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ADVANCED MATERIALS

Supporting Information

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Kinetically Assembled Nanoparticles of Bioactive Macromolecules Exhibit Enhanced Stability and Cell-Targeted Biological Efficacy

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Experimental Section

Materials: All chemicals/materials were purchased from Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA) and used as received unless otherwise noted. Bioactive amphiphilic macromolecules (AMs), termed **AM 1** and **AM 2**, and mucic acid modified with lauroyl chloride (**M12**) were prepared as previously described.[1, 2] D,L-Lactide was recrystallized three times from distilled ethyl acetate. Poly(ethylene glycol)-*block*-poly(lactic acid) (PEG-*b*-PLA; M_n of each block = 5 kDa) was a generous gift prepared and provided by Dr. Howard Bowman of Surmodics Pharmaceuticals (Birmingham, AL). Heterobifunctional PEG (M_n = 5000 g mol^{-1}, PDI =1.11), having primary amine protection with *tert*-butyloxycarbonyl at one terminus and a free primary amine at the other (*t*-Boc-NH_2-PEG_{114}-NH_2), was purchased from JenKem Technology (Beijing, China). The red hydrophobic fluorophore 2,2,10,10-Tetraethyl-6,14-bis-(triisopropylsilylethynyl)-1,3,9,11-tetraoxa-dicyclopenta[*b*,*m*]pentacene (EtTP-5) was a generous gift provided by Prof. John Anthony from the University of Kentucky, Department of Chemistry (Lexington, KY). Deionized (DI) water with a resistivity of 18 MΩ·cm was obtained using Milli-Q Water System (Millipore, Billerica, MA). Macrophage colony stimulating factor (M-CSF) was purchased from PeproTech (Rocky Hill, NJ). Primary Human Coronary Artery Smooth Muscle Cells (CASMC) and Primary Human Coronary Artery Endothelial Cells (HCAEC) were purchased from Lonza (Walkersville, MD). Human embryonic kidney cells stably transfected with human Scavenger Receptor A1 (HEK-SRA) were a generous gift from Dr. Steven R. Post from the University of Kentucky (Lexington, KY).

PLA Homopolymer Synthesis: PLA homopolymer (M_n = 10,600 g mol^{-1}, PDI = 1.02) was synthesized according to literature procedure.[3] Briefly, all glassware was dried prior to use and reactions were carried out under dry argon. D,L-Lactide (9.90 g, 69 mmol) was dried under vacuum for 3 h, and then dissolved in 80 mL anhydrous toluene (0.86 M) under argon at 72 °C, using an oil bath. Dry *n*-octanol (95 mg, 0.73 mmol) was added to the lactide solution and stirred for 30 min. Previously distilled tin(II) 2-ethylhexanoate (294 mg, 0.73 mmol) was dissolved in 2 mL of dry toluene and added slowly to the above solution. The ring-opening polymerization was allowed to react for 60 h at 70 °C and subsequently cooled to room temperature. Toluene was removed via rotary evaporation at 70 °C and 18 mL of dichloromethane (DCM) was added to dissolve the PLA product. PLA was purified by first precipitating into 200 mL cold hexane/ethanol (4/1, v/v) and washed twice with the cold hexane/ethanol mixture. Solvent was removed via rotary evaporation and the product was dried *in vacuo*. The product was then redissolved in 16 mL of DCM and precipitated into 120 mL cold methanol followed by washing with cold methanol. This process was repeated in triplicate, methanol was removed via rotary evaporation and the resulting PLA homopolymer (white solid) was dried *in vacuo* (7.20 g, 72% yield).

Molecular weight characteristics and PDI were determined via size exclusion chromatography (SEC) utilizing a Waters® 515 HPLC Pump, 717 plus Autosampler, 486 Tunable Absorbance Detector, and 410 Differential Refractometer and dimethylformamide (DMF) as eluent.

Fluorescent AM Synthesis: Amine terminal, bioactive AM, specifically **AM 1**, was fluorescently labeled with 5(6)-carboxyfluorescein *N*-hydroxysuccinimide ester. First, **M12** (280 mg, 0.30 mmol) was added to a round bottomed flask and dissolved in 14 mL of a DCM:DMF (5:2) solvent mixture. *t*-Boc-NH_2-PEG_{114}-NH_2 (500 mg, 0.10 mmol) was dissolved in 4mL of DCM and subsequently added to the **M12** solution. Then 320 µL of a 1M *N*,*N*′-dicyclohexylcarbodiimide (DCC) (0.32 mmol) solution in DCM was slowly added to the above reaction solution and triethylamine (TEA; 0.15 mmol) was added as a catalyst. This reaction was allowed to proceed for 48 h at room temperature. After 48 h, the reaction mixture was diluted with 20 ml DCM and washed

once with 0.1N HCl and twice with brine. The organic layer was dried over $MgSO_4$ for 30 min and isolated by vacuum filtration. DCM was removed from the filtrate via rotary evaporation and the resulting oil was diluted in ~ 2 mL of DCM. The product was precipitated into cold diethyl ether and collected by centrifugation. The resulting white solid was washed 4 times with diethyl ether and dried *in vacuo*. PEGylation of the **M12** functionality was confirmed by ^{1}H NMR utilizing a Varian 400 MHz spectrophotometer. **AM 1**-NH_2-*t*-boc: ^{1}H NMR ($CDCl_3$; ppm): 0.88 (t, 12H, CH_3), 1.30 (m, 64H, CH_2), 1.45 (s, 9H, CH_3), 1.65 (m, 8H, CH_2), 2.38 (m, 4H, CH_2), 2.60 (m, 4H, CH_2), 3.63 (m, ~0.45kH, CH_2), 5.42 (m, 1H, CH), 5.50 (m, 1H, CH), 5.59 (m, 2H,CH).

The obtained product **AM 1**-tBoc (200 mg, 0.034 mmol) was added to a 20 mL vial and dissolved in 5 mL DCM and submerged in an ice bath. Next, trifluoroacetic acid (0.050 mL, 0.68 mmol) was added and the deprotection was carried out for 1 h. The reaction mixture was washed once with 0.10 N HCl and twice with brine. The organic layer was dried over $MgSO_4$ for 30 min and separated by vacuum filtration. The solvent was removed via rotary evaporation and the resulting oil was dissolved in 2 mL DCM and precipitated into cold diethyl ether. The resulting white solid was washed twice with diethyl ether and dried *in vacuo*. Removal of the *t*-boc protecting group was verified by ^{1}H NMR, as determined by the disappearance of the singlet peak centered at 1.45 ppm. All other ^{1}H NMR shifts were identical to those listed above.

Following deprotection and isolation, the amine terminated, bioactive AM (30 mg, 5.0 µmol) was dissolved in 400 µL of anhydrous DMSO and 5(6)-carboxyfluorescein *N*-hydroxysuccinimide ester (12 mg, 25 µmol) was dissolved in 600 µL of anhydrous DMSO. In 30 minute intervals, 67 µL of the AM solution was added to the fluorescein solution over a 3 h period to give a final AM and activated fluorescein concentration of 5.0 mM and 25 mM, respectively. A catalytic amount of TEA (5.0 µmol) was added to the AM solution prior to mixing. The reaction solution was allowed to react in the dark for 18 h at room temperature. The resulting fluorophore labeled AM was purified by dialysis against phosphate buffer saline (PBS) for 36 h followed by dialysis against deionized water for 36 h and subsequently lyophilized yielding an orange colored solid (31 mg, 97% yield).

The resulting fluorescently labeled AM was characterized by UV-vis spectroscopy using a Thermo Scientific NanoDrop 2000C UV-vis spectrophotometer. The degree of fluorescein labeling was quantitative and calculated using an extinction coefficient of 68 000 M^{-1} cm^{-1}, as previously reported,[4] for free fluorescein in PBS.

Nanoparticle Fabrication: Kinetically assembled nanoparticles (NPs) comprised of bioactive AMs or PEG-*b*-PLA and an organic core solute were prepared via Flash NanoPrecipitation.[5, 6] Typically, a confined impinging jet mixer was utilized to mix 250 µL of an aqueous stream with 250 µL of a THF stream containing 40 mg mL^{-1} AM and 20 mg mL^{-1} of a chosen core solute, mucic acid acylated with lauroyl groups (**M12**; No PEG) or poly(lactic acid) (PLA). All NP samples were prepared at an AM:core solute weight ratio of 2:1. Upon mixing, the exit stream was immediately introduced into 2 mL of DI H_2O (H_2O:THF of 9:1) and subsequently dialyzed against DI H_2O to remove residual THF. Dual fluorescent NPs comprised of **AM 1** as the AM and **M12** as the core were prepared using the same procedure as above. The shell of the NP was rendered fluorescent using a weight ratio of 5:1 of unlabeled to fluorescein labeled **AM 1** (i.e. fluorescein labeled **AM 1** is 20 wt%) and the core was made fluorescent by co-precipitating 2.3 wt% of ETTP5 relative to **M12** weight. All experiments outlined below utilized NPs within two weeks of fabrication unless otherwise noted.

Dynamic Light Scattering and Zeta Potential: Dynamic light scattering (DLS) and zeta potential measurements of fabricated NPs were performed using a Malvern-Zetasizer Nano Series DLS detector with a 22 mW He-Ne laser operating at λ = 632.8 nm, an avalanche photodiode detector with high quantum efficiency, and an ALV/LSE-5003 multiple τ digital correlator electronics system. NP sizes were determined following dialysis at a concentration of 0.6 mg mL^{-1} in either DI H_2O or PBS at 25 °C. In addition, DLS measurements to determine the size and colloidal stability of the fabricated NPs in the presence of fetal bovine serum (FBS) were carried out in RPMI media with or

without 20 % v/v of FBS at a NP concentration of 0.6 mg mL^{-1} at 37 °C. As a control, size data was also obtained on RPMI media containing solely FBS. Data analysis of DLS measurements was performed using the normal resolution mode and reported hydrodynamic diameters were obtained from intensity distributions. Zeta potential measurements were carried out at a NP concentration of 1.0 mg mL^{-1} in PBS inside a zeta folded capillary cells purchased from Malvern Instruments. Samples were gently mixed with pipettes to ensure homogeneous mixing and equilibrated for 5 min at 25 °C. Both DLS and zeta potential measurements were performed in triplicate.

AM Release Profile: NPs comprised of 20 wt% fluorescein labeled **AM 1**, prepared as described previously, and 80 wt% unlabeled **AM 1** were utilized to determine the release profile of **AM 1**. For the micellar system, fluorescein labeled **AM 1** and unlabeled **AM 1**, both at 1 mM (6 mg mL^{-1}) , were mixed in a volume ratio of fluorescently labeled to unlabeled of 20/80 v/v and sonicated for 15 min. Fluorescently labeled NPs or micelles in either 0.5 mL of PBS or 10% v/v FBS in PBS at 6.0 mg mL^{-1} for NPs or 4.0 mg mL^{-1} for micelles were sealed in a 300 kDa MWCO Float-A-Lyzer (Spectrum Laboratories Inc; Rancho Dominguez, CA) and dialyzed against 80 mL of the equivalent buffer solution at room temperature. The buffer solution was replaced three times within the first 24 h and every 24 h after. Samples were taken from the inner fluid at predetermined time intervals and measured using a Thermo Scientific NanoDrop 2000C UV-vis spectrophotometer. Percent release of the fluorescently labeled **AM 1** was determined by comparing the absorbance spectrum at the predetermined time points to the absorbance spectrum prior to dialysis. UV-vis absorbance measurements were performed in triplicate.

Cell Culture: Peripheral blood mononuclear cells (PBMC) were isolated from human buffy coats (Blood Center of New Jersey; East Orange, NJ) by Ficoll-Paque (1.077 g mL^{-1}; GE Healthcare) density gradient. Monocytes were selected by plate adherence after two hours by washing thrice with PBS and cultured for 7 days in RPMI 1640 (ATCC) with 10% FBS, 1% Penicillin/Streptomycin and

50 ng mL^{-1} M-CSF for differentiation into macrophages (MDM). Media was exchanged every two to three days. Primary Human Coronary Artery Smooth Muscle Cells (CASMC) (Lonza) between passages 3-6 were cultured in SM-GM2 media (Lonza). CASMC were plated on Type I Collagen coated wells for cytotoxicity experiments. Primary Human Coronary Artery Endothelial Cells (HCAEC) (Lonza) between passages 2-6 were cultured in EGM2-MV media (Lonza). HCAEC were seeded on fibronectin-coated wells for cytotoxicity experiments. Human embryonic kidney cells stably transfected with human scavenger receptor A1 (HEK-SRA) were cultured in DMEM high glucose (Lonza) supplemented with 10% FBS, 1% penicillin/streptomycin, Blasticidin and HygromycinB. SRA expression was induced through the addition of 0.25 μg mL^{-1} tetracycline.

Nanoparticle Cytocompatibility: NP cytocompatibility was determined via LIVE/DEAD assay (Invitrogen; Carlsbad, CA) with MDMs, CASMCs and HCAECs. Cells were treated for 24 h under 10 % FBS with several NP concentrations ranging from 9 to 900 μg mL^{-1}, which corresponds to a bulk **AM 1** concentration of 10^{-6} to 10^{-4} M respectively. After treatment, cells were stained with 2 μM calcein AM and 4 μM ethidium homodimer-1, and subsequently imaged with a Nikon Eclipse TE2000-S epifluorescent microscope. Live cells were discerned from dead cells using Image J analysis software 1.43m. For the negative control group, cells were treated with 70% methanol for 10 minutes prior to LIVE/DEAD assay. All conditions were normalized to untreated cells and repeated in triplicate.

oxLDL Uptake by PBMC Derived Macrophages: Human MDMs were co-incubated with 5 μg mL^{-1} of 3,3'-dioctadecyloxacarbocyanine (DiO) labeled oxLDL (Kalen Biomedical; Montgomery Village, MD) and either NPs or micelles for 24 h in the presence of 0 (serum-free), 5, 10 or 20% FBS. **AM 1** concentration in both delivery systems was held constant at 10^{-6} M, which corresponds to a NP concentration of 9 μg mL^{-1}. Cells were fixed and counterstained with Hoechst 33342 before epifluorescent imaging on a Nikon Eclipse TE2000-S. Uptake of oxLDL in MDMs was quantified by

ImageJ and normalized to cell count. Analyzed data were from three independent experiments performed in triplicate.

Foam Cell Formation: Foam cell phenotype was induced by incubating MDMs with 50 μg mL^{-1} of oxLDL (Biomedical Technologies; Stoughton, MA) and either NPs or micelles for 48 h in the presence of 10% FBS. To maintain the **AM 1** to oxLDL concentration ratio used in the DiO oxLDL uptake studies, **AM 1** concentration of 10^{-5} M or 60 μg mL^{-1} and NP concentration of 90 μg mL^{-1} was used. Cells were fixed, stained with Oil-Red-O and counterstained with Hoechst 33342. Brightfield and epifluorescent images were taken on a Nikon Eclipse TE2000-S. Cells with excessive lipid accumulation indicating the foam cell phenotype were counted relative to total population. Analyzed data were from three independent experiments performed in triplicate.

Nanoparticle-Scavenger Receptor Interactions: HEK-SRA cells (basal and induced) were employed to investigate scavenger receptor (SR) interactions with NPs. For uptake/binding interactions cells were incubated with dual fluorescent NPs (90 μg mL^{-1}) for 18 h in 10% FBS media. Dual fluorescent NPs were prepared as described above. For SR-A1 blocking experiments, induced HEK-SRA cells were first incubated with either 10 μg mL^{-1} SR-A1 monoclonal antibody (monoclonal mouse antihuman; R&D Systems; Minneapolis, MN) or polyinosinic acid for 1 h in 10% FBS media. To discern non-specific from specific binding, an IgG isotype control was included where the SR-A1 monoclonal antibody was replaced with purified mouse IgG_1 (Invitrogen; Carlsbad, CA). After 1 h, the media was removed and replaced with 10% FBS media containing dual fluorescent NPs (90 μg mL^{-1}) and SR-A1 mAb, IgG_1 isotype, or polyinosinic acid (10 μg mL^{-1}) for 6 h. Cells for both NP uptake/binding and SR-A1 receptor blocking were fixed and counterstained with Hoechst 33342 before imaging on a Leica TCS SP2 confocal microscope. Images were analyzed for co-localization of ETTP5, encapsulated in the NP core, and fluorescein labeled **AM 1**. Cell

associated fluorescence due to NP binding/uptake was quantified by ImageJ and normalized to cell count.

Estimation of the Number of AM Chains per Nanoparticle: An approximation for the number of **AM 1** chains per **NP 1** was determined by employing previously published results[7] and the following equation:

$$n\pi\left(\frac{\xi_i}{2}\right)^2 = 4\pi\left(\frac{D_{sphere}}{2} + \frac{\xi_i}{2}\right)^2 \quad (1)$$

where *n* is the number of chains per particle, ξ_i is the blob size a polymer chain occupies at the particle surface, and D_{sphere} is the diameter of the polymer coated particle. Eq (1) sets the area occupied by all polymer chains equal to the total available surface area of the particle. Assuming that the attached PEG chains have an equal size to that of a free PEG chain in solution, the minimum number of chains per particle can be determined. This is referred to as the mushroom confirmation and the corresponding size ($\xi_{mushroom}$), equivalent to the "Flory size", can be calculated as follows[7]:

$$\xi_{mushroom} = 0.76\,Mw_{PEG}{}^{0.5}\,[\text{Å}] \quad (2)$$

where Mw_{PEG} is equal to the molecular weight of the PEG chain, which is 5 kDa in this report. Based on Eq (2) the blob size for a mushroom confirmation is equal to ξ = 5.40 nm and the area occupied by each PEG chain is 23 nm^2 $polymer^{-1}$. To estimate the number of polymer chains, in mushroom confirmation, a volume average diameter of 150 ± 10 nm for **NP 1** was used in Eq (1). This provides an estimate of 3300 **AM 1** chains $particle^{-1}$ and assuming a homogenous distribution of **AM 1** and **M12** during NP fabrication, ~10000 **M12** molecules per NP core. It is important to note that the mushroom confirmation approximates a minimum number of chains per particle.

Estimation of a more practical blob size, $\xi_{experimental}$, can be made by utilizing experimental data reported by Prud'homme and coworkers,[7] who first functionalized polystyrene latex spheres with PEG chains and subsequently determined the number of bound chains per particle via a Baleux assay.[8] Accounting for the molecular weight of PEG in this report, 5 kDa, and determining the ratio

of surface area between the previously reported latex sphere and the volume average diameter of **NP 1**, 150 ± 10 nm, a blob size of ξ = 4.1 nm was estimated. Based on Eq 1, this provides an estimate of 5600 **AM 1** chains particle^{-1} and 17000 **M12** molecules per NP core.

Statistical Analysis: DLS, AM release, and cytocompatibility results are presented as means ± S.D, while all other in vitro results are presented as ± S.E.M. Data for inhibition of oxLDL uptake and foam cell formation were evaluated by one-way ANOVA and Tukey's test was used for post-hoc pairwise comparisons between individual conditions. A *p*-value of 0.05 or less was considered statistically significant.

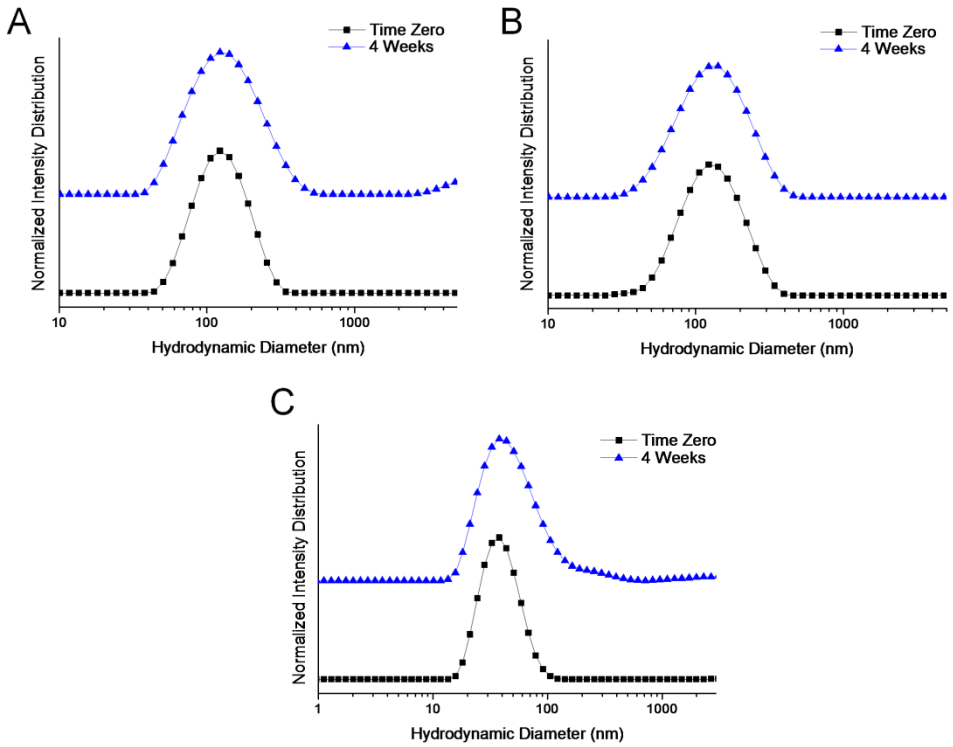

Figure S1. Time zero (black) and 4 week (blue) intensity distributions of A) **NP 3**, B) **NP 4,** and C) **NP 6** stored at 37 °C.

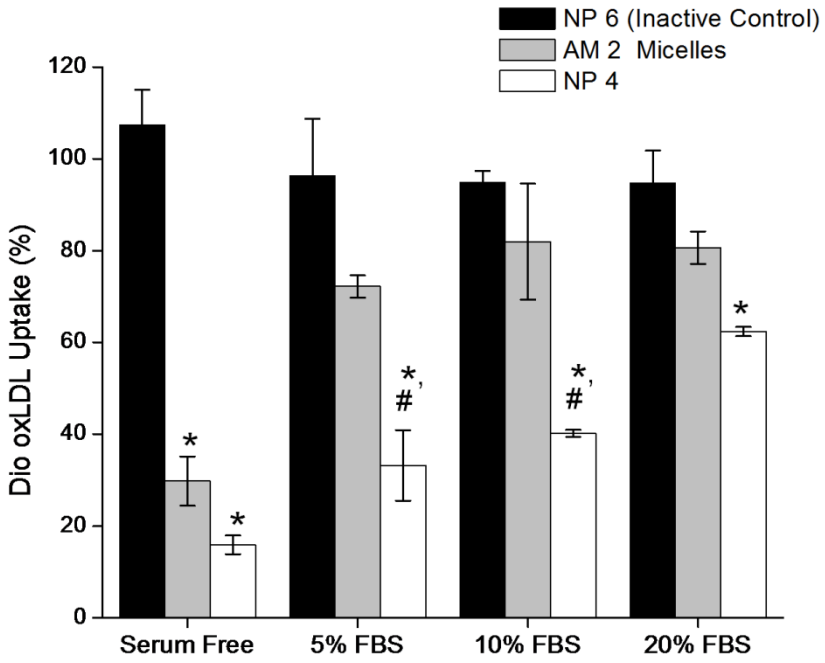

Figure S2. In vitro comparison of kinetic assembled NPs versus thermodynamic assembled micelles for inhibiting oxLDL uptake. Quantification of DiO oxLDL (5 μg mL^{-1}) uptake by MDMs after 24 h incubation with **AM 2** micelles or **NP 4** NPs under 0 to 20% FBS, [**AM 2**] = 10^{-6} M. **NP 6** serve as an inactive control and all treatments are normalized to cells incubated exclusively with DiO oxLDL. Data are from an n = 3 conducted in triplicate (error bars = ± S.E.M.). Statistical significance was evaluated at $p < 0.05$, * indicates significance versus **NP 6** and # versus **AM 2** micelles.

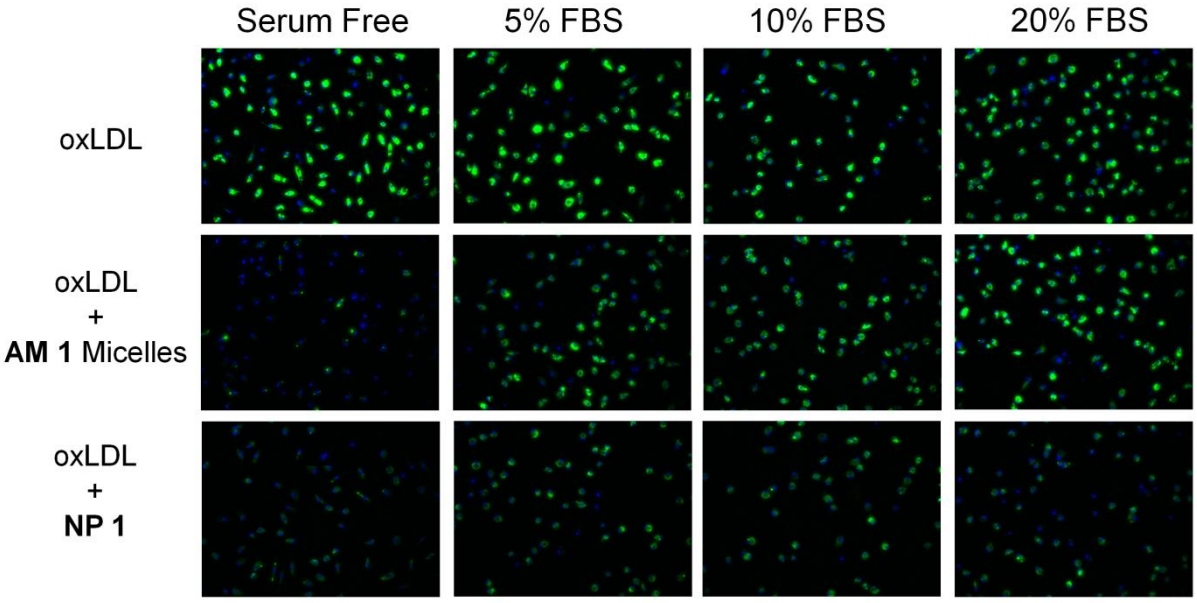

Figure S3. Representative fluorescent images contrasting the level of oxLDL uptake between human MDMs incubated with DiO oxLDL (green; top row) and co-incubated with DiO oxLDL and either **AM 1** micelles (middle row) or **NP 1** (bottom row) under serum-free, 5, 10, and 20 % FBS for 24 h. Nuclei were counterstained with Hoechst 33342.

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