Supplementary Materials for

MK2 inhibitory peptide delivered in nanopolyplexes prevents vascular graft intimal hyperplasia

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METHODS

Synthesis of cell penetrant MK2 inhibitory peptide

An MK2 inhibitory peptide (MK2i) with the sequence YARAAARQARAKALARQLGVAA was synthesized on a PS3 peptide synthesizer utilizing standard Fmoc Chemistry. MK2i peptide was cleaved/deprotected in TFA/Phenol/H_2O/triisopropylsilane (88/5/5/2). Peptides were then further purified by reverse phase HPLC utilizing a phenomenex Luna C18(2) AXIA packed column. HPLC-grade water with 0.05% formic acid and HPLC-grade acetonitrile were used as mobile phases A and B, respectively. The MK2i peptide was purified utilizing a 90% A to 90% B gradient over 25 min. Peptide purity was verified through electrospray ionization mass spectrometry (ESI-MS) on a Waters Synapt ESI-MS.

Monomer and polymer synthesis

All reagents were purchased from Sigma and were of analytical grade unless otherwise stated. 2-propylacrylic acid was synthesized according to (*36*) using diethyl propylmalonate as a precursor. The 4-cyano-4-(ethylsulfanylthiocarbonyl) sulfanylvpentanoic acid chain transfer agent (CTA) was synthesized as described previously (*37*). RAFT polymerization of the poly(propylacrylic acid) (PPAA) homopolymer was carried out in bulk under a nitrogen atmosphere at 70°C for 48 hours using 2,2'-azo-bis-isobutyrylnitrile (AIBN) as the free radical initiator. The reaction mix was put through three freeze-vacuum-thaw cycles and purged with nitrogen for thirty minutes prior to polymerization. The molar ratio of CTA to AIBN was 1 to 1, and the monomer to CTA ratio was set so that a degree of polymerization (DP) of 190 would be achieved at 100% conversion. Following polymerization, the resultant polymer was dissolved in DMF and precipitated into ether 5 times before drying overnight *in vacuo*.

RAFT polymerization of the poly(acrylic acid) (PAA) homopolymer was carried out in distilled dioxane under a nitrogen atmosphere at 70°C for 18 hours using AIBN as the free radical initiator. The reaction mix was purged with nitrogen for thirty minutes prior to polymerization. The molar ratio of CTA to AIBN was 5 to 1 and the monomer to CTA ratio was set so that a DP of 150 would be achieved at 100% conversion. Following polymerization, the resulting polymer was dissolved in dioxane and precipitated into ether 5 times before drying overnight *in vacuo*. Gel permeation chromatography was used to determine molecular weight

and polydispersity of the PPAA and PAA homopolymers using HPLC-grade DMF containing 0.1% LiBr at 60°C as the mobile phase. Molecular weight calculations were performed with ASTRA V software and were based on experimentally-determined dn/dc values determined through offline injections of the polymer through a refractive index detector (calculated PPAA dn/dc = 0.087 ml/g DP = 193 (GPC), PDI = 1.47 (GPC); calculated PAA dn/dc = 0.09 ml/g DP = 150 (GPC), PDI = 1.27 (GPC). Polymer purity and molecular weight were then verified through NMR spectroscopy utilizing D_6MSO as a solvent. (PPAA DP = 190 (H^1 NMR); PAA DP = 106 (H^1 NMR).

pH-dependent membrane disruption hemolysis assay

To assess the endosomal escape potential of MK2i-NPs, a red blood cell hemolysis assay was utilized as described previously (*38*) to measure pH-dependent lipid bilayer membrane disruption. Following approval by Vanderbilt Medical Center's Institutional Review Board, whole human blood was drawn from an anonymous donor, and plasma was removed through centrifugation and saline washes. The remaining erythrocytes were resuspended into phosphate buffers corresponding to physiologic (pH 7.4), early endosome (pH 6.8), early/late endosome (pH 6.2), and late endosome/lysosomal (pH 5.8) environments. MK2i-NPs, NE-MK2i-NPs, MK2i peptide alone (1-40 μg/ml), PBS (negative control), or 1% Triton X-100 (positive control) were added to the erythrocyte suspensions and incubated at 37°C for 1 hour. Intact erythrocytes were pelleted via centrifugation, and supernatant was transferred to a new 96-well plate. The hemoglobin content within the supernatant was then measured by absorbance at 541 nm. Percent hemolysis was determined relative to Triton X-100 and PBS controls.

Scratch wound chemokinesis assay

VSMCs were seeded in Lab-TEK II 8-well chambered coverglass at a density of 20,000 cells/well in 250 μl low serum growth media and allowed to adhere overnight to achieve a nearly confluent (90-95%) monolayer. Cells were treated with MK2i-NPs, NE-MK2i-NPs, MK2i peptide or PBS for 30 minutes. Following treatment, scratch wounds were made with a 10 uL pipette tip through the middle of each cell monolayer. The media was then replaced with low serum growth media containing a CellTracker Green BODIPY dye for 30 minutes to enable visualization of migrating cells. Following treatment with the dye, media was replaced with low

serum growth media containing 50 ng/ml PDGF-BB (or with PBS for the negative control). Scratch wound areas were then imaged at 24 hours using a Nikon Eclipse Ti inverted fluorescence microscope with NIS Elements imaging software. Wound closure was calculated with ImageJ software by quantifying the scratch wound area around the periphery of migrating cells normalized to the original scratch wound area. Scratch wound assays for each treatment group were performed in 3 independent experiments.

Boyden chamber chemotaxis assay

VSMCs or HUVECs were seeded in a 24 well plate at a density of 30,000 cells/well in low serum media and allowed to adhere overnight. Cells were treated for 30 mins with MK2i-NPs, NE-MK2i-NPs, MK2i peptide, or PBS. For 5 day post-treatment migration assays VSMCs were washed 2× with PBS and then cultured in fresh medium for 5 days with the media being replaced every 2 days. Following treatment or 5 days of post-treatment incubation, each well was washed 2× with PBS, trypsinized, resuspended in 100 μl low serum growth media, and plated onto 6.5-mm, 8-μm pore polycarbonate inserts (Corning) in a 24-well plate with 600 μl low serum growth media containing either 50 ng/ml PDGF-BB (VSMCs) or 25 ng/ml VEGF (ECs) in the lower chamber. Cells were allowed to migrate for 8 hours, and then cells on the upper side of each insert were gently removed with a cotton swab. Cells on the lower side of each insert were then fixed and stained using a Modified Giemsa Differential Quik Stain Kit. After staining, 4 images were taken from the four quadrants of each insert, and the number of cells per high-power field were quantified in ImageJ by thresholding each image and manually counting the cells.

Cell proliferation assay

To confirm that VSMC migration results were not influenced by treatment effects on cellular growth rates, a cellular proliferation assay was performed. VSMCs were seeded at 10,000 cells/well in a 96-well plate in low serum media and allowed to adhere overnight. Cells were treated for 30 minutes with MK2i-NPs, NE-MK2i-NPs, MK2i peptide or PBS. Each treatment was then aspirated and replaced with 100 μl low serum growth media ± 50 ng/ml PDGF-BB. After 24 hours of incubation, a CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) was performed according to the manufacturer's protocol.

Tumor necrosis factor-α, interleukin-6 ELISAs

Cells were treated in low serum media (DMEM, 1% FBS, and 1% P/S) with 10 μM ANG-II (for TNF-α ELISA) or 20 ng/ml TNF-α (IL-6 ELISA) for 4 hours followed by treatment with MK2i-NPs, MK2i, or NE-MK2i-NPs for 2 hours. Following treatment, each well was aspirated and supplemented with fresh medium. After 24 hours, 100 μl of supernatant was collected and frozen at -80°C until cytokine analysis was performed.

Monocyte chemoattractant protein-1 ELISA

Cells were treated in low serum media with MK2i-NPs, MK2i, or NE-MK2i-NPs for 2 hours. Following treatment, each well was aspirated and supplemented with fresh medium. After 3 or 5 days cells were stimulated with TNFα (20 ng/ml) for 24 hours. Following stimulation 100 μl of supernatant was collected and frozen at -80°C until cytokine analysis could be performed.

Microscopic analysis of cellular uptake and intracellular trafficking

An amine-reactive Alexa-488 succinimidyl ester was dissolved in DMSO and mixed at a 1 to 3 molar ratio with the MK2i peptide in 100 mM sodium bicarbonate buffer (pH 8.3). Unreacted fluorophore and organic solvent were removed using a PD-10 miditrap G-10 desalting column, and the fluorescently labeled peptide was lyophilized. PPAA and PAA polymers were mixed with fluorescently labeled MK2i peptide at a CR 1:3 and syringe filtered through a 0.45-μm PTFE filter to form fluorescent MK2i-NPs and control NE-MK2i-NPs, respectively. Fluorescent MK2i-NPs, NE-MK2i-NPs, or MK2i peptide alone were applied to VSMCs grown on Lab-Tek II 8-well chambered coverglass at a concentration of 10 μM MK2i peptide in low serum media for 2 hours. Cells were then washed 2× with PBS, and subsequently incubated in fresh medium for an additional 0, 2, 4, 10, or 22 hours. For the final two hours of incubation, 50 nM Lysotracker Red DND-99 was added to each well in order to visualize acidic endo/lysosomal vesicles. Cells were then washed with 0.1% Trypan blue to quench extracellular fluorescence followed by two additional washes with PBS. Cells were then imaged using a LSM 710 META confocal fluorescence microscope with ZEN imaging software. Gain settings were kept constant for all images acquired.

All images were processed using imageJ and colocalization was analyzed using Just Another Colocalization Plugin (JACoP). Mander's overlap coefficients were then calculated for

$n \geq 3$ separate images for each treatment group to quantify colocalization. To determine treatment effects on the size of the compartments where the peptide was found, the free hand selection tool in ImageJ was used to outline $n \geq 50$ individual intracellular compartments for each treatment group, and the area of each was quantified and averaged.

Flow cytometric quantification of intracellular uptake and retention

VSMCs or HUVECs were grown to 80-90% confluence, harvested, and seeded at 20,000 cells/well in a 24 well plate and allowed to adhere overnight in low serum media (DMEM, 1% FBS, and 1% P/S). Fluorescent MK2i peptide, MK2i-NPs, and NE-MK2i-NPs were synthesized as noted above for microscopy analysis, and VSMCs were treated at a concentration of 10 μM MK2i for 2 hours. Following treatment, cells were washed with PBS, washed with CellScrub buffer for 10 minutes at room temperature to remove extracellular polyplexes and/or peptide, washed 2× in PBS, and given fresh complete growth media. ECs were immediately harvested and VSMCs were incubated for an additional 0, 12, 24, 72, or 120 hours. Cells were then washed with PBS, trypsinized, and resuspended in 0.1% Trypan blue in PBS for analysis on a FACSCalibur flow cytometer BD CellQuest Pro software (v 5.2). Data was exported and analyzed with FlowJo software (v 7.6.4). All samples were run in triplicate.

The intracellular MK2i half-life ($t_{1/2}$) was calculated by exponential decay nonlinear regression analysis of intracellular peptide fluorescence at 0 and 5 days following treatment removal using the exponential decay function [where N= intracellular fluorescence and λ = the decay rate]:

$$N(t) = N_o e^{-\lambda t} \qquad (eq.S1)$$

And calculating the $t_{1/2}$ from the decay constant of each exponential decay function as follows:

$$t_{1/2} = {\ln(2)}/{\lambda} \qquad (eq.S2)$$

Human saphenous vein

Upon approval by Vanderbilt Medical Center's institutional Review Board, de-identified, discarded segments of human saphenous vein were collected from consented patients undergoing coronary or peripheral vascular bypass surgeries. Following surgical resection, human saphenous vein segments were stored in saline solution until the end of the surgical procedure, at which

time they were placed in cold transplant harvest buffer (100 mM potassium lactobionate, 25 mM KH_2PO_4, 5 mM $MgSO_4$, 30 mM raffinose, 5 mM adenosine, 3 mM glutathione, 1 mM allopurinol, 50 g/l hydroxyethyl starch, pH 7.4). All human saphenous vein segments were used within 24 hours of harvest.

Using sterile technique, human saphenous vein segments were transferred to a 60-mm Petri dish. The end of each segment (0.5 mm) was removed with a blade, and excess adventitial and adipose tissue was removed with minimal manipulation. Human saphenous vein segments were cut into consecutive rings with an approximate width of 1.0 mm to be utilized in organ culture experiments. Two rings from each segment were immediately fixed in 10% formalin at 37°C for 30 min to obtain pre-culture intimal thickness measurements.

Prior to organ culture experiments, human saphenous vein viability was confirmed. Human saphenous vein rings were weighed and their lengths recorded. Human saphenous vein rings were then suspended in a muscle bath containing a bicarbonate buffer (120 mM NaCl, 4.7 mM KCl, 1.0 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 10 mM glucose, 1.5 mM $CaCl_2$, and 25 mM Na_2HCO_3, pH 7.4) equilibrated with 95% O2 and 5% CO2 at 37°C. The rings were stretched and the length progressively adjusted until maximal tension was obtained. Normalized reactivity was obtained by determining the passive length–tension relationship for each vessel segment. Rings were maintained at a resting tension of 1 g, which produces maximal responses to contractile agonists, as previously determined, and equilibrated for 2 h in buffer. Force measurements were obtained using a Radnoti Glass Technology force transducer (159901A) interfaced with a Powerlab data acquisition system and Chart software. Human saphenous vein rings were initially contracted with 110 mM KCl (with equimolar replacement of NaCl in bicarbonate buffer) and the force generated was measured. KCl causes membrane depolarization, leading to contraction of vessels containing functionally viable smooth muscle.

Human saphenous vein viability

Human saphenous vein rings were prepared and treated as noted above, and following 1 or 14 days of organ culture, human saphenous vein rings were weighed and then placed in 250 µl of 0.01% methyl tetrazolium dissolved in DPBS for 1 hour. The reaction was stopped by placing the rings into distilled water. The rings were then placed into 1 ml of CelloSolve and incubated at 37°C overnight. Rings were then mixed in solution, and the CelloSolve was extracted and

placed into a cuvette where the optical density at 570 nm was determined. Relative viability calculations were based on the optical density normalized to the wet weight of the ring.

Microscopic analysis of MK2i delivery to human saphenous vein

An Alexa-568 labeled MK2i peptide was prepared as stated in section 4.9. After verifying viability, human saphenous vein rings were treated with 100 µM of labeled MK2i peptide, MK2i-NPs, or NE-MK2i-NPs for 30 minutes while suspended in a muscle bath, washed 2× in PBS, and immediately embedded in OCT compound and frozen over dry ice. Five-µm cryosections were cut from the middle of each treated vessel and mounted on microscope slides. Immunofluorescence staining was then carried out with CD31 and α-SMA primary antibodies and a FAM labeled secondary antibody. Microscopy images were obtained using a Nikon Eclipse Ti inverted fluorescence microscope. Gain settings were kept constant for all images acquired for all samples.

Western blot analysis of protein phosphorylation in human saphenous vein

Following 2 hours of treatment with MK2i, a portion of the treated human saphenous vein rings were snap-frozen with liquid nitrogen, pulverized, and homogenized using urea-DTT-CHAPS buffer. For analysis of hnRNP A0 phosphorylation treated human saphenous vein rings were maintained in organ culture in fresh media for 24 hours prior to homogenization. For analysis of CREB and HSP27 phosphorylation human saphenous vein rings were frozen immediately after treatment removal. Lysates were centrifuged (6000 g, 20 minutes), and the supernatant was collected for each sample for evaluation of HnRNP A0, CREB, and HSP27 phosphorylation. Equal amounts of protein (20 µg per lane) were loaded on 15, 10, or 4–20% SDS–PAGE gels; proteins were electrophoretically separated and then transferred to Immobilon membranes. For hnRNP A0 phosphorylation, membranes were probed overnight at 4°C with primary antibodies for phospho-hnRNP A0 (Millipore) and unphosphorylated hnRNP A0 (Santa Cruz). For CREB phosphorylation, membranes were probed overnight at 4°C with primary antibodies for phospho-CREB (abcam) and unphosphorylated CREB (abcam). For HSP27 phsophorylation membranes were probed overnight at 4°C with primary antibodies for phospho-HSP27 (Epitomics) and unphosphorylated HSP27 (Santa Cruz). After washing, the membranes were incubated with appropriate secondary antibodies (Li-Cor) for 1 hour at room temperature. The secondary

antibody was imaged using the Odyssey direct infrared fluorescence imaging system and densitometrically quantified with LiCor Odyssey software v2.1 at 800 and 680 nm wavelengths. For each biological replicate, all treated samples were normalized to untreated control tissue.

SUPPLEMENTARY FIGURES

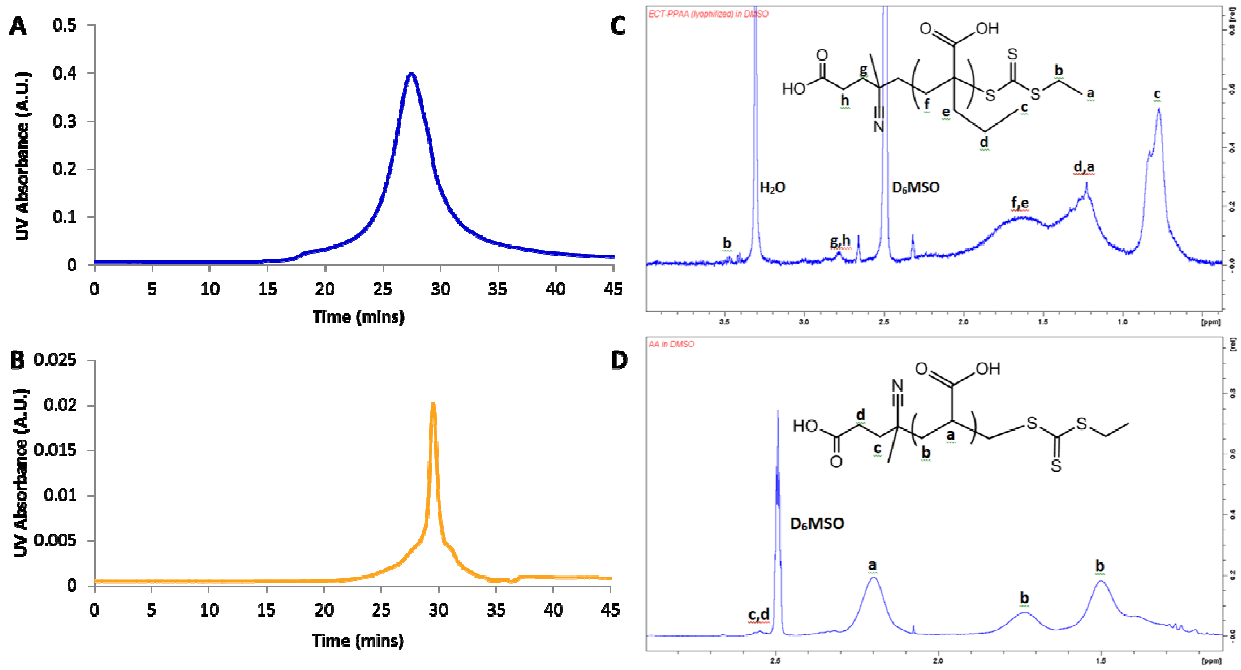

Figure S1: Polymer characterization with GPC and NMR. (**A** and **B**) GPC chromatograms of poly(propylacrylic acid) (PPAA) [degree of polymerization = 193, PDI =1.471, dη/dC = 0.087 (ml/g)] (A) and poly(acrylic acid) (PAA) [degree of polymerization = 150, PDI = 1.27, dη/dC = 0.09 (ml/g)] (B) polymers in DMF. The trace shows UV absorbance at the characteristic absorption peak of the trithiocarbonate moiety (310 nm) present in the 4-cyano-4-(ethylsulfanylthiocarbonyl) sulfanylvpentanoic acid chain transfer agent utilized in the polymerization. (**C** and **D**) ^{1}H NMR spectra of PPAA (C) and PAA (D) in D_6MSO. Molecular weight was determined by comparing the area of peaks associated with the chain transfer agent (peaks c,d for PAA and peak b for PPAA) to peaks associated acrylic acid/propylacrylic acid (peak a for PAA and peak c for PPAA): PAA degree of polymerization = 106; PPAA degree of polymerization = 190.

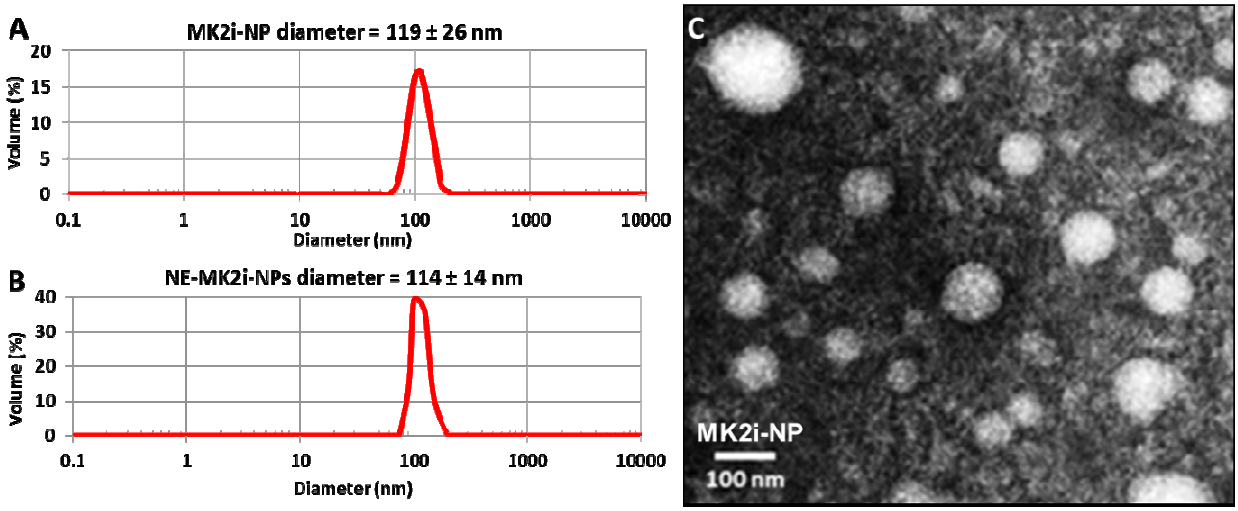

Figure S2: NP size and morphology. (**A** and **B**) Dynamic light scattering analysis of MK2i-NP (A) and NE-MK2i-NP (B) size distributions. (**C**) Representative TEM image of uranyl acetate counterstained MK2i-NPs.

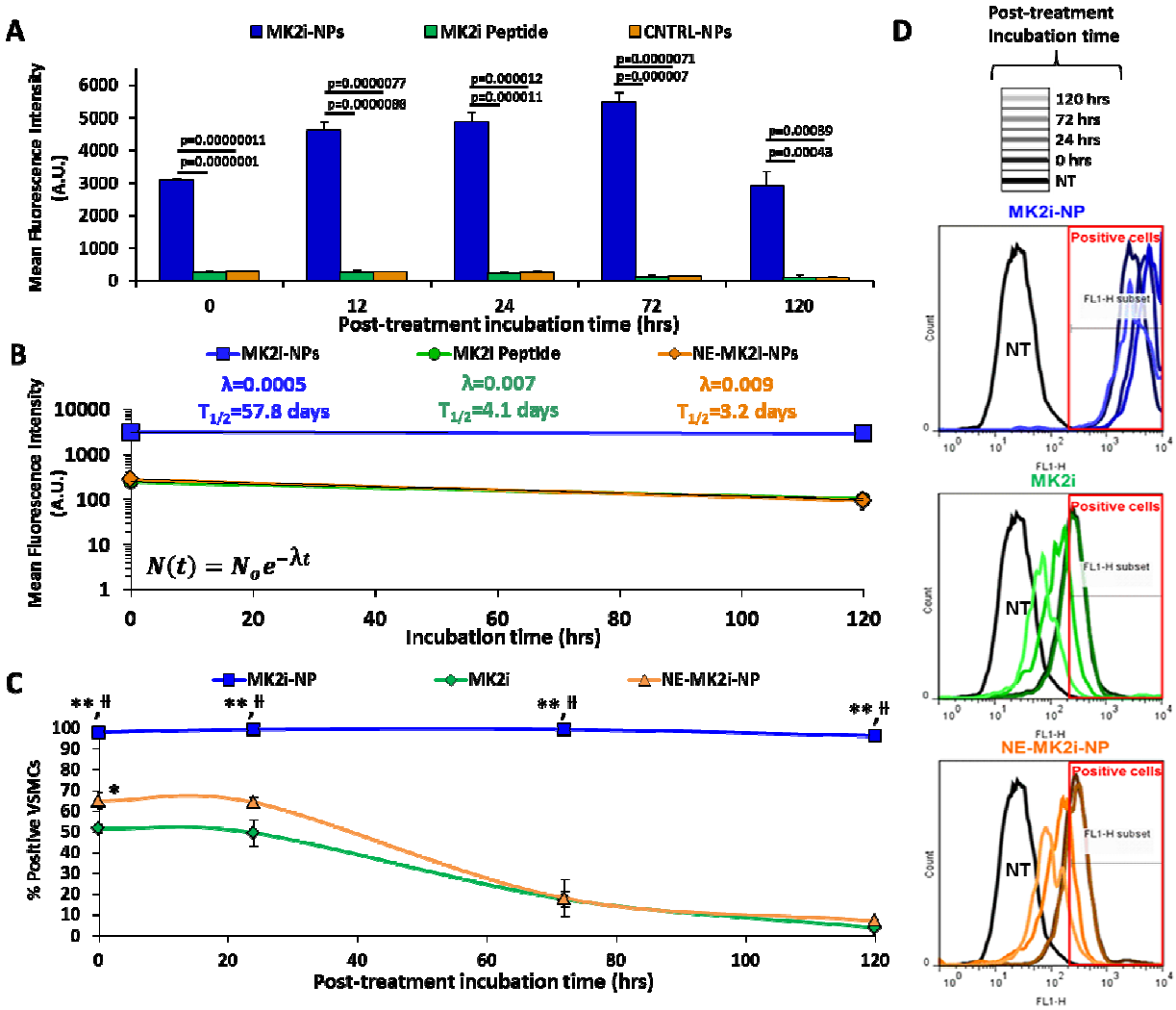

Figure S3: MK2i uptake and intracellular retention in VSMCs. (**A**) Flow cytometric quantification of human coronary artery VSMC uptake and retention of fluorescently labeled MK2i, MK2i-NPs, and NE-MK2i-NPs. Data are means ± SEM (n =3). P values determined by single factor ANOVA. (**B**) Quantification of intracellular MK2i half-life ($_{1/2}$) by exponential decay nonlinear regression analysis of intracellular peptide fluorescence 0 and 5 days following treatment removal. (**C** and **D**) Longitudinal quantification (C) and representative flow histograms and subsets (D) used to calculate the percentage of VSMCs positive for MK2i internalization following removal of treatment with free MK2i, MK2i-NPs, or NE-MK2i-NPs. Data are means ± SEM (n = 3). * P<0.01, ** P<0.001 vs. MK2i; $^{\square}P$<0.01, $^{\square\square}P$<0.001 vs. NE-MK2i-NPs; single factor ANOVA.

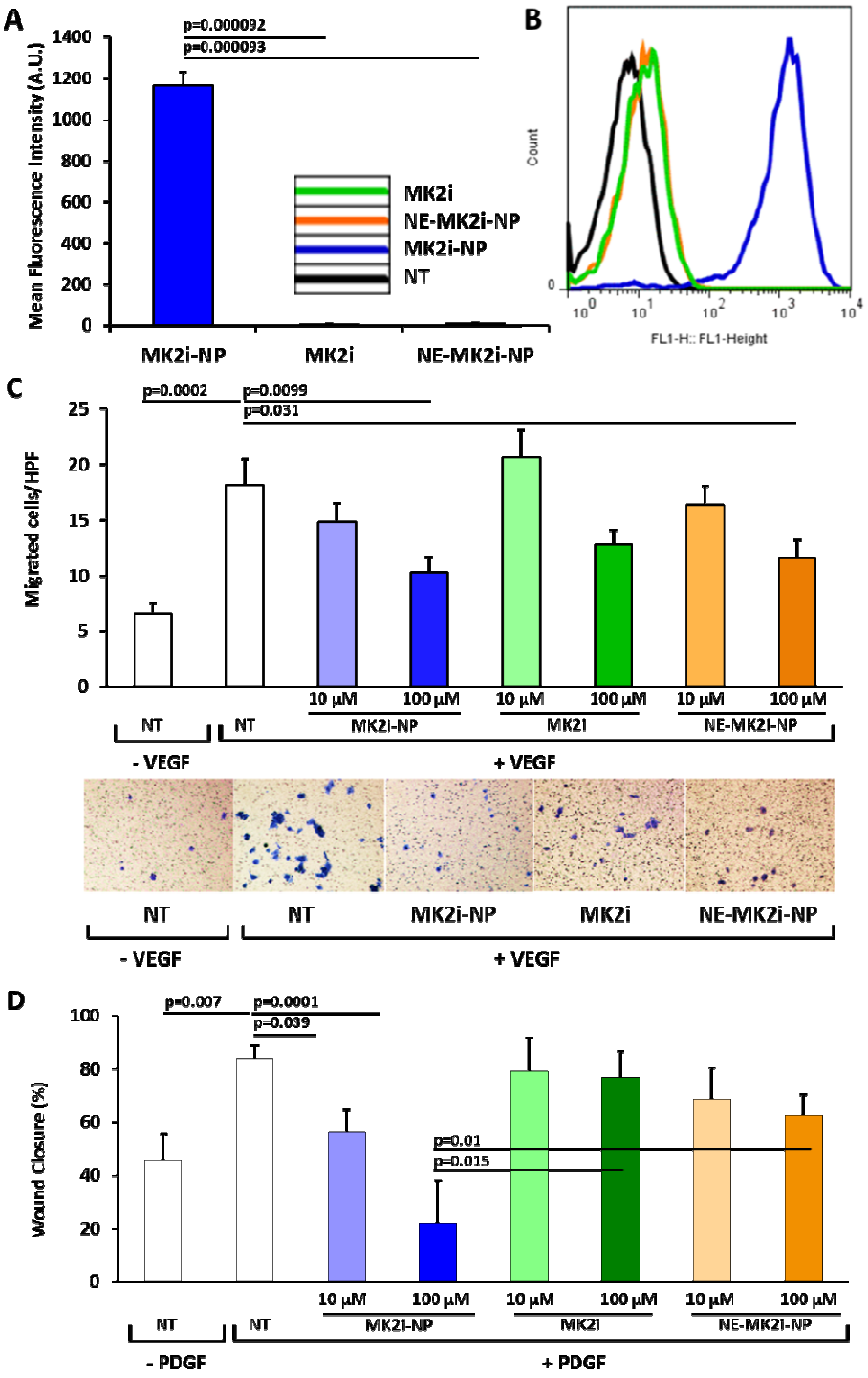

Figure S4: MK2i uptake in ECs and effects on EC and VSMC migration. (**A** and **B**) Flow cytometric quantification (A) and representative flow histograms (B) of EC uptake of fluorescently labeled MK2i, MK2i-NPs, and NE-MK2i-NPs. (**C**) Quantification and representative images of EC migration immediately after treatment removal determined by Boyden transwell migration assay. (**D**) Quantification of MK2i-treated VSMC migration in the presence of the chemoattractant PDGF-BB. Migration was determined by calculating percent wound closure 24 hours after scratch wound application *in vitro*. In (A, C, and D), data are means ± SEM (n = 3). P values determined by single factor ANOVA.

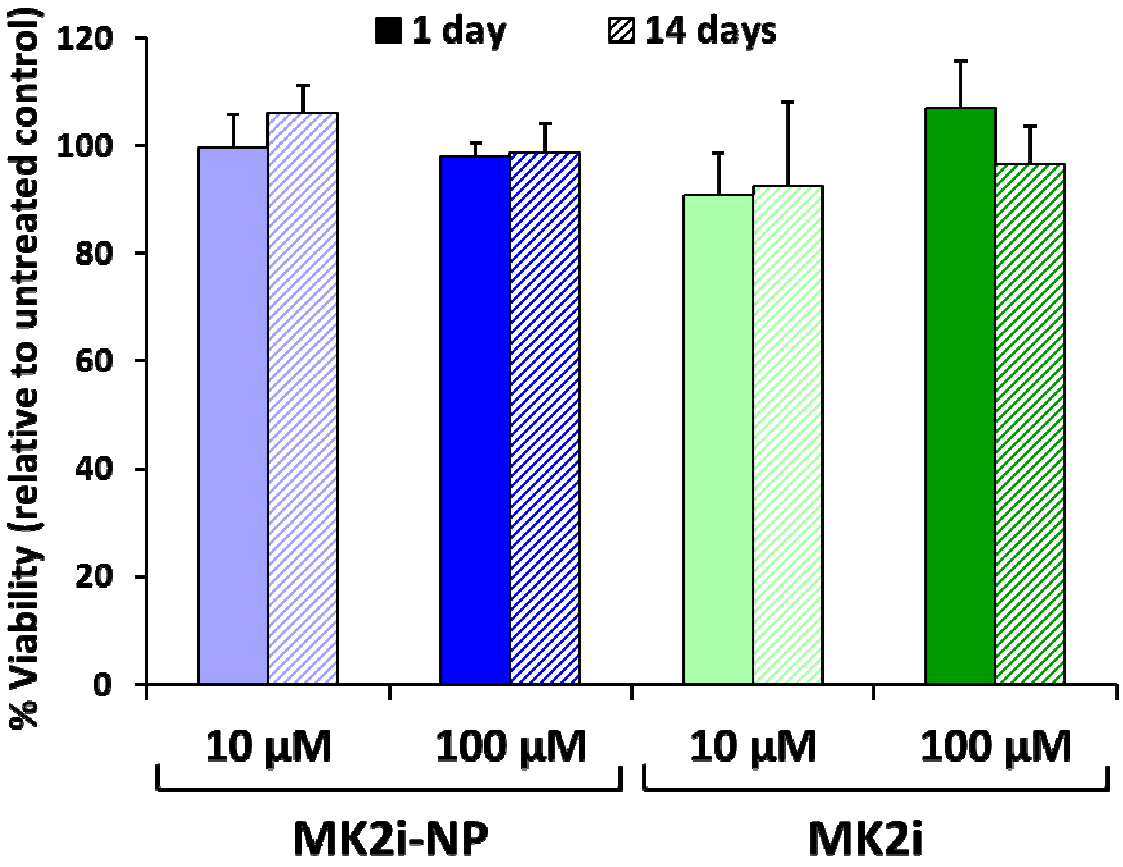

Figure S5: The effects of MK2i-NP and free MK2i on HSV viability. Quantification of tissue viability in human saphenous vein rings treated for 2 hours and subsequently maintained in organ culture for 1 or 14 days as assessed through an MTT assay. Data are means ± SEM (n = 3 biological replicates from 3 separate donors). No statistical differences were observed ($P > 0.05$, single factor ANOVA).

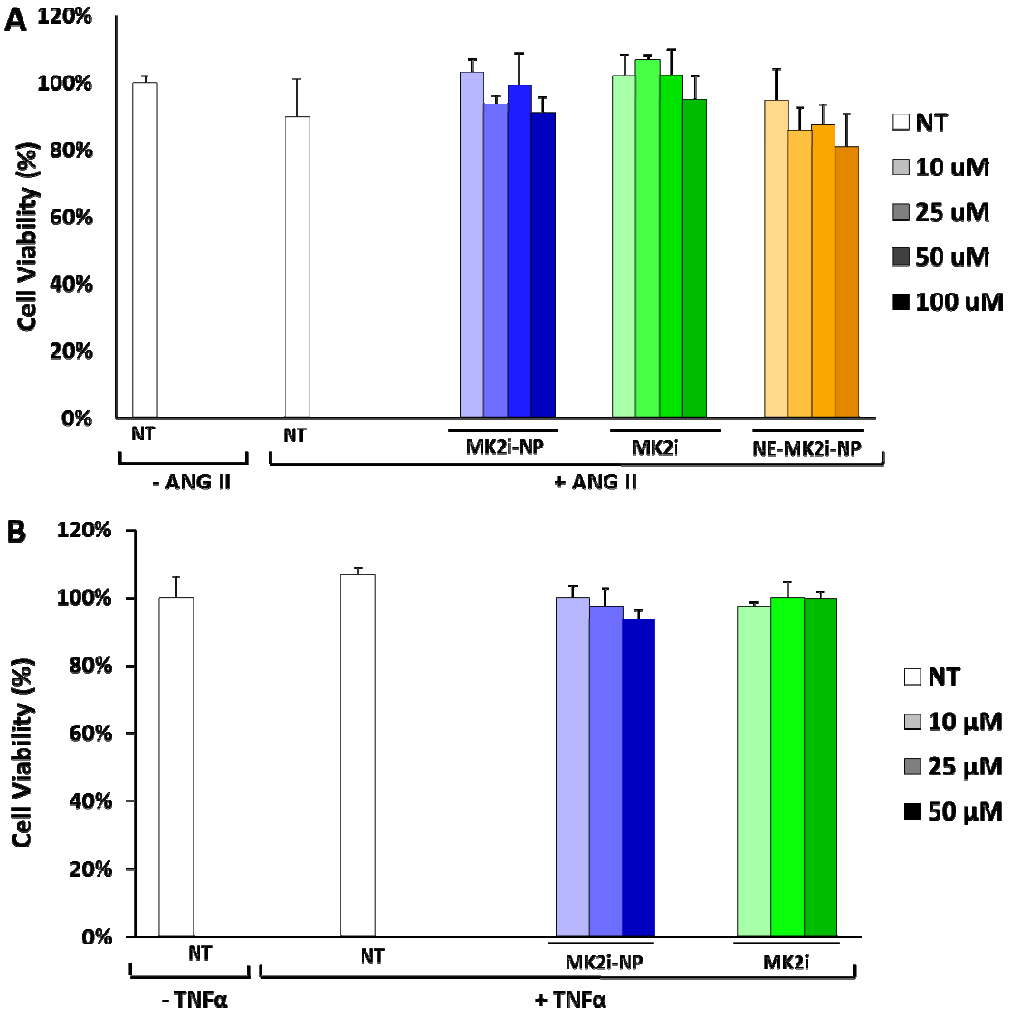

Figure S6: Viability of MK2i-treated VSMCs used for inflammatory cytokine analysis. Cells were stimulated with 10 µM angiotensin II (ANG II) (**A**) or 20 ng/ml TNFα (**B**) for 6 hours, then treated for two hours with MK2i-NPs, NE-MK2i-NPs, or the MK2i peptide alone (concentrations in figure legends) and cultured for 24 hours in fresh media. VSMC viability was determined by LDH cytotoxicity assay. NT, no treatment. Data are means ± SEM (n = 4). No statistical differences were observed (P > 0.05); single factor ANOVA.

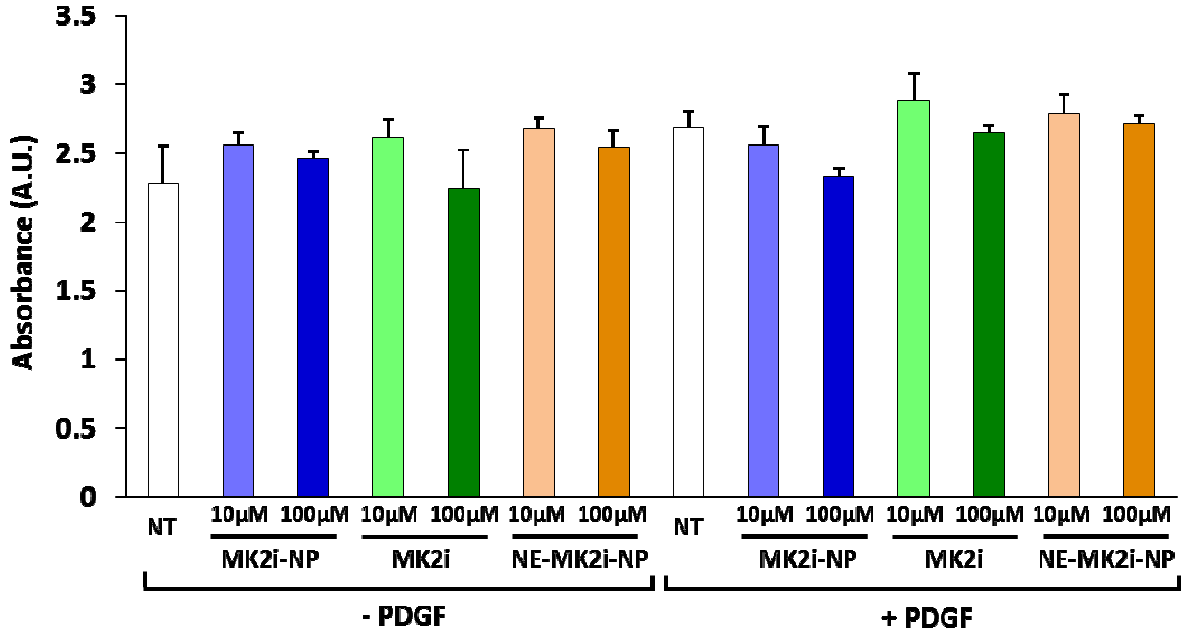

Figure S7. VSMC proliferation assay as a control for migration experiments. Quantification of cellular proliferation in VSMCs treated for 30 minutes with MK2i peptide alone, MK2i-NP, or NE-MK2i-NP and cultured for 24 hours in fresh media with or without 50 ng/ml PDGF-BB. NT = no treatment. Data are means ± SEM (n = 4). No statistical differences were observed ($P > 0.05$, one-way ANOVA).

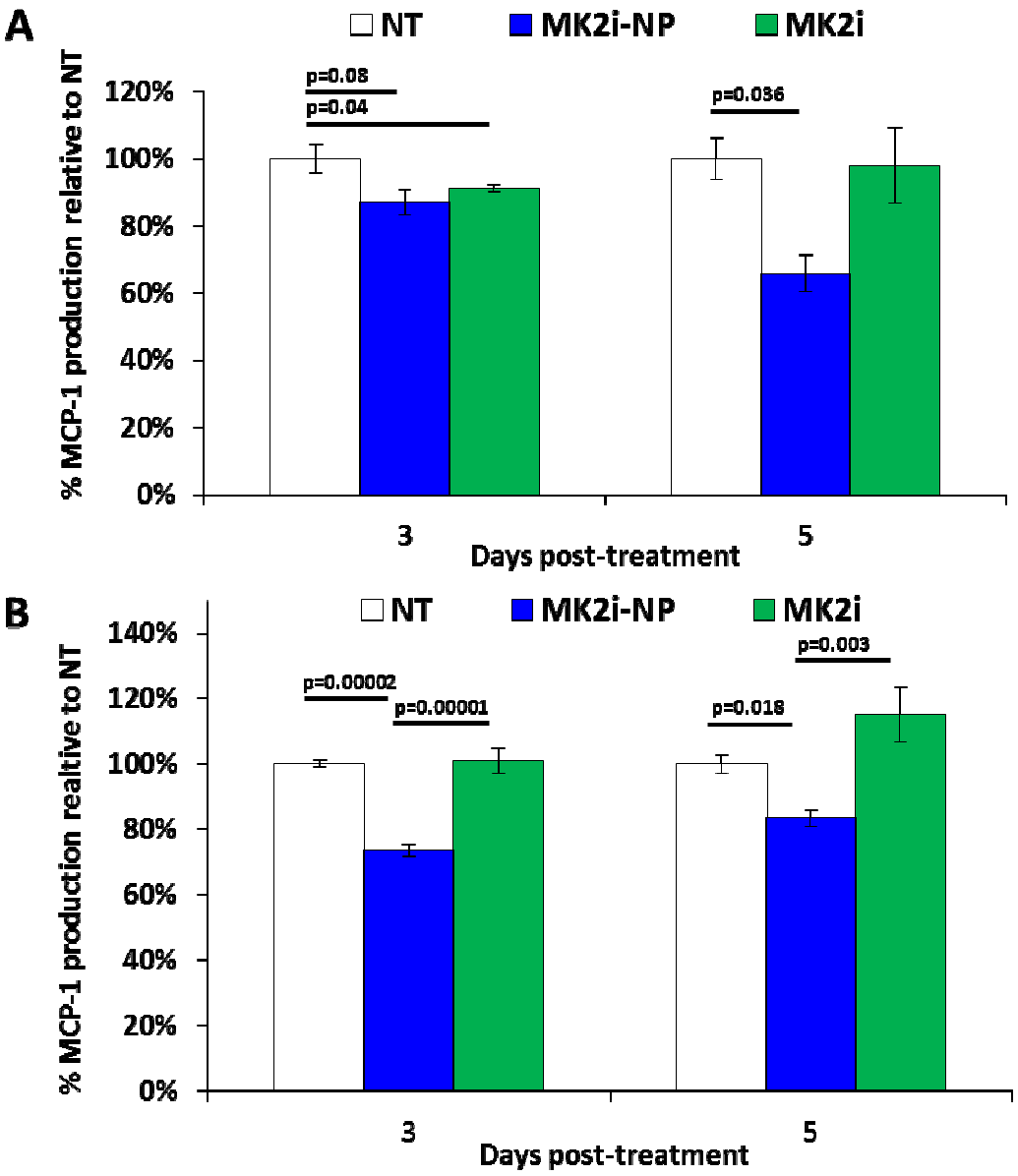

Figure S8: MK2i-NP versus MK2i treatment effects on VSMC and EC MCP-1 production over time. Quantification of MCP-1 production over time relative to untreated controls in both VSMCs (**A**) and ECs (**B**). Cells were treated for 2 hours and then cultured in fresh medium after MK2i treatment removal. After 3 or 5 days, cells were stimulated with 20 ng/ml TNFα for 24 hours and supernatants were collected for cytokine analysis. All treatments used a 10 μM dose of MK2i. Data are means ± SEM (n = 4). P values determined by single factor ANOVA.

SUPPLEMENTARY TABLES

Table S1: NP library characterization. Size summary of MK2i-NPs prepared at different charge ratios ($[NH_3^+]/[COO^-]$) as determined by DLS analysis. Asterisks (*) indicate multimodal size distributions (multiple peaks present). For 1:3 (Alexa), polyplexes were formulated with an Alexa488-conjugated MK2i peptide to use in cellular uptake studies. For 1:3 (NE), polyplexes were formulated with a non-endosomolytic (NE) PAA polymer that does not exhibit pH-dependent membrane-disruptive activity in the endosomal pH range as a vehicle control. Data are means ± peak width.

NH_2:COOH	Z-average diameter (nm)
10:1	10.32 ± 2.63*
2:1	52.1 ± 46.86*
1:1	970.6 ± 662.4
1:1.5	465.1 ± 138.4*
1:2	474.2 ± 32.59
1:3	118.8 ± 26.76
1:4	607.4 ± 285.2*
1:5	213.0 ± 67.95*
1:10	21.57 ± 9.89*
1:3 (Alexa)	168.5 ± 24.63
1:3 (NE)	113.7 ± 14.47
1:3 (NE Alexa)	197.4 ± 12.85