

Supporting Information

Silica-coated Gd(DOTA)-loaded protein nanoparticles enable magnetic resonance imaging of macrophages

Michael A. Bruckman^{#a}, Lauren N. Randolph^{#a}, Neetu M. Gulati^b, Phoebe L. Stewart^b, Nicole F. Steinmetz^{a,c,d,e*}

^aDepartment of Biomedical Engineering, ^bDepartment of Pharmacology and Cleveland Center for Membrane and Structural Biology, ^cDepartment of Radiology, ^dDepartment of Materials Science and Engineering, ^eDepartment of Macromolecular Science and Engineering Case Western Reserve University, Cleveland, OH

Authors contributed equally.

* Corresponding author: nicole.steinmetz@case.edu

TMV manufacturing

Propagation

The TMV lysine mutant S152K was propagated in *Nicotiana benthamiana* plants and recovered, with a yield of 5 mg TMV per gram infected leaf material, using established extraction methods.¹ The concentration of TMV in plant extracts was determined by UV/vis spectroscopy ($\epsilon_{260\text{nm}} = 3.0 \text{ mg}^{-1} \text{ mL cm}^{-1}$) and virus integrity was verified by TEM and SEM imaging.

Bioconjugation

Gd(DOTA)-labeled particles were prepared as previously described.² Briefly, the TMV external surface was modified with a diazonium salt generated from 3-ethynylaniline (25 molar equivalents (eq), pH = 8.5, 30 min) to incorporate a terminal alkyne. Similarly, the internal channel was modified in the same way by mixing propargylamine (50 eq) with ethyldimethylpropylcarbodiimide (EDC, 100 eq) and *n*-hydroxybenzotriazole (HOBt, 50 eq) for 24 h. Gd was chelated to azido-mono amide-1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA-azide, Macrocylics) as previously described.² Briefly, a 1:1 mixture of GdCl₃ and DOTA-azide in water was mixed at room temperature for 3 days while maintaining the pH at 6-7 (tested using pH paper). After 3 days, the pH was increased to 9-10 and the precipitate was removed by centrifugation. Efficient conjugation of Gd(DOTA) azide to TMV terminal alkyne groups was accomplished by copper-catalyzed azide-alkyne cycloaddition (CuAAC) to form TMV particles with Gd-conjugated externally (eGd-TMV) or internally (iGd-TMV). Alkyne-labeled TMV (2 mg/mL) in 0.1 M potassium phosphate buffer (pH 7.0) was mixed with Gd(DOTA) azide (2 eq), aminoguanidine (2 mM), ascorbic acid (2 mM) and copper sulfate (1 mM) for 30 min on ice. The reaction mix was purified by ultracentrifugation in a 10-40% sucrose gradient, and

analyzed by TEM and MALDI-TOF MS. For flow cytometry experiments, sulfo-Cy5 azide (Lumiprobe) was used in place of Gd(DOTA) azide to synthesize internally-labeled Cy5-TMV.

Thermal transition to SNP

The standard protocol for thermal transition from native TMV rods to SNPs involves heating a sample of TMV rods (0.3 mg mL^{-1}) for 60 s at 96°C in a Peltier thermal cycler. SNPs are then recovered by centrifugation at 42,000 rpm for 2 h.

Mineralization

We added 1 mL of TMV or SNP particles (1 mg mL^{-1}) to 18 mL ethanol on ice and mixed in 1 mL TEOS (10% (v/v) in ethanol) and 4 mL 5 M NH_4OH , alternating every 15 min for 1 h. The reaction was incubated at 4°C for 18 h followed by centrifugation at 3000 rpm for 20 min. The samples were washed with water and centrifuged again followed by overnight dialysis against water.

Particle characterization

Inductively-coupled plasma optical emission spectroscopy (ICP-OES)

The Gd loading of modified TMVs or SNPs before and after mineralization was determined by ICP-OES. Samples were diluted to give a protein concentration of 0.1 mg mL^{-1} in pure water and analyzed immediately.

Relaxivity

The ionic relaxivity of the Gd(DOTA)-loaded TMVs and SNPs was tested using a Bruker Minispec mq60 relaxometer at 60 MHz. A standard inversion recovery sequence was used to determine the T_1 .

Transmission electron microscopy (TEM)

Drops of TMV rods or SNPs before and after mineralization (0.2 mg mL^{-1} in 5 mL deionized water) were placed on copper TEM grids, adsorbed for 5 min, washed with deionized water, and negatively stained with 2% (w/v) uranyl acetate for 2 min. Samples were examined by energy dispersive X-ray spectroscopy using a Zeiss Libra 200FE transmission electron microscope operated at 200 kV.

Scanning electron microscopy (SEM)

Samples were dried onto silicon wafers and then mounted on the surface of an aluminum pin stub using double-sided adhesive carbon discs (Agar Scientific). The stubs were then sputter-coated with gold (or palladium) in a high-resolution sputter coater (Agar Scientific, Ltd.) and transferred to a Hitachi 4500 scanning electron microscope.

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

Native and modified TMV particles (10–20 mg in 24 μL water) were denatured by adding 6 μL 6 M guanidine hydrochloride and mixing for 5 min at room temperature. Denatured proteins were spotted onto an MTP 384 massive target plate using mC18 Zip-Tips (Millipore). MALDI-MS analysis was carried out using a Bruker Ultra-Flex I TOF/TOF mass spectrometer.

Particle testing *in vitro*

Cell culture

RAW 264.7 cells (ATCC) were maintained in Dubelco's minimal essential medium (DMEM) at 37°C in a 5% CO₂ humidified atmosphere. The medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) L-glutamine, and 1% (v/v) penicillin–streptomycin. All reagents were obtained from Gibco.

Flow cytometry

RAW 264.7 cells (500,000 cells in 200 µL DMEM per well) were added to an untreated 96-well v-bottom plate. The iGd-TMV, Gd-SNP, iGd-TMV-Si, and Gd-SNP-Si particles were added at a concentration of 100,000 particles/cell in triplicate and incubated for 1, 3, and 8 h at 37°C in a 5% CO₂ humidified atmosphere. Following incubation, the cells were pelleted at 500 x g for 4 min. The supernatant was removed, and the cells were resuspended in FACS buffer (1 mM EDTA, 1% (v/v) FBS, and 25 mM HEPES, pH 7.0 in Ca²⁺ and Mg²⁺ free PBS). This washing step was carried out three times. The cells were then fixed in 2% (v/v) paraformaldehyde in FACS buffer for 10 min at room temperature and washed another three times. Analysis was carried out using the BD LSR II flow cytometer, and 10,000 events per sample were collected.

Magnetic resonance imaging (MRI) of cell pellets

RAW cells (5 x 10⁶ cells in 1 mL DMEM per tube) were added to untreated 1.5 mL Eppendorf tubes. The iGd-TMV, Gd-SNP, iGd-TMV-Si, and Gd-SNP-Si particles were added at a concentration of 1 x 10⁶ particles/cell and incubated for 8 h at 37°C in a 5% CO₂ humidified atmosphere. Following

incubation, the cells were pelleted at 500 x g for 4 min. The supernatant was removed, and the cells were resuspended in FACS buffer. This washing step was carried out three times. The cells were then fixed in 2% (v/v) paraformaldehyde in FACS buffer for 10 min at room temperature and washed another three times. Cells were pelleted in a custom 384 well plate and analysis was carried out using a pre-clinical 7.0T (300 MHz) MRI (Bruker BioSpec 70/30USR). Following multiple scouting scans, a T₁-weighted Multi Slice Multi Echo (MSME) sequence was used with the following parameters: TR/TE=600/8.0 ms, 1 mm thickness, four averages, matrix = 128 x 128, field of view = 2.98 cm. Exported DICOM images were analyzed with the free open software OsiriX.

Contrast-to-noise (CNR) calculations

The contrast-to-noise ratio was determined by dividing the mean intensity of the cell pellet area over the mean intensity of the buffer area.

Immunogold labeling

TMV samples were dried on TEM grids, washed with 10 mM sodium phosphate buffer pH 7.0 and floated on a drop of 1% (w/v) bovine serum albumin (BSA) in Tris-buffered saline pH 7.4 plus 0.1% (v/v) Tween-20 (TBST) for 30 min. Samples were equilibrated with 0.1% BSA for 5 min before binding for 1 h with a rabbit anti-TMV antibody (10 µg mL⁻¹ in 0.1% BSA). The grids were then washed three times with 0.1% (w/v) BSA before binding with goat anti-rabbit secondary antibodies conjugated to 10-nm gold nanoparticles for 2 h. The grids were then washed in phosphate-buffered saline pH 7.4 plus 0.01% (v/v) Tween-20 (PBST), then water, prior to staining with 2% (v/v) uranyl acetate for 1 min. The grids were imaged by TEM.

Supporting Data

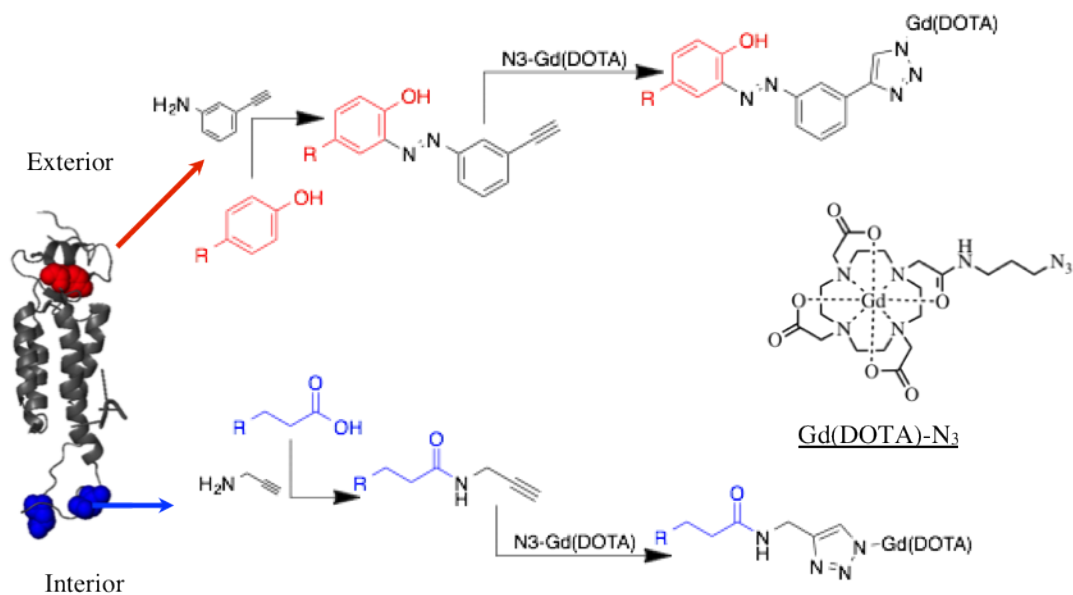


Figure S1 Schematic diagram showing the bioconjugation of TMV rods.

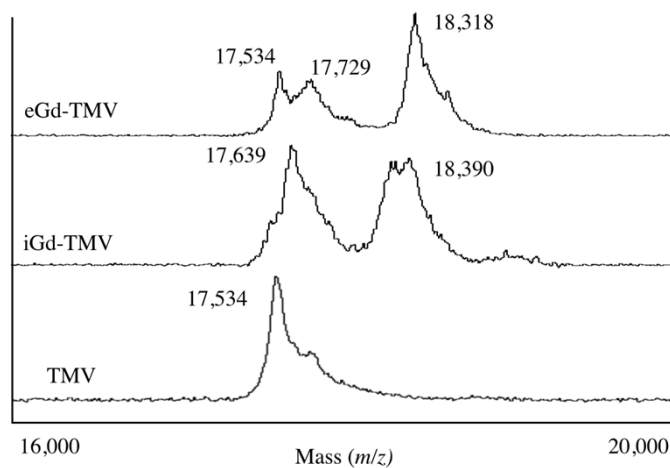


Figure S2 MALDI-TOF MS spectra for modified TMV particles. Peak assignments: native TMV = 17,534 m/z ; iGd-TMV = 17,639 m/z (alkyne-modified) and 18,390 m/z (Gd(DOTA) modified); eGd-TMV = 17,534 m/z (unmodified), 17,729 m/z (alkyne modified), and 18,318 m/z (Gd(DOTA) modified).

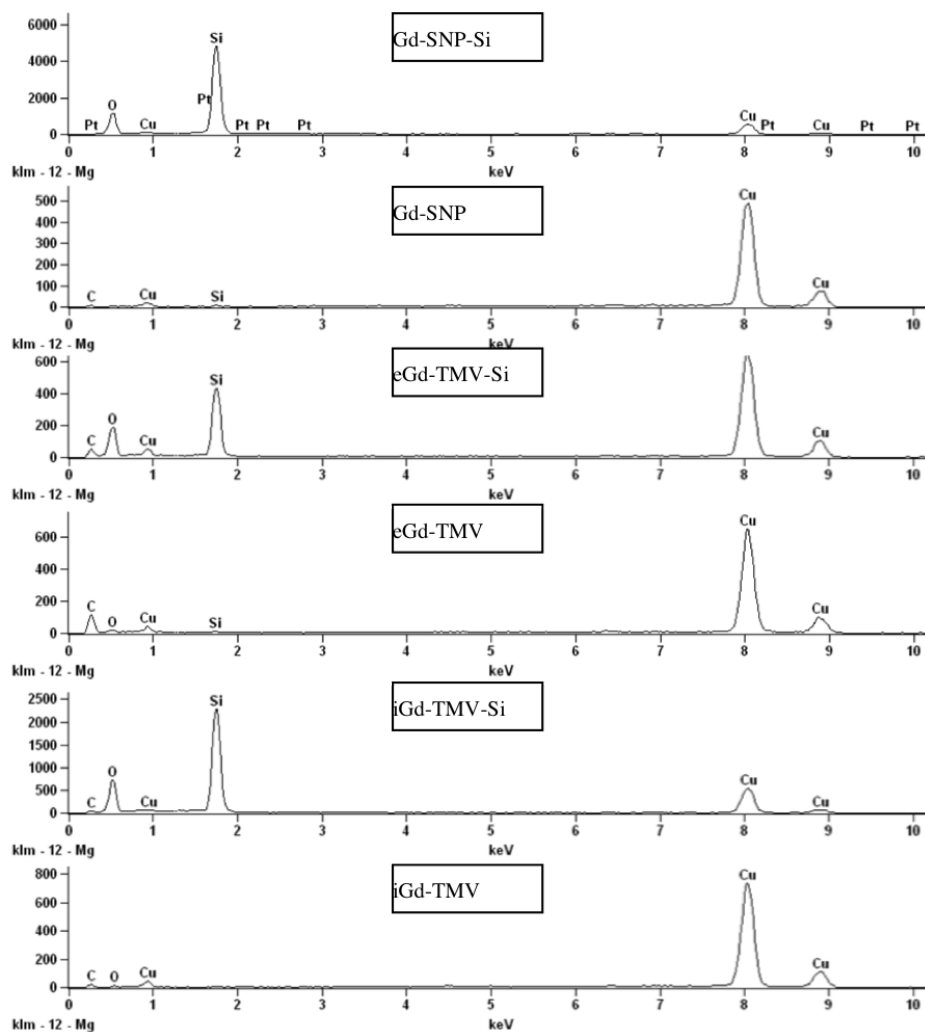


Figure S3 Electron dispersion spectra of TMV before and after mineralization confirming the presence of silica after mineralization.

Sample	Gd per NP	Relaxivity per Gd	Relaxivity per particle
0.5 eq Gd	429	13.2	5,640
1 eq Gd	643	12.5	8,019
2 eq Gd	846	11.7	9,901
4 eq Gd	1,265	10.6	13,406
8 eq Gd	1,477	10.1	14,922

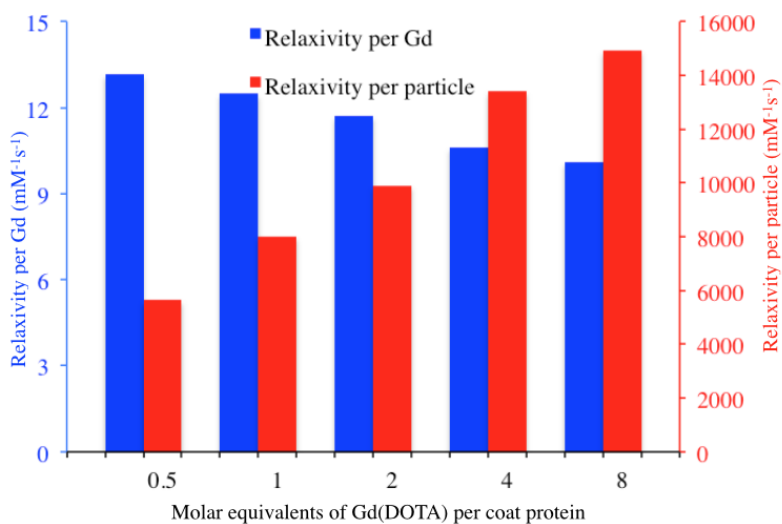


Figure S4 Table and graph showing the relaxivity of TMV rods loaded with varying amounts of Gd(DOTA), where eq = molar equivalents. Measurements at 60 MHz. Relaxivity values in mM⁻¹ s⁻¹.

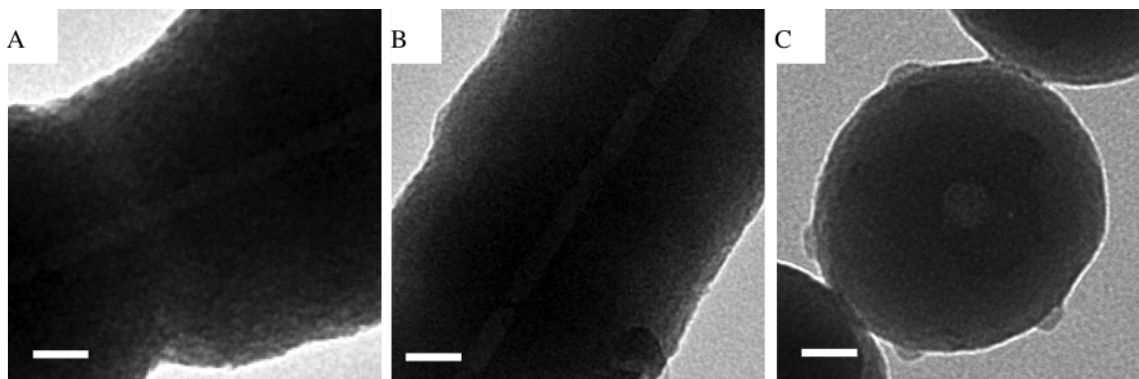


Figure S5 High-resolution TEM images of (A) iGd-TMV-Si, (B) eGd-TMV-Si, and (C) Gd-SNP-Si showing dense silica coat. Scale bar = 25 nm.

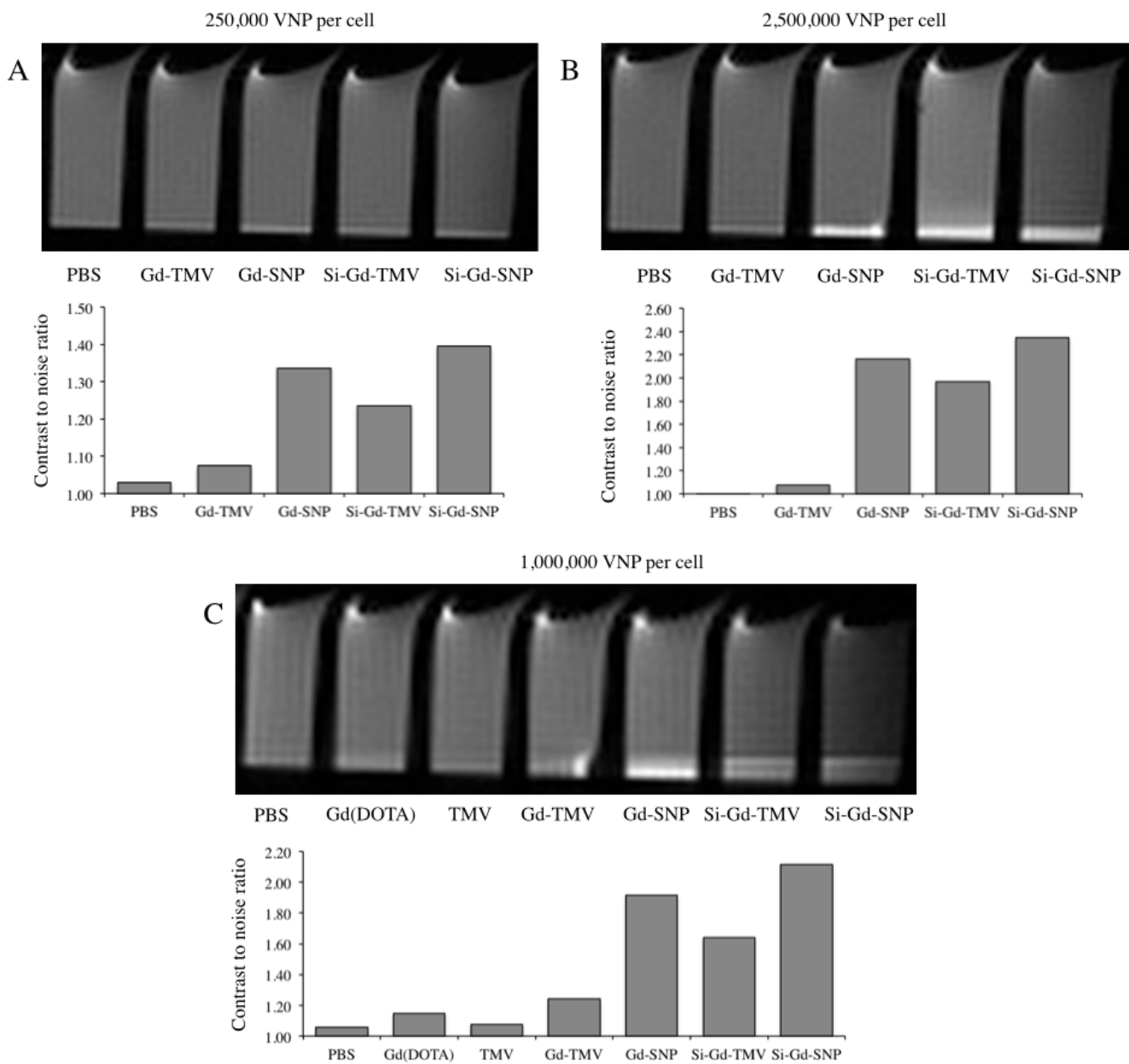


Figure S6 Top panels: MRI phantom images of RAW 264.7 cell pellets 8 h after binding with Gd-TMV, Gd-SNP, Si-Gd-TMV and Si-Gd-SNP. Gd(DOTA)-labeled TMV and SNP formulations were incubated with RAW 264.7 cells for 8 h, then cells were washed and pelleted prior to obtaining MRI images using a 7.0T (300 MHz) MRI (Bruker BioSpec 70/30USR). In A and B, 1,000,000 cells were incubated with (A) 250,000 VNP per cell and (B) 2,500,000 VNP per cell. In panel C, 5,000,000 cells were incubated

with 1,000,000 VNP per cell. Bottom panels: Cell interactions were quantified by contrast-to-noise (CNR) ratio of the MRI phantom image (cell pellets vs. medium).

References:

1. M. A. Bruckman and N. F. Steinmetz, *Methods in molecular biology*, 2014, **1108**, 173-185.
2. M. A. Bruckman, S. Hern, K. Jiang, C. A. Flask, X. Yu and N. F. Steinmetz, *Journal of Materials Chemistry B*, 2013, **1**, 1482.