

Protease-Triggered Unveiling of Bioactive Nanoparticles

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SUPPORTING INFORMATION

Detailed Methods

Unless otherwise stated all reagents were purchased from Sigma-Aldrich and all reactions were performed at room temperature.

Nanoparticle Synthesis: Superparamagnetic iron oxide nanoparticles were synthesized according to published protocols¹. Briefly, dextran-coated iron oxide nanoparticles were synthesized, purified, and subsequently cross-linked using epichlorohydrin. After exhaustive dialysis, particles were aminated by adding 1:5 v/v ammonium hydroxide (30%) and incubated on a shaker overnight. Aminated nanoparticles were subsequently purified from excess ammonia using a Sephadex G-50 column and concentrated using a high-gradient magnetic-field filtration column (Miltenyi Biotec, Auburn, CA). Amine-functionalized particles were labeled with the NHS ester NIR fluorochrome, VivoTag 680 (VisEn Medical, Woburn, MA), by adding 1:20 w/w and incubating on a shaker for one hour. Excess dye was removed by filtration on a Sephadex G-50 column. The particle molarity was determined by the viscosity/light scattering method².

Peptide-PEG Synthesis: Peptides were synthesized in the MIT Biopolymers core to contain sequentially, an amino terminus for PEG attachment, a TAMRA-labeled lysine, an MMP-cleavage sequence, and a cysteine at the carboxyl terminus for particle attachment. The purity of the cleavable MMP2 substrate (NH₂-G-K(TAMRA)-G-P-L-G-V-R-G-C-CONH₂) and the non-cleavable D amino

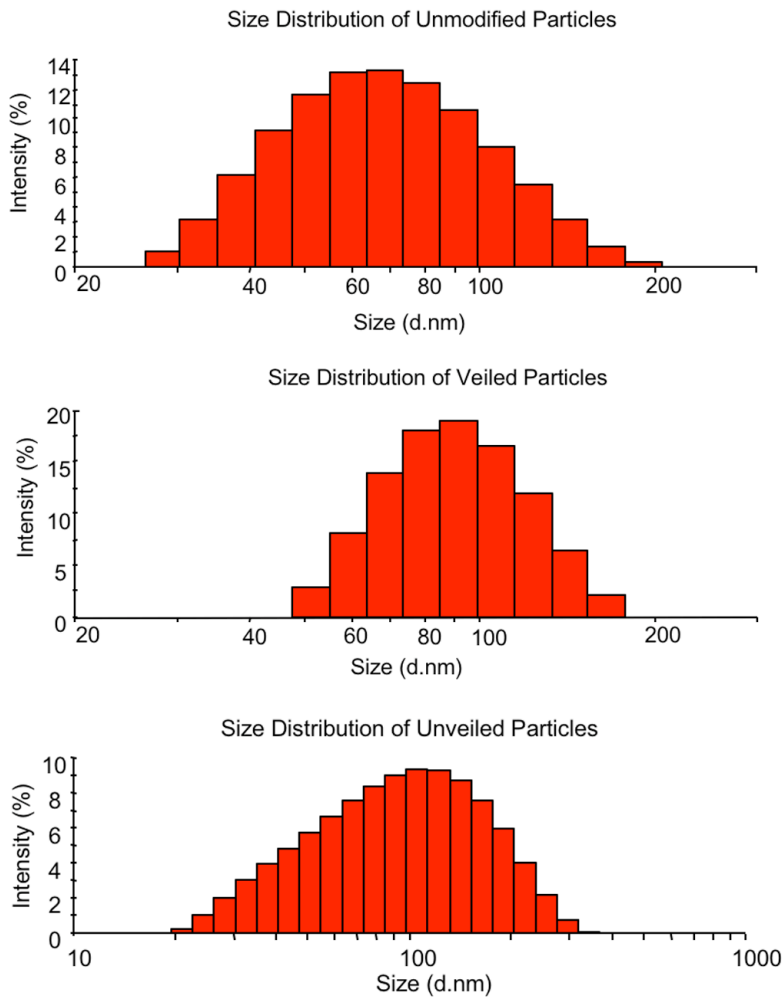
acid analogue (NH₂-G-K(TAMRA)-G-dP-dL-G-dV-dR-G-C-CONH₂) was verified with HPLC and mass spectrometry. Amine-reactive 10 kDa mPEG-SMB reagents (methoxy-polyethylene glycol-succinimidyl α methylbutanoate) were purchased from Nektar Therapeutics. Peptides were reacted with polymers in PBS + 0.005 M EDTA pH 7.2 at 500 μ M and 400 μ M, respectively, for >24 hours with shaking. Free peptide was removed by reducing with 0.1 M TCEP and filtered using a G-50 Sephadex column. The reduced polymer was then quantified using fluorochrome extinction and added to nanoparticle preparations as described below.

Ligand Attachment to Nanoparticles: The attachment of peptide-PEGs to nanoparticles was performed simultaneously with attachment of cell internalizing peptides (NH₂-RRRRGRRRRK(FITC)GC, MIT Biopolymers). The internalizing peptide purity was verified by HPLC and mass spectrometry and its concentration was quantified using the molar extinction coefficient of FITC. Aminated nanoparticles (1.3 mg Fe/ml) were reacted with N-succinimidyl iodoacetate (11 mM) in 0.1 M HEPES 0.15 M NaCl pH 7.2 (HEPES buffer) for 3 hours and filtered using a G-50 Sephadex column into phosphate buffered saline + 0.005 M EDTA pH 7.2 (PBS-EDTA buffer). Purified nanoparticles (0.06 mg Fe/ml) were then combined with stock solutions of reduced peptide-PEG (60 μ M) in PBS-EDTA buffer and internalizing peptide (serial dilutions of 63 μ M, 50.4 μ M, 37.8 μ M, 25.2 μ M, 12.6 μ M, & 0 μ M) in 0.1% TFA at 1:3 and 1:0.1 v/v respectively. The stock concentration selected for the optimized particle was 25.2 μ M. The number of ligands per particle was determined spectrophotometrically using a pre-determined extinction coefficient for iron nanoparticles, FITC-labeled internalizing peptide, and TAMRA-labeled peptide PEG at 400 nm, 495 nm and 555 nm respectively. The optimized particle was determined to have 16 VT 680 dyes, 6 internalizing peptides, and 60 peptide-PEGs.

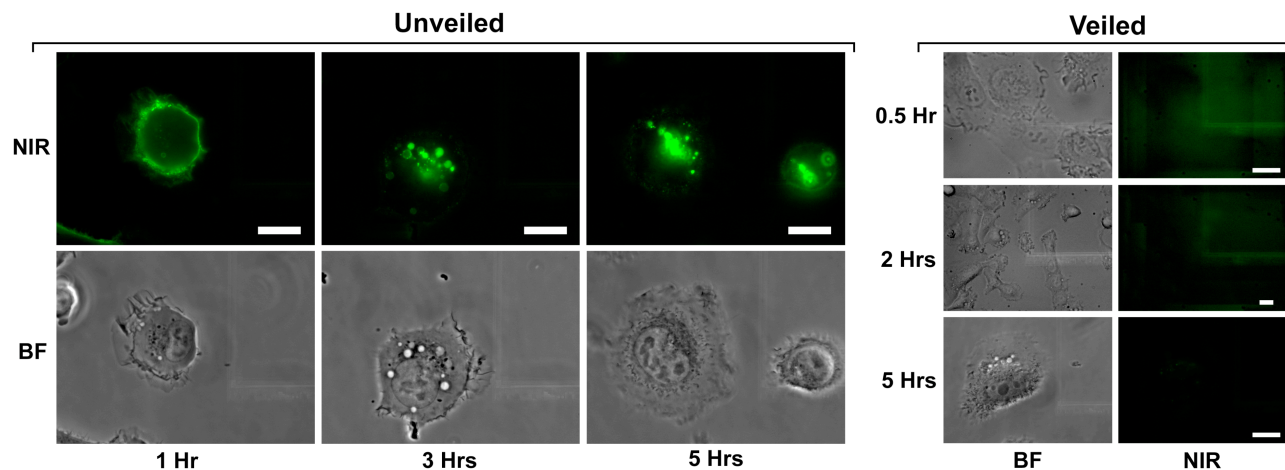
Dynamic Light Scattering: DLS measurements for unmodified and veiled particles were performed in aqueous buffer with 0.1 M HEPES 0.15 M NaCl at 25°C using a Malvern ZS90. Measurements on

unveiled particles were performed in the solution used for activation after incubating with MMPs for 3 hours at room temperature.

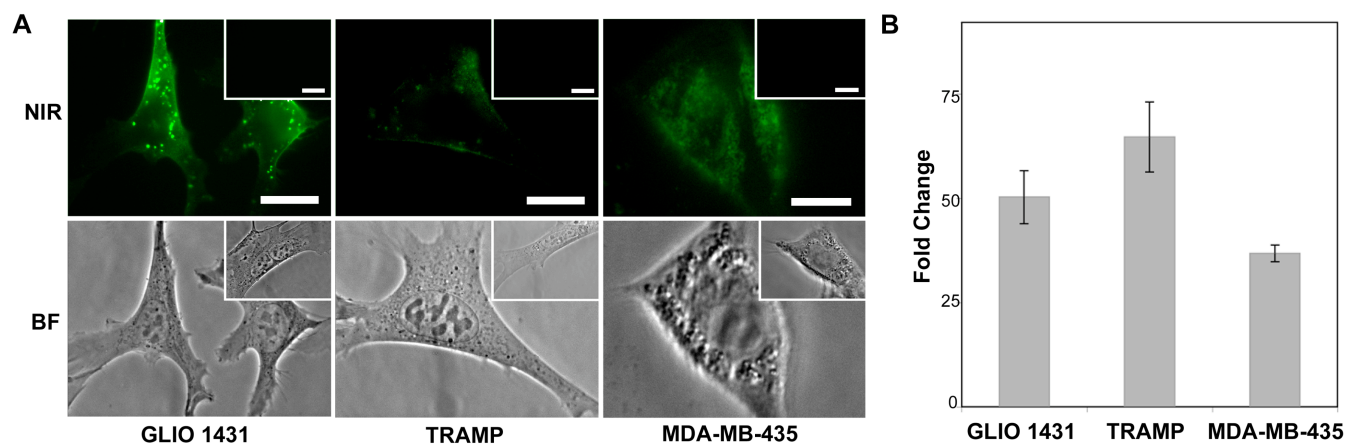
Flow Cytometry and Microscopy: GLIO 1431 (obtained from Al Charest at Tuft's University), TRAMP (obtained from Jianzhu Chen at M.I.T), and MDA-MB-435 (obtained from Erkki Ruoslahti at the Burnham Institute) were cultured in DMEM media with 10% FCS and 1% P/S and grown to 80% confluency. Veiled and MMP-activated (unveiled) nanoparticles (100 μ l at 0.1 mg/ml Fe) were added to 400 μ l cell culture media with 25 μ M Galardin and incubated over cells for various times. For flow cytometry studies, adherent cells were detached from the tissue culture plate with 0.25% trypsin, washed in PBS, and analyzed on a Beckman Dickson LSR II using a 633 nm excitation source and a 690/40 band pass filter to detect VT-680 labeled nanoparticles in cells. Microscopy was conducted on live cells in glass bottom wells using a 100x objective and a cy5.5 filter cube (Chroma).



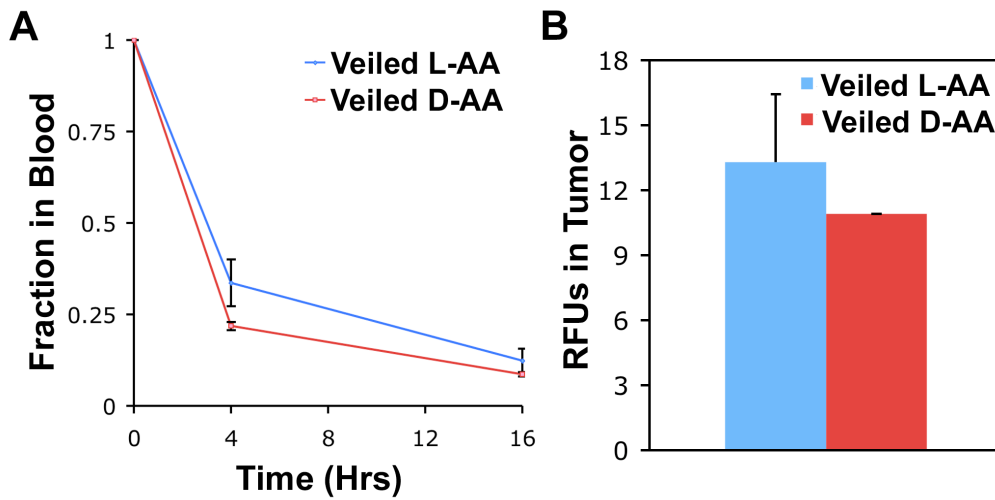
Supplementary Figure 1. Size distribution intensity profiles by dynamic light scattering show unmodified particles with a Z-average of 63.4 nm and a PDI of 0.146. After modification with bioactive peptides and removable polymer, the particle size increases to a Z-average of 85.3 nm and a PDI of 0.08. Unveiled particles do not increase in size having a Z-average size of 84.3 and a PDI of 0.316.



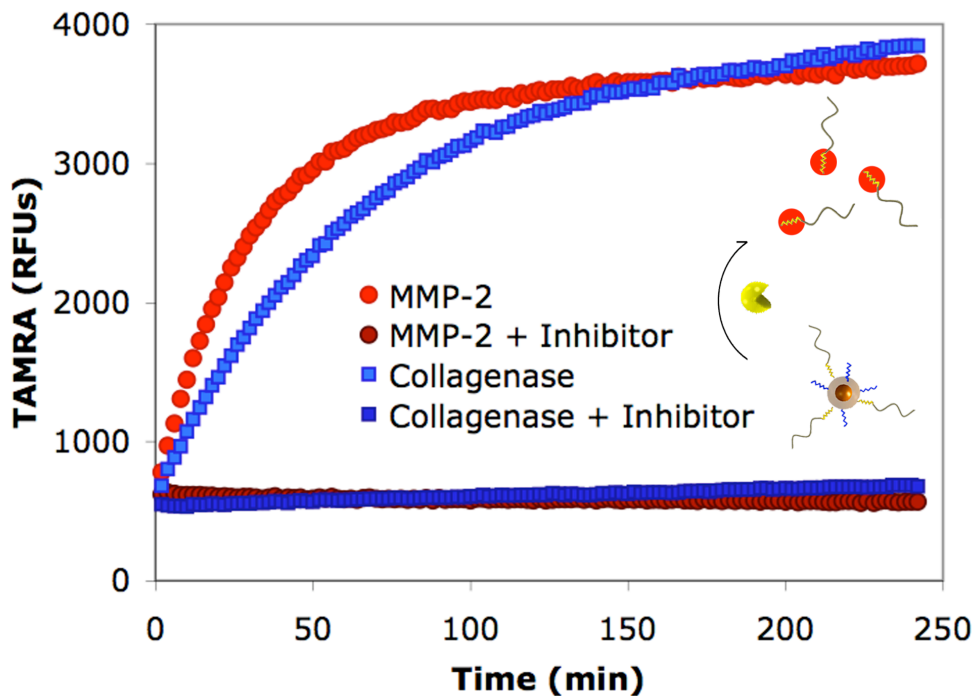
Supplementary Figure 2. Trafficking of unveiled nanoparticles by epifluorescence microscopy. MMP-activated (unveiled) nanoparticles incubated over HT-1080 cells are imaged at 1, 3, and 5 hrs. At 1 hr particles can be seen lining the cell membrane; over longer time points particles appear punctate in intracellular organelles that traffic to the nucleus. Internalization of veiled particles is not visible. Scale bar is 75 μm .



Supplementary Figure 3. Unveiling of nanoparticles initiates cell-uptake in other cell lines. A) MMP-activated (unveiled) nanoparticles internalize in brain (GLIO 1431), prostate (TRAMP), and breast (MDA-MB-435) cancer cell-culture models. Internalization of veiled nanoparticles is not visible (insert). Scale bar is 50 μm . B) Fold increase in mean internalization of unveiled over veiled nanoparticles after incubation for 5 hrs as measured by flow cytometry. Error bars are standard deviations of three separate experiments.



Supplementary Figure 4. Blood clearance and tumor accumulation of nanoparticles bearing MMP cleavable (L-AA) and non-cleavable (D-AA) polymer coatings. A) Nanoparticles bearing cleavable polymer coatings have similar circulation times to non-cleavable controls, suggesting that the cleavable polymer remains intact in the blood. B) Analysis of nanoparticle accumulation in the tumor at 48 hrs by FMT demonstrates similar accumulation of cleavable particles compared to non-cleavable controls. The FMT analysis returned integer values, which were identical for all three animals injected with D-AA particles.



Supplementary Figure 5. Recombinant MMP-2 (2.5 µg/ml) or collagenase- (20 µg/ml) removes peptide-PEG and relieves TAMRA-iron quenching interactions enabling monitoring of protease activation. Incubation with the broad-spectrum inhibitor, Galardin (25 µM, Biomol), inhibits activation by both enzyme formulations.

1. Palmacci, S.; Josephson, L. *U.S. Patent Vol. 5* **1993**, p. 176.
2. Reynolds, F.; O'Loughlin, T.; Weissleder, R.; Josephson, L. *Analytical Chemistry* **2005**, *77*, (3), 814-817.