Supporting Information

Dual-modal magnetic resonance and fluorescence imaging of atherosclerotic plaques in vivo using VCAM-1 targeted tobacco mosaic virus

Michael A. Bruckman[1], Kai Jiang[1], Emily J. Simpson[5], Lauren N. Randolph[1], Leonard G. Luyt[5] Xin Yu[1,2], and Nicole F. Steinmetz[1,2,3,4]*

[1]Department of Biomedical Engineering, [2]Department of Radiology, [3]Department of Materials Science and Engineering, and [4]Department of Macromolecular Engineering, Case Western Reserve University Schools of Medicine and Engineering, 10900 Euclid Ave., Cleveland, OH 44106, USA

[5]Departments of Chemistry, Oncology, Medical Imaging, The University of Western Ontario, London, ON, N6A 4L6 Canada

Supporting Information Methods

Isolation of TMV

TMV particles were isolated from N. benthamiana plants using a previously established protocol[1]. The TMV concentration was determined based on UV-visible absorbance at 260 nm with an extinction coefficient of 3.0 mL mg^{-1} cm^{-1}.

VCAM Peptide synthesis

All chemicals were purchased from Sigma-Aldrich, Novabiochem, Peptides International and Aapptec and were used without further purification. Dry CH_2Cl_2 was prepared by distillation from CaH under argon. For LC-MS, a Waters, Inc. system was used, consisting of a Waters 2998 Photodiode Array Detector and a Waters 2767 Sample Manager. For analytical LC-MS studies, a Sunfire RP-C18 4.6 x 250 mm, 5 μm column was used. For preparative LC-MS, a Sunfire RP-C18 19 x 150 mm, 5 μm column was used. In both cases, absorbance was detected at wavelengths of 220 nm and 254 nm. A gradient solvent system consisting of CH_3CN + 0.1% of TFA (solvent A) and H_2O + 0.1% of TFA (solvent B) was used. For electro-spray ionization (ESI) mass spectra a Micromass Quattro Micro API mass spectrometer was used.

The peptide was synthesized on-resin using standard Fmoc solid-phase peptide chemistry by manual synthesis using Fmoc (9-fluorenylmethoxycarbonyl) protected rink amide MBHA (4-methylbenzhydrylamine) resin (0.1 mmol, loading 0.51 mmol/g). The Fmoc protecting goup was removed by treatment with 20% piperidine in DMF for 5 and 15 minutes, followed by rinses with DCM (dichloromethane) and DMF (N,N-dimethylformamide). Fmoc-Lys(alloc)-OH was then coupled twice for 30 min and 1 h using 3 eq. of Fmoc amino acid, 3 eq. of HCTU (2-(6-

chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate) and 6 eq. of DIPEA (N,N-diisopropylethylamine) in 5 mL DMF. Fmoc-AEEA-OH linker was then coupled overnight using 3 eq of the linker, 3 eq HCTU and 6 eq DIPEA in 5 mL DMF. This was followed by couplings of Fmoc-Arg(Pbf)-OH, Fmoc-His(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-His(Trt)-OH and Fmoc-Val-OH, respectively, using 3 eq of Fmoc amino acid, 3 eq HCTU and 6 eq DIPEA in 5 mL DMF for 30 min and 1 hr. The alloc group was then removed from the lysine side-chain by treatment with $PhSiH_3$ and $Pd(PPh_3)_4$ in distilled DCM for 30 min. Finally, a 550 MW N_3-$(PEG)_7$-COOH spacer was coupled overnight using 3 eq of the spacer, 3 eq HCTU and 6 eq DIPEA in 5 mL DMF. Once complete, the N-terminal Fmoc group was removed using the same conditions as above. The peptide was then deprotected and cleaved from the resin by treatment with TFA (trifluoroacetic acid) containing water (2.5% v/v) and triisopropylsilane (2.5% v/v) as scavengers for 4 h. Purification was performed by preparative high-performance liquid chromatography (HPLC, linear gradient of 5-70% solvent A in B). The purity of the resultant peptide was determined to be 98% by analytical HPLC (linear gradient of 10-80% solvent A in B). This resulted in a 12% yield of VCAM peptide (20.8 mg, 0.012 mmol). MS (ESI): m/z calculated for $C_{73}H_{129}N_{24}O_{23}$, 1710.97 $[M+H]^+$, 855.99 $[M+2H]^{2+}$; observed 1710.97 $[M+H]^+$, 855.94 $[M+2H]^{2+}$.

Bioconjugation of TMV particles

Targeted (VCAM-TMV) and non-targeted (PEG-TMV) rods were synthesized using the following sequence of established reactions[2].

The exterior was labeled with a terminal alkyne by targeting tyrosine 139 residues. Wt-TMV was mixed with 25 molar equivalents (eq) per coat protein (CP) in situ generated diazonium salt

(3-ethynylaniline mixed with sodium nitrite in an acidic solution) in 100 mM borate buffer pH=8.8 for 30 minutes on ice. The reaction was purified via 40% sucrose cushion ultracentrifugation (all sucrose cushion ultracentrifugations were performed at 160,000 xg for 2.5 hours with a 3 mL sucrose cushion).

The resulting exterior alkyne TMV (eAlk-TMV) underwent a copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction by mixing with 1 eq per CP of VCAM azide, 1 mM copper sulfate ($CuSO_4$), 2 mM aminoguanidine (AMG), and 2 mM ascorbic acid (Asc) in 10 mM phosphate buffer pH 7.4 on ice for 30 minutes. The reaction was purified by sucrose cushion ultracentrifugation yielding a pellet of VCAM labeled TMV (eVCAM-TMV).

The interior was then labeled with terminal alkynes by targeting glutamic acids 97 and 106 with a primary amine and EDC. eVCAM-TMV was mixed with propargyl amine (50 eq per CP) with ethyldimethylaminopropylcarbodiimide (EDC, 45 eq per CP), and n-hydroxybenzotriazole (150 eq per CP) in 100 mM HEPES buffer pH=7.4 for 24 hours at room temperature. The resulting product (eVCAMiAlk-TMV) was purified by sucrose cushion ultracentrifugation.

Finally, contrast agents (Gd(DOTA) azide and sulfo-Cy5 azide) were attached to TMV using the CuAAC reaction. eVCAMiAlk-TMV was mixed with 2 eq per CP of Gd(DOTA) azide and 1 eq per CP of sulfo-Cy5 azide in the presence of 1 mM $CuSO_4$, 2 mM AMG, and 2 mM Asc in 10 mM phosphate buffer pH=7.4 for 30 minutes on ice. The reaction was purified by 10-40% (w/v) sucrose gradient ultracentrifugation (made using a Biocomp Instruments Gradient Master) at 96,000 xg for 2 hours. The light scattering region was collected and diluted to fill the centrifuge tube for final ultracentrifugation at 160,000 xg for 2.5 hours to yield the final product eVCAMiCy5Gd-TMV, referred to as VCAM-TMV.

PEG-TMV was synthesized using the same methods but replacing VCAM azide with PEG_{2000} azide.

Modified TMV particles were characterized for labeling efficiency, structural integrity, and magnetic T_1 relaxivity. Labeling efficiency was confirmed using matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS), denaturing gel electrophoresis (SDS-PAGE), induced coupling plasma optical emission spectroscopy (ICP-OES) (for Gd), and UV-Vis absorbance (for Cy5). The particle integrity was confirmed by transmission electron microscopy (TEM) and size exclusion chromatography (SEC). The ionic relaxivity of engineered Gd-loaded TMV particles was tested using a Bruker Minispec mq60 relaxometer.

Size exclusion chromatography (SEC):

All labeled particles were analyzed by SEC using a Superose 6 column on the ÄKTA Explorer chromatography system (GE Healthcare). Samples (100 μg/100 μL) were analyzed at a flow rate of 0.5 mL/min using 0.1 M potassium phosphate buffer (pH 7.4).

Transmission electron microscopy (TEM):

Drops of TMV formulations in DI water were placed on carbon coated copper TEM grids (5 μL, 0.1 mg/mL), allowed to adsorb for 5 minutes, washed with DI water, and negatively stained with 2% (w/v) uranyl acetate for 2 minute. Samples were examined using a Zeiss Libra 200FE transmission electron microscope operated at 200 kV.

UV-visible spectroscopy

UV-visible absorbance measurements were obtained using Thermo Scientific NanoDrop 2000 Spectrophotometer.

Induced coupling plasma optical emission spectroscopy (ICP-OES)

The Gd per TMV ratio was determined using an ICP-OES (Agilent 730 Axial ICP-OES).

Gel electrophoresis:

Denaturing gel electrophoresis was used to analyze protein modifications. Specifically, 10 μg of TMV was loaded on denaturing 4-12% NuPAGE gels (Invitrogen). After separation, the gel stained with Coomassie Blue was photographed using an AlphaImager (Biosciences) imaging system. ImageJ software (rsbweb.nih.gov/ij/, Supporting Information) was used for band analysis and to estimate the number of PEG and VCAM molecules attached.

Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS):

For MALDI-TOF MS analysis, native and modified TMV (24 μL, 10-20 μg, dialyzed in pure H_2O for best results) were denatured by adding guanidine hydrochloride (6 μL, 6 M) and mixing for 5 min at room temperature. Denatured proteins were spotted on MTP 384 massive target plate using Zip-$Tips_{\mu C18}$ (Millipore). MALDI-TOF MS analysis was performed using a Bruker Ultra-Flex I TOF/TOF mass spectrometer.

Relaxivity measurements:

The ionic relaxivity of the Gd(DOTA)-modified TMV was determined using a Bruker Minispec mq60 relaxometer (60 MHz). The Gd concentration was determined using an ICP-OES. Multiple concentrations of TMV were used with a standard inversion recovery sequence protocol to determine the T_1 values.

Animal protocols:

All experiments were carried out using IACUC approved procedures. $ApoE^{-/-}$ (B6.129P2-$ApoE^{tmv1Unc}$/J, 002052) mice were purchased at The Jackson Laboratory. $ApoE^{-/-}$ mice were fed a western diet (1.25% cholesterol, 20% fat, Research Diets Inc.) for 14-18 weeks prior to imaging experiments. For imaging experiments, $ApoE^{-/-}$ mice were injected via the tail vein with virus particles at an amount of 10 mg/kg in 100 µL PBS. Healthy C57BL/6 mice of the same age served as negative controls.

Ex vivo fluorescence imaging

Mice were euthanized and dissected to remove aortas 3 hours post administration of TMV and respective controls. The aortas were fixed in 4% (v/v) paraformaldehyde in 30% (w/v) sucrose overnight at 4°C. After fixation, the aorta was cleaned to remove fatty connective tissue. Next, the cleaned aortas were imaged using a Maestro fluorescence imaging system to detect Cy5 fluorescence signal. Image cubes were obtained with an exposure time of 800 ms per step. The obtained image cubes were background subtracted prior to quantitative image analysis.

Immunofluorescence Imaging

Immediately after *ex vivo* fluorescence imaging, the cleaned aortas were cut into 10-12 2-4 mm long sections and embedded in OCT medium and flash frozen. The frozen samples were cryosectioned to 10 μm sections and mounted on Fisherbrand ColorFrost Plus microscope glass slides for staining. Freshly sectioned aortas were stained for macrophage cell marker CD68 (antibodies purchased from BioLegend) at 250:1 dilution followed by Cy3 labeled secondary antibody labeling (500:1 dilution) for fluorescence detection and mounted using mounting media containing DAPI (Fluoroshield with DAPI, Sigma).

MRI analysis

In vivo MRI scans were performed using a Bruker BioSpin 7.0T 70/30USR MRI system. This system has been outfitted with an RF mouse coil. Mice were anesthetized for all procedures (isoflurane 1.5%;O_2 2.5 L/min) and their respiration, body temperature, and heart rate (ECG) were monitored in real-time. Following multiple scouting scans, a T_1-weighted Multi Slice Multi Echo (MSME) black-blood fat-suppression sequence was optimized to detect the aorta wall with the following parameters: TR/TE=600/8.0 ms, 8 axial slices 1 mm thickness with 1.5 mm slice separation, two averages, matrix = 256x256, field of view = 2.98 cm, acquisition time = 10:14 minutes. Respiration and ECG triggering was applied per slice. Images were taken prior and post TMV administration; formulations were injected while the animal remained in the MRI machine. Sequential scans were performed for up to 150 minutes. Statistical analysis of MRI results was performed using MatLab program to determine the contrast to noise ratio (CNR).

Supporting data

Peptide synthesis Supporting data:

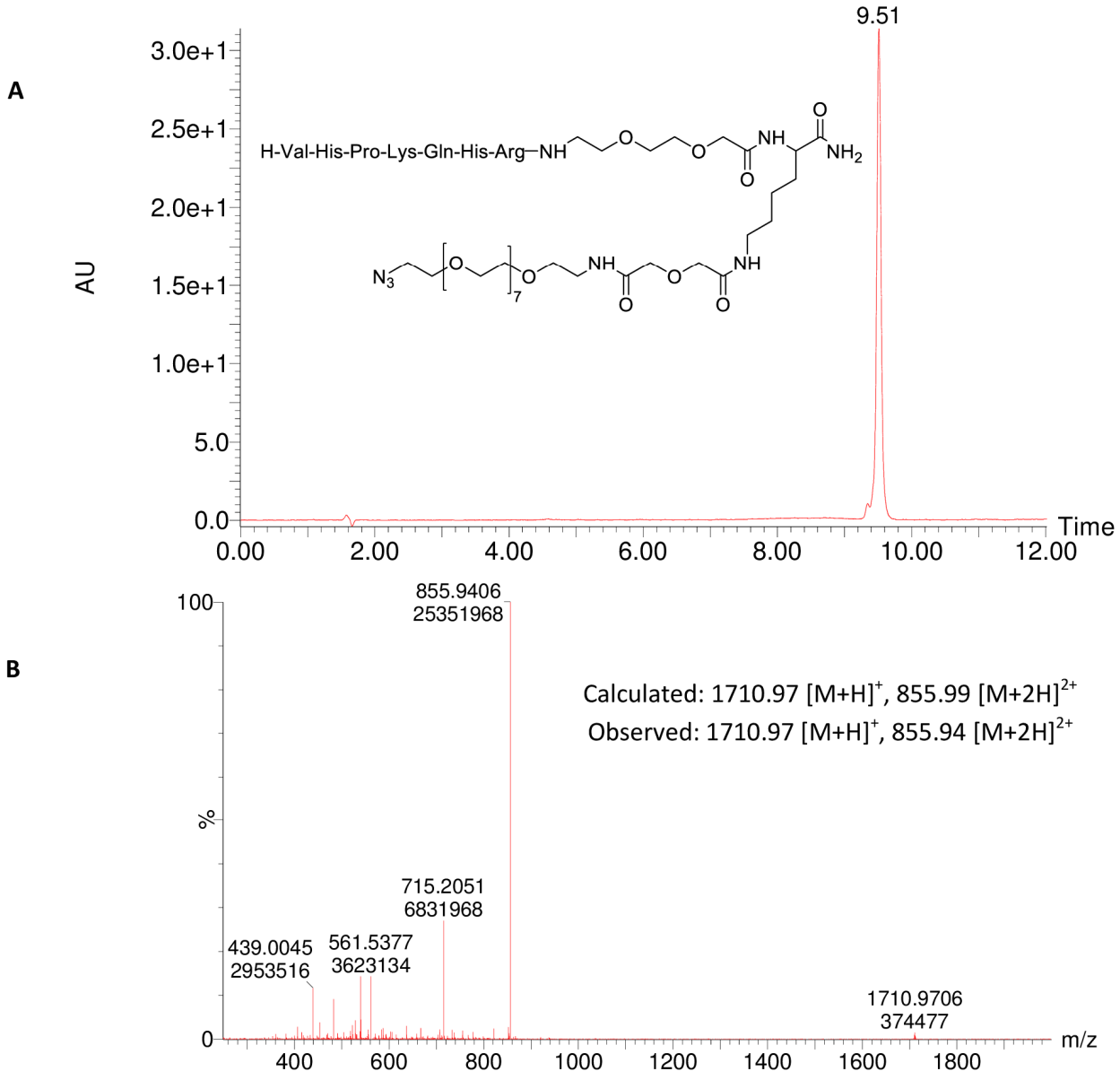

Figure S1 (A) Analytical HPLC trace, UV absorbance detected from 210-800 nm (RP-C18 4.6 x 250 mm, 5 µm), and (B) ESI+-MS of VCAM peptide (chemical structure shown in (A)).

MALDI-TOF MS Supporting data:

Quantitative analysis of VCAM-TMV and PEG-TMV indicated that 25% of the coat proteins (CPs) were modified with either a fluorophore, Gd(DOTA), or PEG/VCAM peptide. While dual modification of the CPs with targeting ligand and contrast agents was not observed; data indicate collective labeling of the TMV rod consisting of a mix of coat proteins labeled with either a Cy5 NIR dye, Gd(DOTA) MR contrast agent, and the VCAM-1 targeting ligand or PEG. Therefore, a mix of CPs is present in each particle formulation; the lack of 100% reactivity in each bioconjugation step opens the probability for multiple combinations of modified CPs as observed by peak broadening in the MALDI TOF MS spectra (Supporting Figures S2+S3). Broadening of spectral peaks obtained from MALDI TOF MS can also be a result of matrix binding, salt binding, low sample concentration, etc.

Analysis of the MALDI-TOF MS spectra is consistent with theoretical peak assignments (Supporting Table T1+T2): The predicted mass of unmodified TMV CP is 17,534 m/z. The step-by-step bioconjugation additions outlined in Figure 1 for VCAM-TMV are:

addition of an exterior alkyne (128 m/z) yielding eAlk-TMV

addition of a VCAM peptide (1,711 m/z) yielding eVCAM-TMV

addition of an interior alkyne (37 m/z) yielding eVCAMiAlk-TMV

addition of the contrast agents Cy5 (778 m/z) and Gd(DOTA) (596 m/z) yielding eVCAMiCy5Gd-TMV or in short VCAM-TMV.

This sequence is also applied for PEG-TMV (PEG MW = 2,000 m/z). The final reaction yielding VCAM-TMV loaded with Gd(DOTA) and Cy5 fluorescent contrast agents has 18 potential CP modification combinations (Supporting Table T1). The mass spectra obtained by MALDI-TOF MS of TMV products after each bioconjugation step leading to VCAM-TMV are shown in Supporting Figure S2; the accompanying Supporting Table T1 shows the theoretical

mass/charge (m/z) for each conjugate and the peak assignments. Similarly, the mass spectra and peak assignments are shown for PEG-TMV (see Supporting Figure S3 and Table T2).

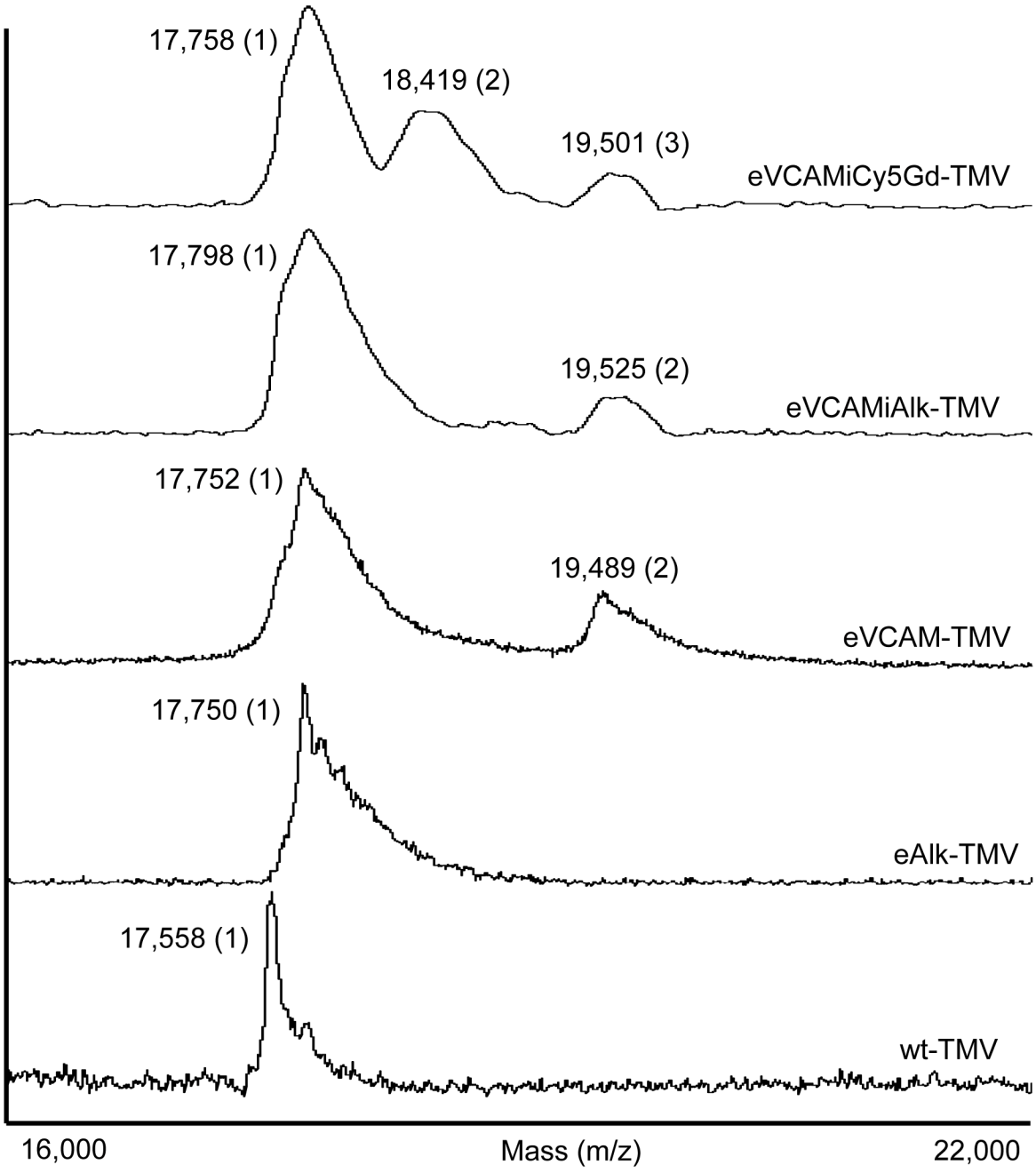

Figure S2: MALDI-TOF MS spectra of TMV product after each bioconjugation step leading up to VCAM-TMV. The peak labels identify m/z values with peak number in parenthesis. **See also Table S1.**

Table S1: Peak assignments for TMV intermediate and final VCAM-TMV (eVCAMiCy5Gd-TMV) products. Tabulated are the measured mass and peak number obtained by MALDI-TOF MS (see Supporting Figure S1) and the peak assignments based on the theoretical mass (predicted m/z).

Sample	**Mass (peak number)**	**Peak assignments (predicted m/z)**
wt-TMV	17,558 (1)	wt (17,534)
eAlk-TMV	17,750 (1)	wt (17,534) eAlk (17,662)
eVCAM-TMV	17,752 (1)	wt (17,534) eAlk (17,662)
	19,489 (2)	eVCAM (19,373)
eVCAMiAlk-TMV	17,798 (1)	wt (17,534) eAlk (17,662) iAlk (17,571) eAlk+iAlk (17,699)
	19,525 (2)	eVCAM (19,373) eVCAM+iAlk (19,410)
eVCAMiCy5Gd-TMV	17,758 (1)	wt (17,534) eAlk (17,662) iAlk (17,571) eAlk+iAlk (17,699)
	18,419 (2)	iGd (18,167) eGd (18,258) e/iGd+e/iAlk (18,295) iCy5 (18,349) eCy5 (18,440) e/iCy5+e/iAlk (18,477)
	19,501 (3)	eVCAM (19,373) eVCAM+iAlk (19,410)

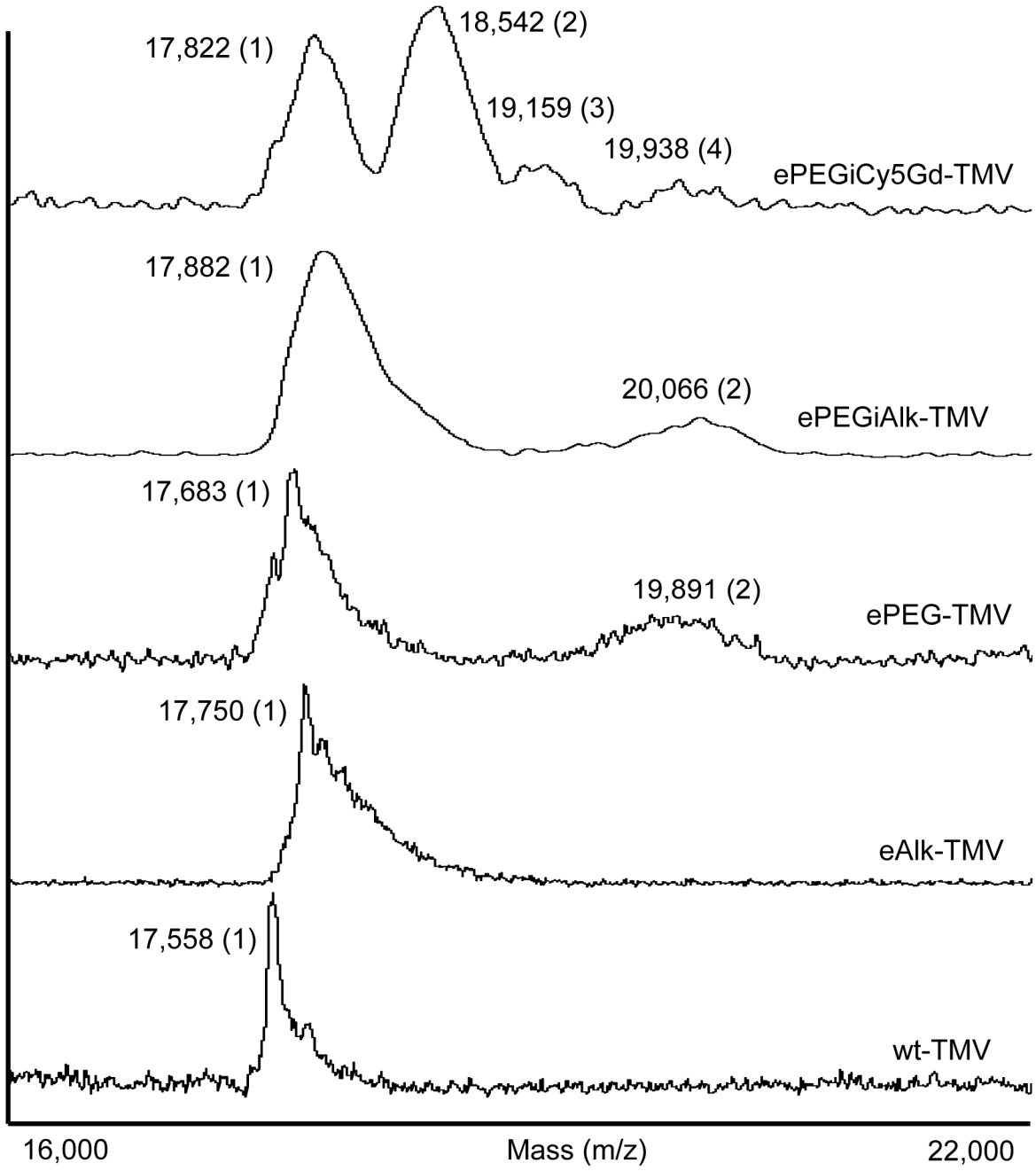

Figure S3 MALDI-TOF MS spectra of the product of each bioconjugation step leading to PEG-TMV. The peak labels identify m/z values with peak number in parenthesis.

Table S2: Peak assignments for TMV intermediate and final PEG-TMV (ePEGiCy5Gd-TMV) products. Tabulated are the measured mass and peak number obtained by MALDI-TOF MS (see Supporting Figure S1) and the peak assignments based on the theoretical mass (predicted m/z).

Sample	Mass (peak number)	Peak assignments (predicted m/z)
wt-TMV	17,558 (1)	wt (17,534)
eAlk-TMV	17,750 (1)	wt (17,534) eAlk (17,662)
ePEG-TMV	17,683 (1)	wt (17,534) eAlk (17,662)
	19,891 (2)	ePEG (19,662)
ePEGiAlk-TMV	17,882 (1)	wt (17,534) eAlk (17,662) iAlk (17,571) eAlk+iAlk (17,699)
	20,066 (2)	ePEG (19,662) ePEG+iAlk (19,699)
ePEGiCy5Gd-TMV	17,822 (1)	wt (17,534) eAlk (17,662) iAlk (17,571) eAlk+iAlk (17,699)
	18,542 (2)	iGd (18,167) eGd (18,258) e/iGd+e/iAlk (18,295) iCy5 (18,349) eCy5 (18,440) e/iCy5+e/iAlk (18,477)
	19,159 (3)	e/iGd+e/iGd (18,891) e/iGd+e/iCy5 (19,073) e/iCy5+e/iCy5 (19,255)
	19,938 (4)	ePEG (19,662) ePEG+iAlk (19,699)

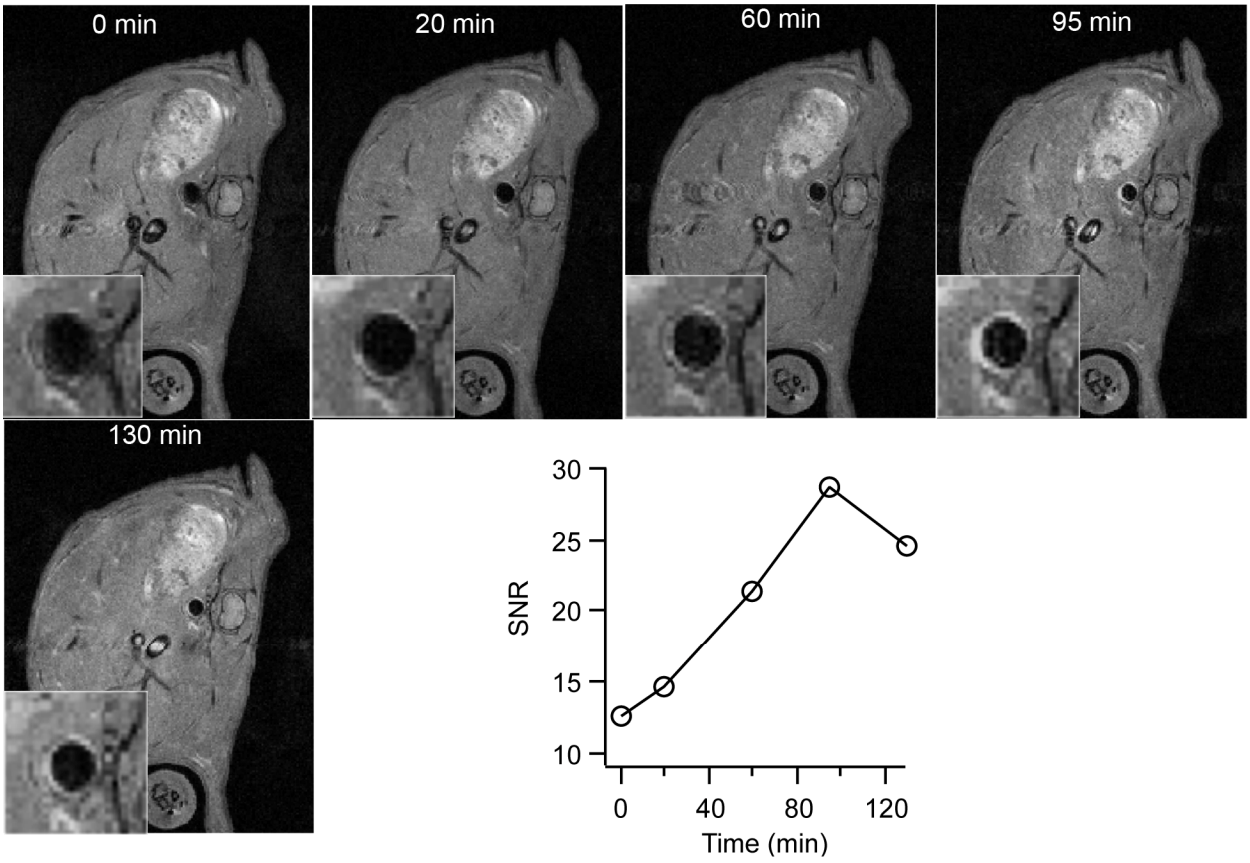

Figure S4 MRI scans of ApoE-/- mice pre- and post-injection with VCAM-TMV. Insets are magnified images of the abdominal aorta regions of interest. Shown is a signal to noise ratio (SNR) graph against time for the aorta vessel wall; data were analyzed using image analysis and Matlab software.

References

1) Boedtker, H.; Simmons, N. S. *Journal of the American Chemical Society* **1958,** 80, (10), 2550-2556.
2) Bruckman, M. A.; Hern, S.; Jiang, K.; Flask, C. A.; Yu, X.; Steinmetz, N. F. *J Mater Chem B Mater Biol Med* **2013,** 1, (10), 1482-1490.