Supplemental Information

Nonionic Peptide Amphiphiles and their Supramolecular Co-Assemblies Tune Charge Density and Bioactivity

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EXPERIMENTAL SECTION

PA synthesis and purification: All PA molecules were synthesized using standard Fmoc synthesis on a Rink amide resin as previously reported.[38] For standard couplings, four equivalents of the amino acid was added in dimethyl formamide with six equivalents of diisopropylethylamine (DIEA) and 3.95 equivalents 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). For the PEG-10 amino acid (ChemPEP, Wellington, FL, USA), 1.5 equivalents were added in dimethyl formamide with an equal concentration of (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and 2 equivalents of DIEA overnight. Following coupling of the palmitic acid tail, peptides were cleaved from the resin in a 95:2.5:2.5 trifluoroacetic acid (TFA)/triisopropylsilane/water mixture for 3 h and precipitated in cold diethyl ether. The crude peptide was dissolved in 0.1% TFA (PA1, PA3, and PA5) or 0.1% ammonium hydroxide (PA2 and PA4) and purified by high-performance liquid chromatography (HPLC). PA1 was purified against a 5% to 95% gradient of water to a mixture of 75% acetonitrile and 25% tetrahydrofuran (THF), while all other PA molecules were purified over a 5 to 95% gradient of water to acetonitrile. All PA molecules were lyophilized following purification. For PA1, the dehydrated peptide was rehydrated and then lyophilized twice to remove any remaining THF. The purity of the PA molecules was confirmed to be above 95% by liquid chromatography - electrospray ionization mass spectrometry using an Agilent 6520 quadrupole time-of-flight (Q-TOF) ESI-MS instrument over a 5% to 95% water to acetonitrile gradient.

Solubilization and heat treatment: PA powders were resuspended to 10 mM in water for experiments at pH 7, in 10 mM hydrochloric acid for pH 2 and 10 mM sodium hydroxide for pH 12. After resuspension, 1 M sodium hydroxide or 1 M hydrogen chloride were added to achieve the desired pH. To co-assemble PA molecules, PA solutions were mixed volumetrically, bath sonicated for 1 hour and then left on the bench to age for at least 2 h. A solution of 150 mM NaCl was added to all samples to achieve a final NaCl concentration of 30 mM. All samples were heat treated at 80 °C for 30 minutes followed by gradual cooling by 1 °C per minute to 25 °C.

Nile red assay: Following heat treatment, PA solutions were serially diluted in water (0 mM NaCl), or a buffer of 15 mM NaCl, or 150 mM NaCl. A solution of 100 μM Nile red was added to the PA solutions to one thousandth the total volume and incubated for 1 h. The fluorescence spectrum was read from 600 nm to 700 nm using a BioTek Cytation 3 microplate reader using 560 nm excitation and the fluorescence shift was determined by subtracting the maximum excitation wavelength for each sample from that of the dye solution diluted in each buffer alone.

X-ray scattering: SAXS and WAXS measurements were obtained simultaneously at the Dupont-Northwestern-Dow Collaborative Access Team Synchrotro n Research Center at the Advanced Photon Source at Argonne National Lab using beamline 5ID-D. Heat treated PA samples were flowed through a 1.5 mm glass capillary at 1 mm/s during x-ray exposure for consistent background subtraction with buffer only samples. Five exposures of 10 seconds were obtained using 17 keV monochromatic X-rays using a CCD detector which was 245 cm behind the sample. The collected two-dimensional scattering images were averaged by azimuthal integration using FIT2D software. Intensities were plotted background subtracted and plotted against the wave vector $q = (4\pi)\sin(\theta/2)$ where $d = 2\pi/q$. Data were fit to a Gaussian function to determine the position of the first minimum in the SAXS pattern.

Circular dichroism: For ionic strength experiments, heat treated PA solutions were diluted 200 times in water, 15 mM NaCl, or 150 mM NaCl prior to measurement. For all other experiments, PA solutions were diluted in a buffer of either 10 mM HCl and 10 mM NaCl, 10 mM NaCl, or 10 mM

NaOH and 10 mM NaCl for measurements at pH 2, pH 7, and pH 12 respectively. CD spectra were acquired on a J-815 CD spectrophotometer (Jasco Analytic Instruments, Easton, MD) in a 2 mm quartz cuvette. The average of 3 measurements over 250 nm to 190 nm was recorded.

Zeta-potential: Heat-treated PA solutions were diluted 20 times in 30 mM NaCl with 10 mM HCl, 30 mM NaCl, or 30 mM NaCl with 10 mM NaOH for pH 2, pH 7, and pH 12 respectively. For each PA, three separate samples were prepared at each pH. Samples were loaded in a disposable folded capillary cell and zeta-potential was measured at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The average of the three measurements for each sample was plotted.

Transmission electron microscopy: TEM was performed on PA solutions preserved in vitreous ice on a JEOL 1230 TEM with a Gatan 831 CCD camera using an accelerating voltage of 100 kV. Samples were diluted with milli-Q water to 1 mM immediately prior to blotting. Copper mesh TEM grids with lacey carbon support (Electron Microscopy Sciences) were treated with glow discharge and then 7 µL of sample was pipetted onto the grid. Samples were blotted twice and plunged into liquid ethane using a Vitrobot Mark IV instrument (FEI) with 95-100% humidity in the chamber at 20 °C. Samples were then transferred to a Gatan 626 cryo-holder under liquid nitrogen for imaging.

Fourier transform infrared spectroscopy: Following heat treatment, PA solutions were lyophilized and then reconstituted in D_2O after which samples were heat treated a second time. For measurement, PA solutions were placed between two CaF_2 windows spaced 50 µm apart and a Bruker Tensor 27 spectrometer was used to measure transmittance. Solvent background was subtracted from the obtained spectrum and plotted against the wavenumber. Data were fit to a Gaussian function to determine the position of the Amide I peak.

Cytotoxicity: Normal human lung fibroblasts were obtained from Lonza and maintained in growth medium consisting of DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells (passage 5) were trypsinized and resuspended in media containing 2% FBS and 150 µL of the cell suspension was added to each well of a 96 well microplate for a total of 2,500 cells per well. After 2 h attachment, 50 µL PA solutions diluted in PBS to 4 µM, 20 µM, 100 µM, or 400 µM were added to each well. After 36 h culture, the media was removed and replaced with a solution of 2 µM calcein and 4 µM ethidium homodimer in PBS. After 30 min, cells were imaged using a Cytation 3 microplate reader. Live and dead cells were counted using the MATLAB image Processing Toolbox to determine viability.

Alkaline phosphatase activity: Human mesenchymal stem cells were obtained from Lonza and maintained in Mesenchymal Stem Cell Growth Media (Lonza). Cells were used for experiments prior to passage 6. Cells were trypsinized and resuspended in growth media containing high glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and 50 mg/L sodium ascorbate. 500 µl of the cell suspension was placed in each well of a 24 well plate for a total of 10,000 cells per well and incubated for 24 hours. Media was removed and replaced with 540 µL of osteogenic media containing low glucose DMEM supplemented with 10% FBS, 50 mg/L sodium ascorbate 10 mM β-glycerophosphate, and 100 nM dexamethasone. 60 µL of a 10 times concentrated PA solution in PBS was then added to each well. Every 3-4 days, media was replaced by removing 300 µL media and adding 270 µL growth media or osteogenic media and 30 µL of the 10 times concentrated PA solution in PBS. Alkaline phosphatase (ALP) activity was determined using the SensoLyte pNPP ALP Assay Kit (Anaspec, Fremont, CA) according to the manufacturer's instructions. Briefly, cells were washed once with the assay buffer and the 200 µl of the assay buffer supplemented with Triton-X was added to each well. The plate was incubated for 1 h at 4 °C under mild agitation, cells were scraped with a pipette tip and collected, and the suspension was centrifuged for 10 min at 5,000 g.

The supernatant was mixed with the assay's detection buffer in a microplate and the optical absorbance was read after a four-hour incubation using a Cytation 3 microplate reader. The supernatant was also used to determine DNA concentration using a Quant-iT PicoGreen dsDNA kit (Molecular Probes, Eugene, OR). ALP concentration was normalized to DNA concentration for each sample. Results were compared using a 2-way ANOVA with Bonferroni post-hoc analysis to compare ALP activity relative to the no PA control sample at each timepoint.

Fluorescence microscopy: Human MSCs were cultured on glass coverslips and treated as described for ALP assay experiments for two weeks. Cells were then fixed in 4% paraformaldehyde in PBS and were permeabilized with a solution 2% horse serum and 0.2% Triton X-100 in PBS for 2 h. Cells were stained for 6 h with phalloidin (100x dilution) and for 2 h with propidium iodide (500x dilution). Imaging was performed using a TissueGnostics microscope with a 20x objective.

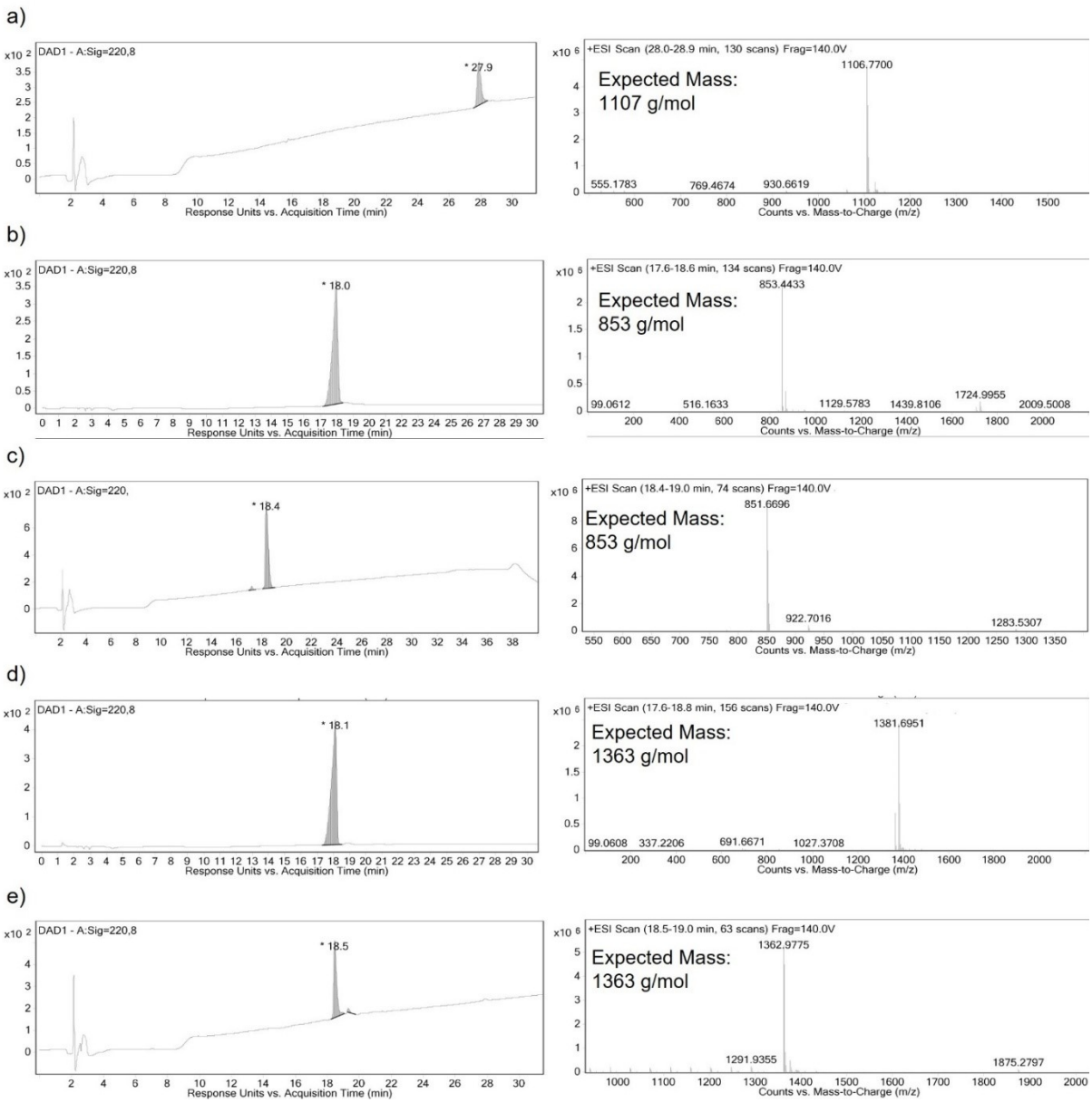

Figure S1. LC-MS and MS of highlighted peak to confirm purity of (a) PA1, (b) PA2, (c) PA3, (d) PA4, and (e) PA5.

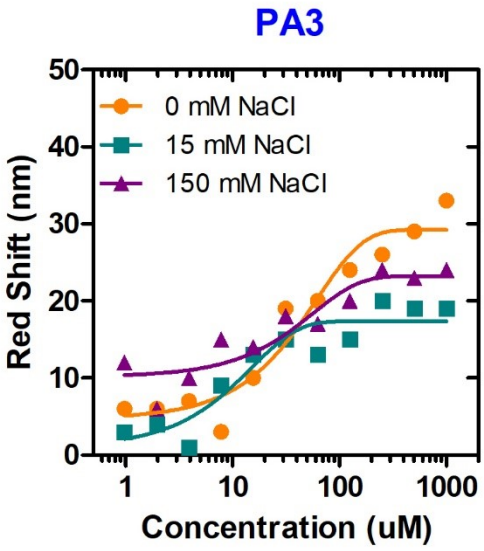

Figure S2. Blueshift of peak Nile red fluorescence as a function of PA3 concentration in buffers of 0 mM NaCl, 15 mM NaCl, and 150 mM NaCl.

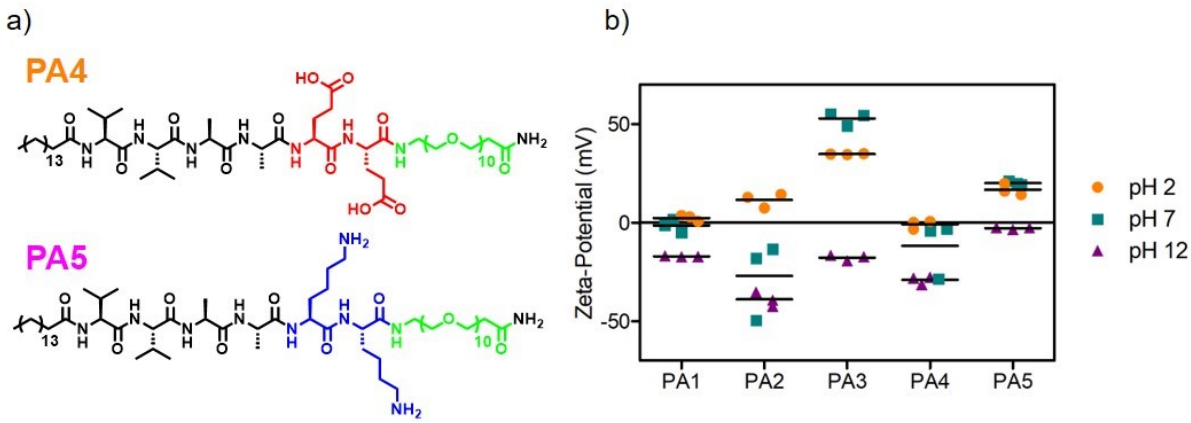

Figure S3. (a) Structures of anionic, PEG-appended PA4 and cationic, PEG-appended PA5. (b) Zeta-potential measurements for all PA molecules tested.

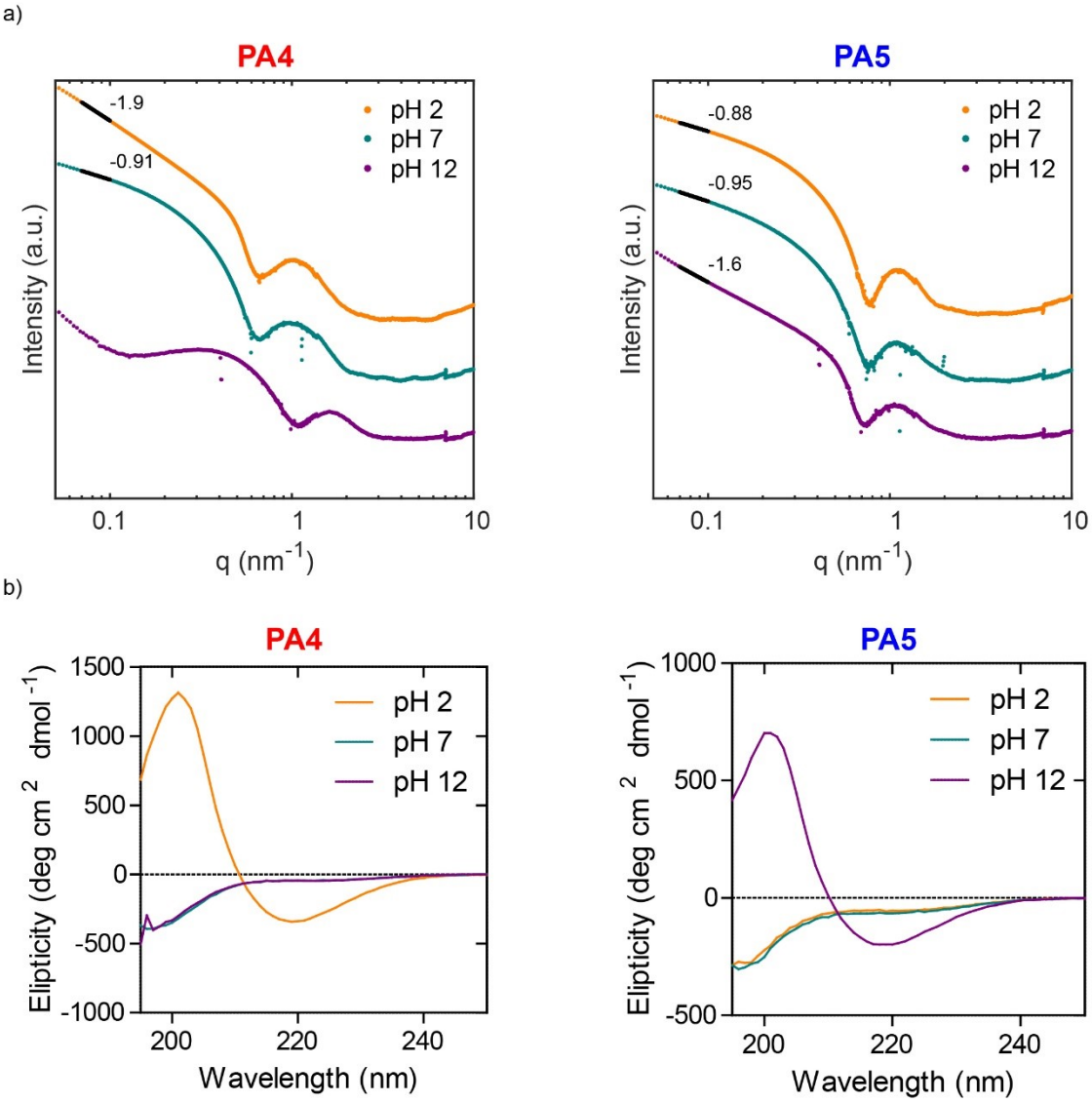

Figure S4. (a) Small-angle X-ray scattering intensity as a function of the wave vector for PA4 and PA5 solutions at pH2, pH 7, and pH 12. (b) Circular dichroism as a function of wavelength for PA4 and PA5 solutions at pH2, pH 7, and pH 12 in 30 mM NaCl.

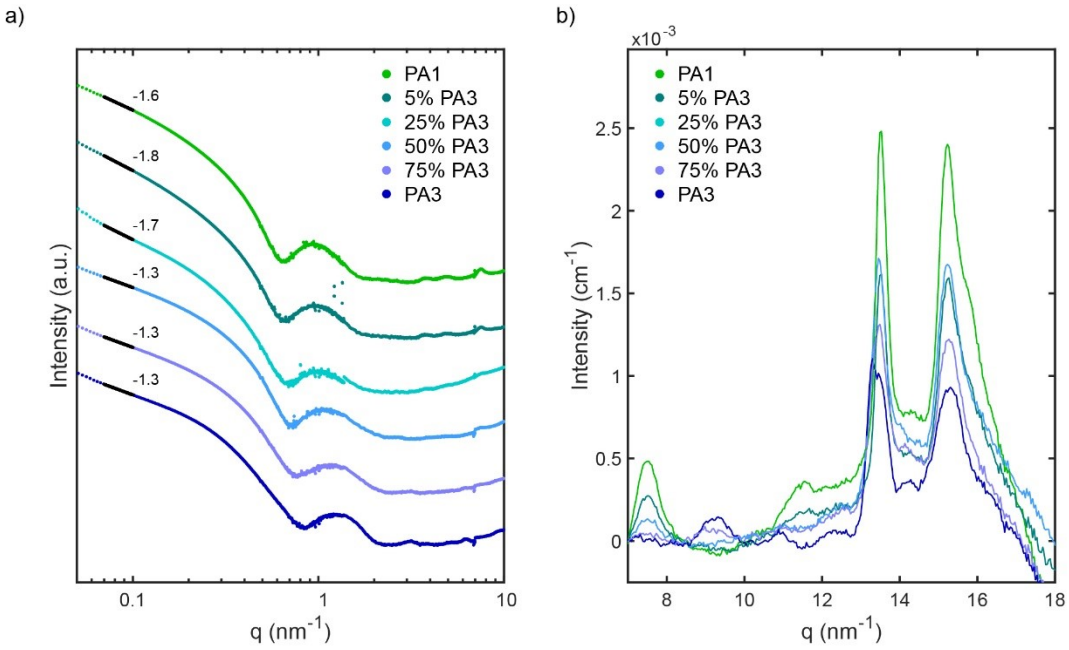

Figure S5. (a) SAXS intensities of co-assembled systems of PA1 and PA3 as a function of the wave vector. (b) WAXS intensities of the co-assembled systems of PA1 and PA3 as a function of the wave vector.

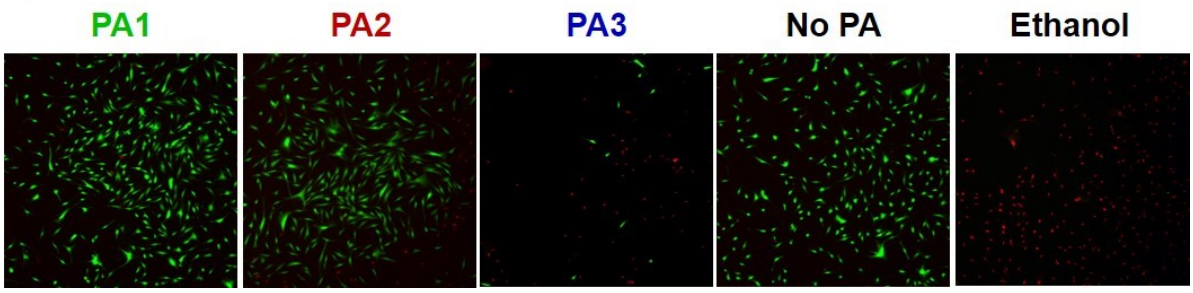

Figure S6. Fluorescence imaging of nHLF cultures with live cells stained with calcein (green) and dead cells stained with ethidium homodimer (red) after 36 h treatment with 100 µM PA1, PA2, or PA3 compared to cells with no PA treatment and cells with no PA treatment treated with 20% ethanol 30 min prior to staining.

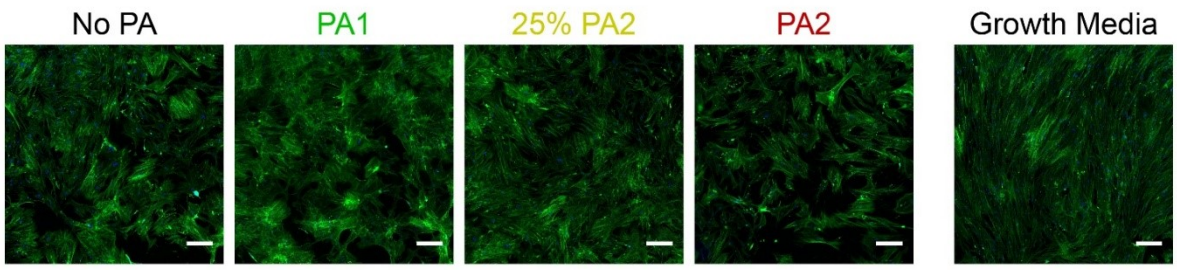

Figure S7. Fluorescence microscopy images of human MSCs cultured for 14 days in osteogenic media supplemented with 100 µM PA solutions or in growth media with nuclei stained in blue and actin stained in green; scale = 200 µm.

Table S1. Parameters obtained by fitting Nile red assay results to an exponential function of the form $Shift = Shift_0 + (Plateau - Shift_0)(1 - e^{-K \times Conc.})$ where Shift is the blueshift as a function of the PA concnentration, Conc., $Shift_0$ is the y-intercept of the function, K is a constant, and Plateau is the maximum blueshift. The Half-shift is the PA concentration where half the total blueshift occurs.

	PA1 + 0 mM NaCl	PA1 + 15 mM NaCl	PA1 + 150 mM NaCl	PA2 + 0 mM NaCl	PA2 + 15 mM NaCl	PA2 + 150 mM NaCl
$Shift_0$	**-0.6048**	**-5.197**	**11.75**	**12.42**	**10.06**	**11.75**
Plateau	**36.32**	**29.91**	**33.41**	**46.41**	**42.07**	**44.2**
K	**0.1099**	**0.08027**	**0.03334**	**0.006842**	**0.009093**	**0.04009**
Half-shift	**6.306**	**8.635**	**20.79**	**101.3**	**76.23**	**17.29**
$Shift_0$ Std. Error	**2.585**	**2.064**	**1.047**	**1.643**	**1.529**	**1.912**
Plateau Std. Error	**1.093**	**1.041**	**0.8078**	**2.699**	**2.147**	**1.357**
K Std. Error	**0.0203**	**0.01369**	**0.005867**	**0.001779**	**0.002151**	**0.008229**
R square	**0.9705**	**0.9755**	**0.9754**	**0.9507**	**0.9581**	**0.9666**