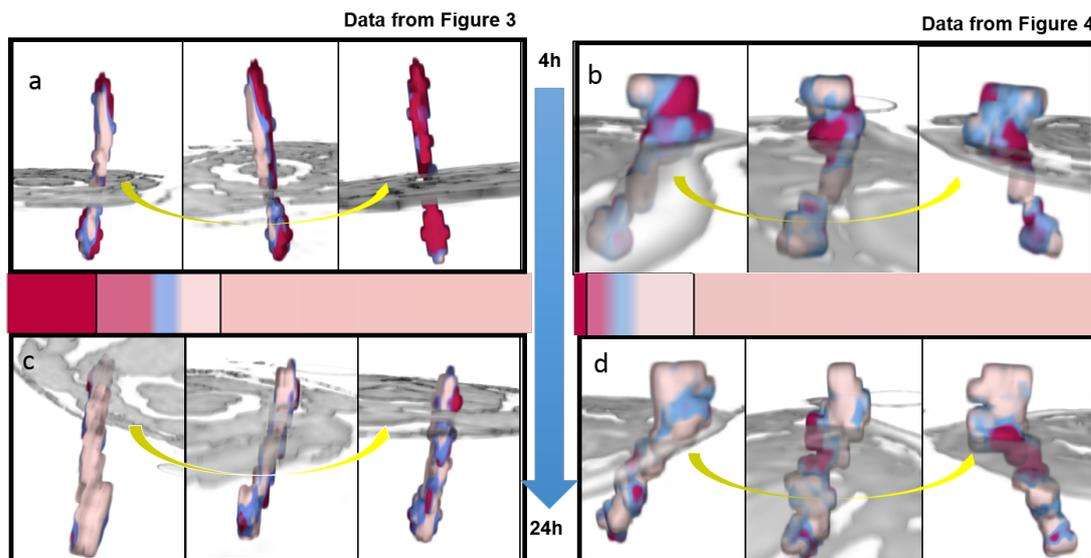


209  
210 **Figure 5.** PET/MR imaging of dual-mode VDIO-DOTA accumulation in carotid artery of ApoE<sup>-/-</sup>  
211 mouse with vascular inflammation (atherosclerotic plaques) induced by ligation. MRI-only images  
212 preinjection (a), after 4h(b) and 24h(e) injection, PET/MRI co-registered images after 4h(c) and 24h(f)  
213 injection and PET-only images after 4h(d) and 24h(g) injection are shown. The image slices were  
214 from the same cross section of the mouse and showed spinal cord (grey oval area in the middle), left  
215 carotid (after ligation) artery (denoted by yellow arrow) and right carotid artery (denoted by red  
216 arrow). The color maps for the PET images were set such that the highest values (as indicated by  
217 red) reflected the highest signal within the animal image volume for the particular time point. The  
218 white circles are markers for MRI intensity standards. (Scale bar = 5 mm).

219 The lower resolution of PET provides decreased accuracy in determining inflamed regions due  
220 to partial volume effects, and subtle inflammatory lesions may be missed. This is suggested by the  
221 fact that although analysis of the MRI data suggested there was accumulation of the NP at the  
222 ligated site, this was not reflected by the simultaneously acquired PET data. The ratios of the PET  
223 signal at the ligated artery compared to the control artery were  $1.05 \pm 0.04$  at 4 hours and  $0.82 \pm 0.33$   
224 at 24 hours. This highlights the need for high-resolution 3D renderings of the affected volume to  
225 better identify regions of high inflammatory activity and assess degree of instability. For improved  
226 volume visualization, we performed three-dimensional rendering of the MRI data as shown in

227 Figure 6. MRI rendering is able to define regional accumulations and concentration differences for  
228 VPIO-DOTA in the vessel wall, allowing us to better localize VCAM-1 expression to specific  
229 regions in the inflamed vessel. Figure 6a and b show rendering of the MRI only data from the 30 mg  
230 Fe/kg dose injection for a representative animal, with a slice from the anatomical MRI included for  
231 anatomical context; this is the same animal and slice view used for the cross section image shown in  
232 Figure 4. The color map (transfer function) for the image was set to define three major color zones  
233 representing low (red), medium (blue), and high (pink) NP uptake. Greater susceptibility from  
234 T<sub>2</sub>-weighting indicates regions of greater particle accumulation (more red). The volume renderings  
235 suggest that VCAM-1 expression, which is targeted by the NPS and indicative of activated  
236 endothelial cells and inflammation, is diffuse along the vasculature and that expression patterns  
237 vary across the vessel. In this particular animal there is a greater accumulation of contrast agent on  
238 one side of the vessel as seen in the 4 h still images in panel a. At 24h (panel b) the trend persists  
239 and one can observe accumulation on one side that is consistent with the 4-hour data.

240 **Figure 6.** Three-dimensional rendering of MRI data (a – b). Medium dose study, MRI data (from  
241 Figure 3). (a) Still frames from video depicting a rotating view of the inflamed vessel 4 h after  
242 injection of contrast agent. (b) Similar data for the vessel at 24 h. A similar trend for higher  
243 accumulation of contrast agent on one side of the vessel is preserved at both time points. (c – d)  
244 PET/MRI study, MRI data (From Figure 4). (c) Still frames from video depicting a rotating view of  
245 the inflamed vessel 4 h after injection of contrast agent. (d) Similar data for the vessel at 24 h. A  
246 trend for a “hot spot” (red) accumulation of contrast agent at the same location is seen at both  
247 time points. Transfer functions used for color assignments are displayed as color bars shown between  
248 the 4 and 24h data sets.

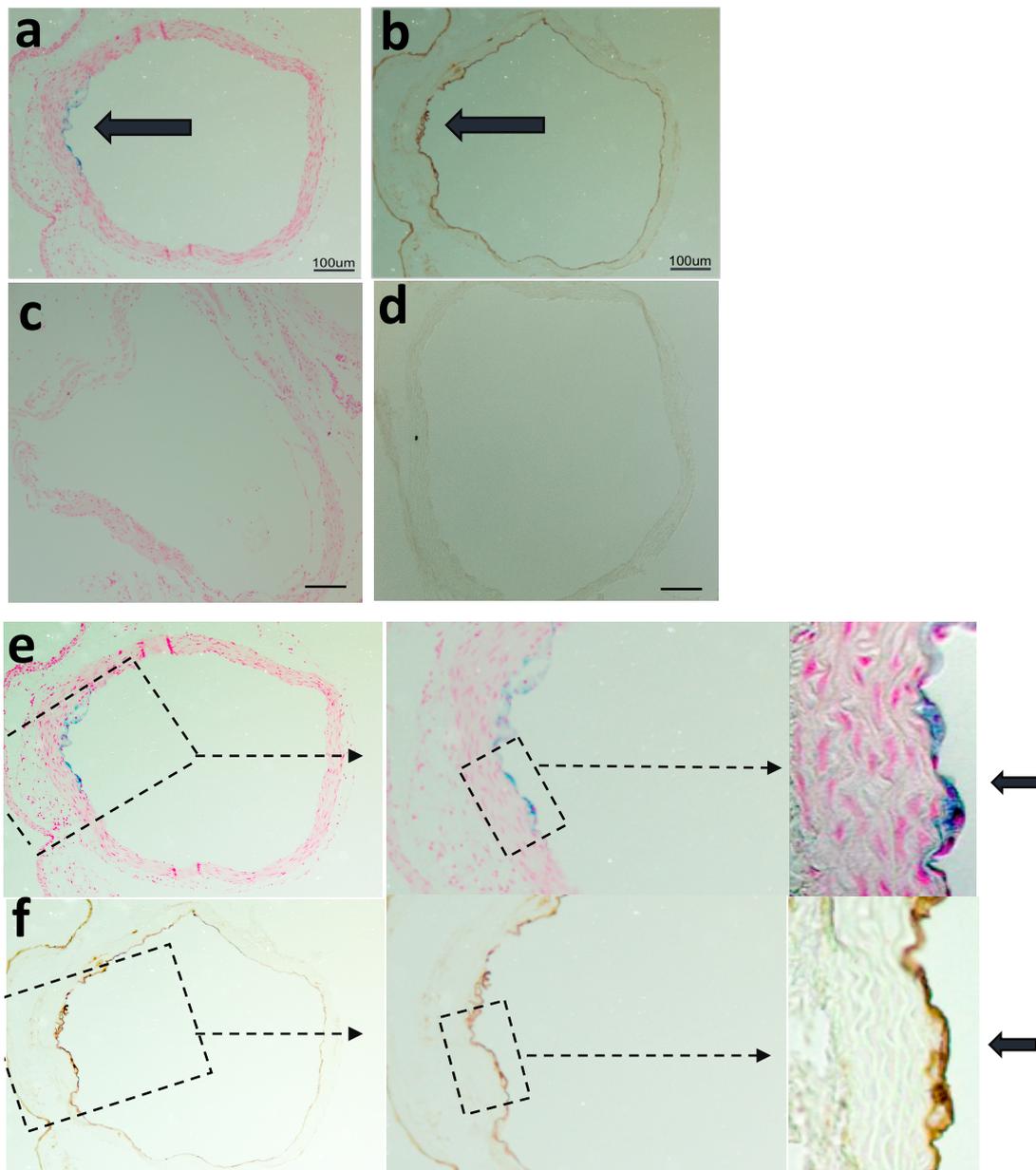


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250 Figures 6c and d display rendering of the MRI data for the animal shown in the PET/MRI  
251 images of Figure 5. Inflammation is even more heterogeneous in this animal and NP accumulates in  
252 patches throughout the vessel. A local “hot spot”, appears in the 4 h data (panel c) and persists but  
253 is decreased through the 24 h time point (panel d) commensurate with the PET and MRI CR results.  
254 At 24 h most of the hot spot lies below the plane of interrogation that was shown in the slice in  
255 Figure 4; this may explain the lack of PET observed in the slice shown and also underscores the  
256 value of evaluating 3D data sets as volume renderings. The rendering provides insight into the  
257 heterogeneity of the NP accumulation, which is not readily apparent from slice by slice  
258 visualization of the MRI image set. This data correlates with the histological data, described in the  
259 next section, which showed inhomogeneous regional accumulations of the nanoparticles that  
260 overlap with regions showing high VCAM-1 expression. The morphology of the local plaque load  
261 can have prognostic implications.[24] Thus, this additional information from the high resolution  
262 MRI may provide vital information for clinical management.

263 The addition of a 3D visualization is very useful for interpreting these types of data sets where  
264 the disease is diffuse and lesions can span across multiple slices. One of the challenges for 3D  
265 rendering is the ability to create images and videos without the need for extensive manual  
266 segmentation, i.e. user delineation of pathology. The renderings shown here were created using  
267 software created at UC Davis that performs intensity-based segmentation by applying a  
268 user-defined color map to all voxels in the data set. This removes some degree of subjectivity from  
269 the assignment of pathological features.

#### 270 *2.4 Iron co-localizes with VCAM-1 expression*

271 Mice were sacrificed after *in vivo* MRI only or PET/MRI study and organs of interest were  
272 collected for further analysis. For the MRI only studies, the tissues from mouse carotids were  
273 embedded into paraffin sections for histological study to examine iron accumulation (Figures 7a, c,  
274 e) and VCAM-1 expression (Figures 7 b, d, f) in the tissues. Prussian Blue staining was used to  
275 detect ferric ion ( $Fe^{3+}$ ) in the tissue. As shown in Figure 7a blue staining demonstrated that iron  
276 could be found in the intima in the inflamed carotid artery (arrow). Anti-VCAM-1 antibody  
277 staining was performed on an adjacent section (Figure 7b), which confirms the presence of VCAM-1  
278 in the same region, as indicated by dark brown stain of the intima on the left side of the vessel  
279 lumen (arrow). The dark blue iron staining co-localized in the regions of darkest brown VCAM-1  
280 staining, supporting that the nanoparticles were co-localized with VCAM-1 expression. There  
281 appears to be a thin layer of VCAM-1 staining on the rest of the lumen that does correlate with  
282 some iron staining. The faint brown stain over the entire intima represents nonspecific staining that  
283 is also found in the control vessel. Non-inflamed contralateral vessel showed no iron (Figure 7c) or  
284 dark brown VCAM staining (Figure 7d).



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**Figure 7.** Iron colocalizes with VCAM-1 expression. (a). Prussian Blue staining shows iron on tissue cross section from inflamed mouse carotid artery. (b). Immunohistochemical staining shows VCAM-1 (brown) on a tissue slice adjacent to that shown in A. (c). Prussian Blue staining showed no iron in tissue cross section from normal mouse carotid artery and (d) no VCAM-1 staining on neighboring tissue slice. (e) Higher magnification view of Prussian Blue staining in panel a at 10X, 20X and 40X. (f) Higher magnification views of VCAM-1 staining in panel b. Dashed boxes indicate the area of interest with increasing magnification, moving left to right. Staining appears localized to the monolayer of endothelial cells lining the vessel.

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VCAM-1, triggered by inflammatory cytokines such as TNF- $\alpha$ , is expressed by endothelial cells as a prelude to the recruitment of macrophages into plaques. In our previous work to label macrophages in the injury model, animals were placed on fat diet for 2 weeks after ligation; the current studies were performed 8 days after ligation. At this earlier time point, plaque size and macrophage involvement are minimal as can be seen in the higher magnification histological images. Figure 7e shows iron staining at increasing magnifications from left to right and iron

300 staining localized to cells of the intima, which is still generally a monolayer of endothelial cells.  
301 Figure 7f shows VCAM-1 staining at increasing magnifications from left to right, which shows  
302 VCAM-1 localized to the same monolayer. There was no intimal thickening, or obvious  
303 macrophage accumulation observed in the histological slices. The iron stain also appeared confined  
304 to a monolayer of "cobblestone-like" endothelial cells and there was no evidence of macrophages,  
305 which display surface ruffles and blebs. These results support that the NPs were binding to  
306 VCAM-1 expressed on endothelial cells.

#### 307 4. Conclusions

308 In this study, we evaluated the use of a simultaneous hybrid PET/MRI system and multimodal  
309 nanoparticle contrast agent to visualize distribution of an early marker of inflammation. We found  
310 robust spatial-temporal co-localization of the PET and MRI signal, and identified regions of  
311 inflammation, which were confirmed by histological staining. PET, limited by its lower spatial  
312 resolution, could not specifically map inflammation in the vessels. PET also missed elevated  
313 contrast that was detected by MRI, likely due to signal averaging effects of diffuse inflammation in  
314 the tissues. But the sensitivity of PET allowed detection of inflammation in a larger volume map  
315 and served to guide MR imaging. Careful analysis of the MRI data, aided by 3D volume rendering,  
316 demonstrated a heterogeneous pattern of VCAM-1 expression extending beyond the site of vessel  
317 injury. The morphology of the local inflammation patterns can have prognostic implications.[24]  
318 This high-resolution mapping of early stage inflammation would not have been possible with MRI  
319 alone as lesions are difficult to detect in small vessels against anatomical background. Whole body  
320 MRI screening in humans would be even more challenging. Thus, the combination of PET and MRI  
321 provided complementary functions in this imaging application.

322 Simultaneous PET/MRI imaging of the multimodal NP afforded a number of advantages over  
323 serial imaging including: 1) avoiding movement of the subject between scanner-, which allowed for  
324 facile spatial co-registration to do the lack of soft tissue movement experienced with scanning with  
325 separate instruments; 2) eliminating time delay between PET and MRI scans, which avoided  
326 complications with subject transport under anesthesia; 3) reduced exposure to personnel associated  
327 with handling and transporting the subject between instruments in different facilities. Given that  
328 many high-resolution MRI techniques are often time consuming and have a limited field of view,  
329 the availability of hybrid instrumentation can allow us to localize the ROI using PET without loss of  
330 spatial fidelity that would come with moving the subject to a separate scanner. This would be  
331 especially important in the clinical scenario whereby the site of injury may not be known a priori.

332 The approach of using hybrid PET/MRI instrumentation along with multimodal imaging  
333 probes provides a powerful platform for both research and clinical pursuits. From a research  
334 perspective, hybrid imaging can provide direct information about the efficacy and distribution of  
335 multimodal molecular imaging agents, providing vital information to refine and improve the signal  
336 characteristics of these agents. From a clinical perspective, PET/MRI hybrid instruments are  
337 available now in a limited number of locations and their optimal applications are under  
338 investigation. Oncological applications have been some of the earliest imaging studies to  
339 demonstrate beneficial results from hybrid PET/MRI.[39, 40] For example, a study of PET/MRI for  
340 clinical prostate cancer imaging found that PET/MRI may demonstrate improved sensitivity to  
341 metastatic lesions compared to PET/CT.[41] Hope for improved diagnoses through hybrid PET/MRI  
342 is high for neurological applications, where the temporal alignment of hybrid instruments could be  
343 an advantage for examining brain processes in real-time.[16]

344 Cardiovascular applications that could benefit from PET/MRI are still under investigation [22,  
345 42]; however, the simultaneous acquisition capability could offer advantages for imaging fast  
346 moving hearts and arteries. Most reports with PET/MRI have focused primarily on unimodal  
347 contrast agents such as <sup>18</sup>FDG using MRI as an anatomic reference.[15, 43, 44] We demonstrate that  
348 PET-guided MRI using multimodal agents can facilitate high-resolution visualization of molecular  
349 targets, and establish utility for mapping a marker associated with inflammation. This can serve as  
350 a powerful research tool for drug validation by allowing monitoring of biomarker targets. Early

351 diagnosis and patient stratification by PET/MRI could provide clinical benefit as drugs to  
352 ameliorate plaque vulnerability come available.

353 In summary, we have demonstrated an approach to identify early inflammatory changes in  
354 vessel injury using a targeted multimodal probe coupled with hybrid PET/MRI imaging *in vivo*.  
355 Such approaches may be able to provide important insights into the pathophysiology and clinical  
356 management of vulnerable plaques. Furthermore, simultaneous PET/MRI allowed direct  
357 comparison of the PET and MRI signal, providing insights into the signal derived from the  
358 multimodal agent that can guide continued development of the instrument as well as the imaging  
359 agent.

360

## 361 5. Materials and Methods

### 362 5.1 Materials

363 Materials were obtained from commercial suppliers and used directly, unless otherwise noted.  
364 Dextran (from leuconostoc, average mol. wt. 9,000–11,000) and ferric chloride hexahydrate  
365 ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , Fw 270.29 g/mol) were purchased from Sigma-Aldrich. Ferrous chloride tetrahydrate  
366 ( $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ , Fw 198.81 g/mol) was from Fluka. Ammonium hydroxide (28–30%), sodium  
367 bicarbonate, and sodium hydroxide were from Fisher Scientific. p-SCN-Bn-DOTA was from  
368 Macrocyclics, Inc (Dallas, TX). N-succinimidyliodoacetate (SIA), purchased from Pierce (Rockford,  
369 IL). Spectra/por® dialysis membrane (mol. wt. cut-off 50,000) was acquired from Spectrum  
370 Laboratories, Inc. The VHPKQHR(MiniPEG1)C peptide was from Genscript ( $\geq 95\%$  purity). Water  
371 was purified using a Millipore Milli-Q Synthesis purifier (18.0 M $\Omega$  cm, Barnstead).

### 372 5.2 Nanoparticle synthesis and characterization

#### 373 5.2.1 Synthesis of dextran coated iron oxide (DIO) nanoparticles

374 Dextran was reduced by published methods as summarized here and outlined in Figure 1.[25]  
375 Nanopure water (18.0 M $\Omega$  cm, Barnstead) was degassed with argon and used throughout the  
376 synthesis process. A mixture of dextran (molecular weight 10,000) and sodium borohydride (26  
377 equivalents) was stirred at room temperature for 12 hours. The solution was adjusted to pH=7,  
378 dialyzed against degassed nanopure water and lyophilized to form reduced dextran as a white  
379 solid. A mixture of reduced dextran and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in a molar ratio of 1:27 (total polysaccharide:  
380  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) was dissolved in deionized nanopure water. The solution was bubbled with argon and  
381 cooled to 4 °C in an ice-water bath.  $\text{Fe}^{2+}$  solution was freshly prepared by dissolving  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$  in  
382 degassed water (with a  $\text{Fe}^{3+}:\text{Fe}^{2+}$  ratio in a range from 1.47 to 1.5) and stored on ice. The  $\text{Fe}^{2+}$  solution  
383 was added to  $\text{Fe}^{3+}$  mixture using a syringe followed by adding chilled (4 °C)  $\text{NH}_4\text{OH}$  ( $\text{NH}_4\text{OH}:\text{Fe}^{3+} =$   
384 16:1) dropwise with vigorous stirring. The ice-water bath was removed and the mixture was heated  
385 to 85 °C and kept at  $85 \pm 5$  °C for 2 hours (argon flow may stop 2–3 min after the temperature  
386 reaches 85 °C). After cooling to room temperature, the solution was dialyzed against deionized  
387 water in a dialysis bag with a molecular weight cut-off of 50,000 Da for 72 hours with 8-10 changes  
388 of water to remove reactants. The resulting product, DIO, was lyophilized and stored at 4 °C.[26]

#### 389 5.2.2 Cross-linking and amination of the DIO nanoparticles

390 The DIO nanoparticles were cross-linked and aminated for the attachment of two ligands:  
391 DOTA (chelator of radioactive  $^{64}\text{Cu}$ ) and the VCAM-1 targeting peptide (Figure 1). The  
392 cross-linking and amination were performed as previously reported with slight modifications.[27]  
393 DIO (2.0g), NaOH (4.02g) pellets and deionized water (40mL) were added to a 100mL  
394 round-bottomed flask and stirred for 30 min. Then epichlorohydrin was added to the mixture and  
395 the solution was stirred for 24h. The solution was dialyzed against deionized water with 8-10  
396 changes of deionized water and lyophilized to yield brown solid. The solid (2.0 g), together with  
397 ammonium hydroxide (250mL), was then transferred to a 500mL round-bottomed flask and stirred

398 for 36h. Excess ammonium hydroxide was removed by dialyzing the solution against deionized  
399 water for 72h with 8-10 changes of deionized water.

### 400 5.2.3 Conjugation of DOTA and VCAM-1 targeting peptide to the nanoparticle surface

401 DOTA was conjugated to the aminated DIO surface based on a literature method. Briefly,  
402 p-SCN-Bn-DOTA (6.71mg), aminated nanoparticle (135mg) and 0.1 M sodium borate buffer  
403 solution (2mL) were added to a 10 ml round-bottomed flask (Figure 1b)[28]. Approximately five  
404 drops of sodium hydroxide aqueous solution (1N) was used to bring the solution pH to 8.5. The pH  
405 of the mixture was monitored during the reaction. The mixture was stirred for 24h, then dialyzed  
406 against deionized water for 72h with 8-10 changes of water in a dialysis bag with a molecular  
407 weight cut-off of 50,000 Da and then lyophilized to give a brown solid of DOTA conjugated  
408 aminated DIO (DIO-DOTA). The conjugation of DOTA to the aminated DIO was confirmed by  
409 Fourier Transform Infrared (IR) Spectroscopy. DIO-DOTA was then used for anti-VCAM-1 peptide  
410 attachment based on previous report[29], described here briefly.

411 DIO-DOTA (22mg Fe in 1.5mL DMSO) was added to 0.5mL of 0.1M Na<sub>2</sub>HPO<sub>4</sub> in water and  
412 0.5mL of 15mM SIA in DMSO in a 10mL round-bottomed flask. The mixture was stirred for 1h at  
413 room temperature followed by another addition of 0.5mL of 15mM SIA  
414 (N-succinimidyliodoacetate) in DMSO. Iodoacetyl-DIO was separated from iodoacetic acid using a  
415 Sephadex G-25 column equilibrated with 0.025M citrate buffer pH 6.5 at 4 °C. Then 6-7 mg of  
416 polymer-modified peptide (VHPKQHR(MiniPEG1)C) in 0.6mL of citrate buffer was added to 5mL  
417 of iodoacetyl-DIO solution and the mixture was incubated overnight at room temperature. The  
418 purchased peptide, C<sub>49</sub>H<sub>84</sub>N<sub>18</sub>O<sub>14</sub>Si<sub>1</sub>, had a molecular weight of 1181.37 Da and an isoelectric point of  
419 pH 9.84. MiniPEG1 (8-amino-3,6-dioxaoctanoic acid) spacer was inserted to distance the peptide  
420 from the surface of the nanoparticle to allow for proper tertiary folded structure and allow better  
421 VCAM binding. Unreacted peptide was removed by using a Sephadex G-25 column equilibrated  
422 with 0.025M citrate buffer pH 6.5 at 4°C. The purified solution was lyophilized to give the final  
423 product that is the DIO nanoparticle with two ligands (DOTA and VCAM-1 targeting peptide) on  
424 the surface (VDIO-DOTA).

### 425 5.2.4 Copper-64 labeled VDIO-DOTA

426 Copper-64 was employed due to its relatively long half-life (12.7 h)[30] and comparatively  
427 stable coordination with multidentate macrocyclic compounds such as DOTA (log K<sub>ML</sub> = 22.3).[31]  
428 VDIO-DOTA (25 mg) was dissolved in 150 µL of 0.2 M pH 5.5 sodium acetate-acetic acid buffer.  
429 Copper-64 (~ 2.5 mCi) was added to the vial and the mixture was vortexed for 5 seconds to obtain a  
430 uniform solution. The solution was incubated at 55-60 °C for 45 minutes. EDTA aqueous solution  
431 (16 µL, 100 mM) was added to the vial and vortexed for 5 seconds to get the solution uniform; then  
432 solution was incubated at 55-60 °C for 15 minutes. The crude product was purified by centrifuge  
433 filtration with 10K Da nanosep filtration tube (Millipore Inc., Billerica, MA, 30 min @14,000 rpm)  
434 and washed 3 times with pH 5.5 sodium acetate-acetic acid buffer. Each time the washing was  
435 removed by centrifuge filtration with a 10-kDa Nanosep filtration tube (10 min at 14,000 rpm). After  
436 three washings the filtration tube was turned over and inserted into a new vial, and the  
437 nanoparticles, VDIO-<sup>64</sup>Cu-DOTA, were collected by centrifuge (2 min @ 1,000 rpm). The radioactive  
438 nanoparticles were diluted to 650 µL with saline (0.9%) and radioactivity of the solution was  
439 measured with a Fluke Biomedical Dose calibrator (34- 162 CAL/RAD MARK IV, Cleveland, OH).  
440 The radiolabeling yield (%) for the NP, percent incorporation of radioisotope, was determined by  
441 dividing the radioactivity of the collected NP by the total activity applied to the column and  
442 multiplying by 100. For injection to animals VDIO-<sup>64</sup>Cu-DOTA (~ 15 µL) was passed through a  
443 sterile 0.22-micron filter before use.

### 444 5.2.5 Characterization of VDIO-DOTA nanoparticles

445 The iron concentration (mg) per unit mass of nanoparticles in DIO or VDIO-DOTA was  
446 measured with a Varian AA 220FS atomic absorption (AA) spectrophotometer using an  
447 air/acetylene flame. The iron-oxide core size of the VDIO-DOTA was measured by Transmission  
448 Electron Microscopy (TEM) on a Philips CM-12, operating at 80 kV and equipped with a  
449 GatanMegascan 795 digital camera. The core size was found by averaging the measurements of 500  
450 particles after drying a dilute drop of VDIO-DOTA particles over a lamp on a copper grid. The  
451 average hydrodynamic particle size (mean volume diameter) and distribution was measured using  
452 Dynamic Light Scattering (DLS) with a Nanotracs 150 particle size analyzer (Microtrac, Inc.,  
453 Montgomeryville, PA) and geometric eight-root regression, with no residuals, was used to fit the  
454 data. The nano-range option was selected and a scan time of 90 seconds was used.

455 Transverse relaxation times ( $T_2$ ) of the VDIO-DOTA particles were measured at 60 MHz (1.4 T)  
456 and 37 °C on a BrukerMinispec mq60. The relaxivity was given as the slope of the straight line  
457 plotted as the function of  $1/T_2$  vs iron concentration. DIO and VDIO-DOTA were diluted in pH 7.0  
458 deionized water to give five aqueous solutions (300  $\mu$ L each): 10.5, 5.25, 2.625, 1.313, and 0.656 mg  
459 Fe/L, respectively. Iron concentrations in each dilution were determined using Atomic Absorption  
460 Spectroscopy.  $T_2$  values were measured using a Carr-Purcell-Meiboom-Gill (CPMG) sequence with  
461  $\tau = 1$  ms, and 200 data points. Each solution was incubated at 37°C for 5 minutes before  
462 measurement.

### 463 5.3 Cytotoxicity

464 Cytotoxicity of VDIO-DOTA was evaluated on HepG2 liver cells using  $C_{12}$  – Resazurin  
465 viability assays. HepG2 liver cells were cultured and maintained in Minimum Essential Medium  
466 containing 10% FBS, 200 U/mL penicillin, 200  $\mu$ g/mL streptomycin, 1 mM sodium pyruvate, and 1  
467 mM nonessential amino acids at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were plated in  
468 96-well dishes at a concentration of  $1 \times 10^4$  cells per well and incubated overnight (5% CO<sub>2</sub>, 37°C).  
469 After overnight incubation, media was replaced with fresh media containing VDIO-DOTA  
470 nanoparticles of varying concentration (0, 0.04, 0.2, 1, 4 and 10mM iron). Each concentration was  
471 performed in triplicate for statistical relevance. Cells were also treated with DIO nanoparticles  
472 under the same conditions as control. Cells were plated in three 96-well plates for measurements at  
473 time points of 4h, 24h and 48h. At each time point, nanoparticle solutions were removed and cells  
474 were washed with 1X PBS for three times. Then fresh media containing  $C_{12}$ – Resazurin (5 $\mu$ M) was  
475 added to each well of cells. After 15min of incubation, fluorescence was measured using a Safire  
476 monochromator microplate reader (Tecan Austria G.M.B.H., Austria) with excitation of 563nm and  
477 emission of 587nm.

### 478 5.4 Iron staining

479 Slides from the inflamed carotid were stained to evaluated for iron within the plaques using  
480 Prussian Blue Solution[32], a 1:1 mixture of 3% hydrochloride acid solution and 3% potassium  
481 ferrocyanide solution, to detect ferric ion ( $Fe^{3+}$ ) in the tissue. Any ferric ion present in the tissue  
482 reacted with the ferrocyanide and results in the formation of the bright blue pigment, Prussian blue,  
483 or ferric ferrocyanide. First, slides were deparaffinized by toluene and rehydrated through changes  
484 of ethanol with decreasing concentrations (100%, 95% and 75%). After rinsing, slides were placed in  
485 Prussian blue solution for 30 min followed by a rinsing step to remove excess staining. Then  
486 Nuclear Fast Red Staining was used to counterstain other tissue (pink) for 10 min. After  
487 dehydration with alcohol and clearing with toluene, tissue sections were placed under coverslips on  
488 slides with mounting media.

### 489 5.5 Immunohistochemistry (IHC)

490 The tissue sections were stained for inflammatory marker VCAM-1. The deparaffinization and  
491 dehydration process were the same as that of iron staining. Antigen retrieval was performed to  
492 unmask binding sites of the primary antibodies. Heat Induced Epitope Retrieval (HIER) was

493 performed using a Decloaking Chamber. 500 ml of deionized water was added into the chamber. A  
494 plastic staining jar with tissue slides immersed in Antigen Retrieval Solution (Sigma 10X Tris-HCl  
495 buffer, pH10, product #T6455) was placed in the Decloaker chamber. The Decloaker was programed  
496 for 30 seconds at 125 °C followed by 10 seconds at 85 °C at 22.5 psi. After the heating process was  
497 done, the staining jar was removed from the Decloaker and cooled for 15 minutes followed by TBST  
498 rinse (Fisher 20X Tris buffered saline with Tween-20 used as 1X diluted solution). Then endogenous  
499 peroxidase block was performed by using 3% H<sub>2</sub>O<sub>2</sub> in water to cancel the interference of peroxidase  
500 in the final step.[33] Protein block buffer (Dako Protein Block) was used to treat the tissue slides to  
501 mask the non-specific binding sites. Samples were labeled using an indirect method. Anti-VCAM-1  
502 antibody (Rabbit monoclonal [EPR5047] to VCAM-1, Abcam Inc., MA) was used as primary  
503 antibody to detect the VCAM-1 expression. Ready-to-use polymers carrying horseradish peroxidase  
504 (Dako North America, Inc., Carpinteria, CA) were then used as the secondary antibody. In the final  
505 staining step, peroxidase on the polymers reacted with hydrogen peroxide to reduce the DAB  
506 (3,3'-Diaminobenzidine) substrate and generate a brown product in regions of VCAM-1 expression.  
507 Slides were examined under microscope.

## 508 5.6 Animal Studies

### 509 5.6.1 Animal model

510 All animal studies were performed under protocols approved by the Animal Care and Use  
511 Committee of the University of California, Davis and the California Institute of Technology. Female  
512 C57BL/6 ApoE<sup>-/-</sup> (10 weeks old, Jax West Laboratories, West Sacramento, CA) mice were used for  
513 the experiments as described in previous studies.[14] Eight days prior to imaging, the left carotid  
514 artery of each mouse was ligated. Eight days was chosen because prior studies showed that  
515 VCAM-1 expression peaks between 7 and 10 days post-ligation in ApoE<sup>-/-</sup> mice.[34] At this stage  
516 pronounced plaques have not yet formed, but inflammation is evident. To perform the ligation a  
517 medial incision was made between the mandible and clavicle, exposing the glands and vessels of  
518 the neck. The carotid artery was singled out from the surrounding tissue, while protecting and  
519 excluding the parallel-running vagus nerve. A 6/0 silk suture was threaded under the dorsal side of  
520 the carotid artery and was tied off to cause injury to the site. The procedure was concluded with  
521 five to six interrupted 4/0 Ethicon (Ethicon Inc) suture to re-approximate the skin of the original  
522 ventral incision. The mice were monitored twice a day for approximately four days to check for  
523 irritation and to administer analgesics when appropriate. Subsequent to ligation, mice were placed  
524 on a high fat diet for seven days. (TD 88137, Harlan Laboratories Inc, Madison, WI).

### 525 5.6.2 *In vivo* MRI-only studies

526 MRI experiments were performed to determine the optimal injection dosages for the NPs prior  
527 to radiolabeling. Prior to NP injection, a pre-scan was taken as baseline. Then VDIO at dosages of 6  
528 mg Fe/kg body mass, 30 mg Fe/kg and 60 mg Fe/kg, were injected intravenously via the tail vein  
529 catheter (N = 4 per concentration). Images were acquired at 4 and 24 hours post-injection.

530 All images were acquired on a 7T (Bruker Biospec) small animal scanner using a home built  
531 quadrature RF volume coil (Cleveland, OH). For all time points, the animal was anesthetized with a  
532 1.5% isoflurane: air mixture and kept at 35–37 °C with warm air flowing through the bore while the  
533 respiration was monitored (MP150, Biopac, Goleta, CA). After localizing the region of interest (ROI)  
534 around the neck using a RARE spin echo sequence (TR/TE = 4000/22 ms, matrix size = 128 × 128,  
535 FOV = 35.35 × 35.35 mm<sup>2</sup>, slice thickness = 0.754 mm), the common carotid arteries were located  
536 with a time-of-flight angiography sequence with venous saturation (FL2D\_ANGIO method,  
537 Paravision 4.0: TR/TE = 13.7/3.5 ms, matrix size = 150 × 100; zero-filled to 256 × 100, FOV = 30 × 20  
538 mm<sup>2</sup>, slice thickness = 0.754 mm). A T<sub>2</sub>\* weighted multiple-gradient echo sequence was then utilized  
539 to visualize the uptake of nanoparticles (TR/TE = 718/3, 7, 11, 15, 19, 23 ms, F.A. = 25°, matrix size =  
540 175 × 100; zero-filled to 234 × 133, FOV = 35 × 20 mm<sup>2</sup>, slice thickness = 0.754 mm) at the region of  
541 the common carotid arteries.

### 542 5.6.3 *In vivo* PET/MRI imaging

543 Studies were performed using an integrated small animal PET/MRI system, consisting of a  
544 first-generation MR-compatible PET insert (constructed by Simon Cherry et. al at UC Davis) that is  
545 fitted within a 7T small animal MRI scanner. [16, 17] This enabled simultaneous PET/MRI images to  
546 be acquired.

547 Mice (N = 4) were surgically prepared as described above. Pre-scans were obtained with MRI  
548 as baseline. Next, mice were injected intravenously via the tail vein with 30 mg Fe/kg  
549 VDIO-<sup>64</sup>Cu-DOTA (~700uCi per mouse, 92% radiation yield) followed by a 150  $\mu$ L of saline flush.  
550 The activity of the injected dose was confirmed by measuring the difference in radioactivity  
551 contained in the syringe before and after injection on a dose calibrator. Imaging was subsequently  
552 performed at 4 and 24 hours post-injection. MRI images were acquired identically to the MRI-only  
553 studies. PET images were acquired with scan duration of 600 seconds at the 4 hour time point and  
554 300 seconds at the 24 hour time point. Images were reconstructed and co-registered to the MRI  
555 dataset as previously described.[17, 35]

556 To compare the focal NP uptake between time points, dosage and subjects, we calculated a  
557 contrast ratio (CR) metric as previously described.[36] This normalizes particle uptake at the  
558 ligation site between subjects and factors out the signal contribution due to blood borne particles by  
559 comparison with the contralateral control.

560 Briefly, CR is defined as:

561

$$CR = \frac{\left[ \frac{[I_{ligated}] - [I_{background}]}{[I_{control}] - [I_{background}]} \right]_i}{\left[ \frac{[I_{ligated}] - [I_{background}]}{[I_{control}] - [I_{background}]} \right]_{prescan}}$$

562 Where  $I_{ligated}$  is the mean intensity of the ROI drawn around the ligated carotid artery,  $I_{control}$  is the  
563 mean intensity of the ROI drawn around the contra-lateral carotid.  $I_{background}$  is the mean intensity of  
564 the ROI drawn in the spinal cord at the same image slice as the other ROIs and  $i$  is either 4 or 24  
565 hours post-injection time points. ROIs were drawn manually at slice levels approximately located at  
566 the common carotid arteries. These were matched between time points. Angiography images were  
567 used to guide ROI delineation around the carotid arteries. Because previous reports noted that the  
568 carotid vessels along with the wall are ~1 mm in diameter,[36] all arterial ROIs had diameters of 1.5  
569 mm.

570 Representative region of interests used for CR calculation are indicated in Figure 3. Values  
571 greater than 1 indicate localization at the ligation site. The  $T_2^*$  weighted image sets at TE = 11 ms  
572 were used. All images were analyzed using ImageJ.

### 573 5.6.4 Visualization of image volumes

574 We developed a hardware accelerated volume rendering system with enhanced rendering  
575 quality to provide better visualization of the PET and MRI signal at the site of the inflamed arteries.  
576 To provide anatomical context, we superimpose a cross section of the data. Illustrative  
577 rendering[37] is applied to this cross section for relative spatial position of the target vessel. The  
578 vessel ROIs were manually segmented from the MRI images to identify the vessel walls. A  
579 pre-integrated transfer function[38] was used to visualize the thin layer of vessel wall as a smooth  
580 and continuous surface. To highlight the NP uptake on the surface of the arteries, we used a color  
581 map to sort the surface MR signal ranging from red (low MR signal), through blue (medium) to  
582 light pink (high MR).

### 583 5.7 Histology

584 After the *in vivo* animal study, organs known to be involved in disease or clearance, i.e. hearts,  
585 carotid arteries, kidneys, spleens and livers, were collected and used for histology. The tissue was

586 fixed in 4% formaldehyde, and then dehydrated by passing tissue through increasing  
587 concentrations of ethanol (75%, 95% and 100%). Then the tissue was placed in warm paraffin wax,  
588 and the melted wax filled the spaces that used to contain water. After cooling, the tissue hardened  
589 into a paraffin block from which 5-micron tissue slices were sectioned and mounted on glass slides.  
590 The tissue sections were stained for iron and VCAM-1 as detailed in Supplemental Information.

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600 experiment design, performed experiments and contributed to data analysis; LZ prepared computer programs  
601 to render the imaging data; JP, IE performed and provided training for histology; REJ, KP contributed to  
602 experimental design and data analysis.

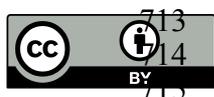
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