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Materials and General Methods. Fmoc-protected amino acids were purchased from Novabiochem. PL-Rink resin was purchased from Polymer Laboratories. 1H-Benzotriazolium 1-[bis(dimethylamino) methylene]-5chloro-hexafluorophosphate (1-),3-oxide (HCTU) was obtained from Peptides International. Trifluoroacetic acid was obtained from Acros organics, and 1,2-ethanedithiol was purchased from Fluka. Oregon Green 514 Phalloidin, 6 and 24 well cell culture plates, polystyrene microcuvettes, diethyl ether, dimethylformamide (DMF), acetonitrile (ACN), N-methylpyrrolidone (NMP), Slide-A-Lyzer™ dialysis cassettes (MWCO 3.5K) and 96-well half area high content imaging glass bottom microplates were purchased from Fisher Scientific. Perfluoro-n-pentane (PFP) was purchased from Strem Chemicals. Thioanisole, anisole, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 2,2'-dithiodipyridine (DTP), dimethyl sulfoxide (DMSO), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 200 mM glutamine solution were obtained from Sigma-Aldrich. RPMI-1640 media, Hanks Balanced Salt Solution (HBSS) and Hoechst 33342 trihydrochloride dye was purchased from Invitrogen. Heat inactivated fetal bovine serum (FBS) and trypsin EDTA were obtained from Hyclone Laboratory Inc. HPLC solvents consisted of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in ACN). The A549 cancer cell line was obtained from the NCI-60 repository. All peptides utilized for experiments were prepared with an amidated C-terminus.

Peptide Synthesis. Fmoc-based solid-phase peptide chemistry was used to prepare the peptides, with HCTU activation on PL-Rink resin using an automated ABI 433A peptide synthesizer. Peptides were cleaved from the resin and simultaneously side-chain deprotected using a trifluoroacetic acid/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2) cocktail for 2 hours under argon atmosphere. The crude product was precipitated with cold diethyl ether and then lyophilized. Peptides were purified via reverse-phase HPLC equipped with a FluoroFlash® semi-preparative column composed of silica gel bonded with perfluorooctylethylsilyl ($Si(CH_2)_2C_8F_{17}$). A gradient of 0-50% solvent B over 25 min., followed by 50-100% solvent B over an additional 50 min. was utilized. All peptides were lyophilized to collect the pure product, and the purity verified by analytical HPLC-MS.

Nano-peptisome Formation. Peptides were weighed out as a dry fluffy solid in a round bottom flask, and dissolved in a volume of 1:1 DMF:ACN containing 1% TFA to a final concentration of 0.5 – 2.0 mg/mL. The solution was stirred at 1,000 rpm on ice for 15 min. before addition of 1% – 2% (v/v) cold PFP. After an additional 5 min. of stirring to properly mix the components and create an emulsion, an equal volume of cold MilliQ water was slowly added dropwise. During this solvent exchange procedure the solution turned opaque due to self-assembly of the peptides at the interface of the water-PFP emulsion. The mixture was stirred at 1,000 rpm for 1 hour on ice, over which time the solution clarified. Unincorporated peptide was removed by dialyzing the mixture against MilliQ water containing 2.5% (v/v) DMSO to oxidize the cysteines and facilitate disulfide cross-linking of the peptisome nanodroplets. In addition, a Pasteur pipette was used to gently bubble air into the media to further promote oxidation. Dialysis was performed for 12 hours, with exchanges every four hours. Two final exchanges of the dialysis media to pure MilliQ water, for 2 hours each, removed residual DMSO. The purified nanodroplets were removed from the dialysis cassette, placed into a clean glass vial and used for experiments within 48 hours.

Physicochemical Characterization. Particle size and zeta potential measurements were performed via dynamic light scattering using a Zetasizer Nano-ZS instrument (Malvern, Worcestershire, UK). For size determination, a solution of peptisomes in water was diluted times into characterization buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.4) to reach a final volume of 1mL in a clean polystyrene microcuvette. The size of pure 1-2 vol% PFP emulsions prepared in 1:1 DMF:ACN containing 1% TFA were also measured as controls. Three independent measurements, ten runs each, were taken at a 175° scattering angle, a sample position of 4.65 mm and an attenuation of 11. Particle size was recorded at both 25°C and 37°C, with a 2 min. sample equilibration time. Material refractive index (RI) was set at 1.59 (25°C) and 1.45 (37°C) using pre-defined settings provided by the manufacturer. Dispersant RI of 1.332 and viscosity [cP] equal to 0.9103 (25°C) and 0.7096 (37°C) were calculated using the 'Solvent Builder' tool in the Zetasizer software. Phase analysis light scattering (PALS) assisted zeta potential measurements were performed by adding the solution of peptisomes to MilliQ water to achieve a ten-fold dilution, and loading 700 µL of the sample into a disposable folded capillary cell (Malvern, DTS1070). Three independent measurements were taken at 25°C, with twenty runs each.

In separate studies, the stability of nano-peptisomes during storage was evaluated via dynamic light scattering. Here, purified particles (formulation #5) were dispersed into milliQ water and left at room temperature. At defined time points over 15 days an aliquot was removed, diluted ten times into characterization buffer, and particle size and count rate recorded at 25°C. Of note, count rate was used as a qualitative indicator of particle density and thus an estimate of stability over time. In parallel experiments, the same particles were initially diluted ten times into blank characterization buffer, or buffer supplemented with 5% fetal bovine serum, and incubated at 37°C to evaluate their stability under physiologic conditions. At defined time points over 48 hours a 1mL aliquot was directly added to a clean polystyrene microcuvette and particle size measured at 37°C. For both experiments, three independent measurements were taken with twenty runs each.

Peptisome Visualization. Differential interference contrast (DIC) microscopy was used to image the nanodroplets in solution. Briefly, peptisomes were diluted two times into characterization buffer and added to 96-well glass bottom high-content imaging microplates. The plates were then loaded onto an LSM 710 confocal microscope (Zeiss, Thornwood, NY) equipped with a temperature controlled humidified chamber. Images were collected at 25°C and 37°C, with a 15 min. sample equilibration time, using a 63x Plan-Apochromat oil objective.

Disulfide Formation Assay. A 1.5 mL solution of freshly prepared peptisomes in water (0.5 mg/mL peptide and 2% PFP) was placed in a round bottom flask and slowly stirred with gentle bubbling of air. A 2.5% volume of DMSO was added to oxidize the thiols and initiate cross-linking. At specific time points, a 30 µL aliquot of the mixture was diluted into 200 µL of 0.1 mM DTP in characterization buffer, and allowed to react for 10 min. The solution was then transferred to a quartz cuvette (1 cm pathlength) and concentration of the free thiolate was determined via absorption at 343 nm (ε_{343} = 7600 cm^{-1} M^{-1})[1] using an Agilent 8453 UV-Vis spectrophotometer (Santa Clara, CA). In separate control experiments, the same procedure was followed without the addition of 2.5% (v/v) of DMSO to evaluate its influence on thiol oxidation and disulfide cross-linking of nano-peptisomes. All values were corrected for background DTP hydrolysis. Percentage

of disulfide formation was calculated by subtracting the concentration of free thiolate from the initial cysteine concentration. Studies were performed in triplicate.

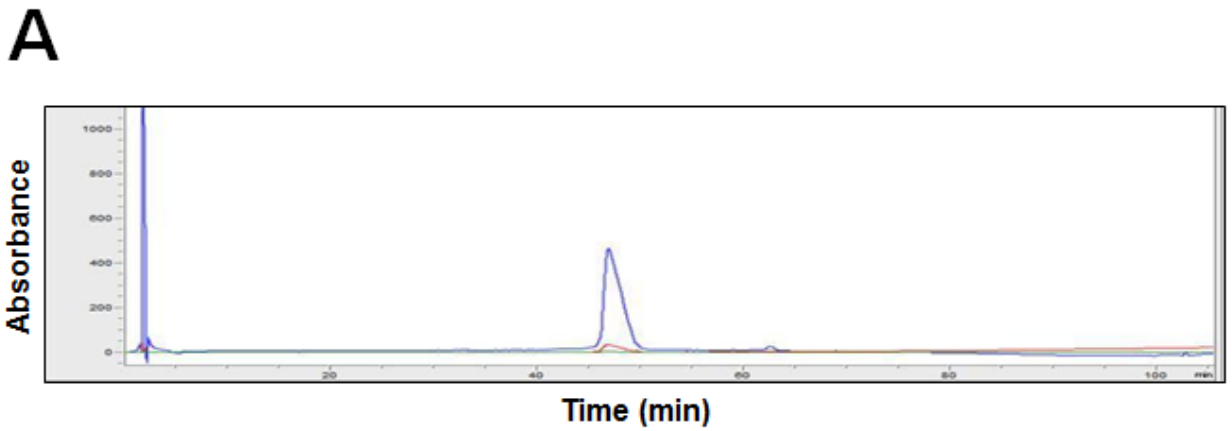
Phalloidin Loading and Ultrasound Delivery. Nano-peptisomes were prepared at 0.5 mg/mL peptide and 2% PFP containing fluorescently-labeled Phalloidin and used for *in vitro* experiments within 24 hours of their preparation. Briefly, 500 µL of cold PFP was added to 300 U of Oregon Green 514 Phalloidin and stirred vigorously on ice for 3 hours. An aliquot of the PFP-Phalloidin mixture was then added to the peptide solution, and peptisomes formed and purified as described above. To evaluate encapsulation efficiency, a 100 µL aliquot of phalloidin-loaded nano-peptisomes in water was added to a clean quartz microcuvette and UV spectra collected from 200 – 1000 nm. Absorbance maxima of Oregon green 514 at 506 nm allowed for the calculation of phalloidin concentration using the manufacturer reported extinction coefficient for the fluorescent label (ThermoFisher Scientific, ε = 85,000 $M^{-1}cm^{-1}$). To calculate per particle loading of phalloidin, nano-peptisome concentration (formulation #5) was measured in water using a Malvern NanoSight LM10 with a camera level of 9, slider shutter of 607, slider gain of 15 and 25 FPS. Analysis settings included a detect threshold of 5 and auto blur size. For these experiments, un-loaded particles were measured to avoid interference of the fluorescently-labeled cargo with the NanoSight particle tracking software.

For flow cytometry studies, A549 cells were suspended in HBSS and added to 24 well plates at 2 x 10^5 cells/well. Phalloidin-loaded peptisomes, diluted in HBSS, were added to the cell suspension to achieve a final density of 2.24 x 10^7 particles/mL in a total volume of 500 µL. Plates were rocked in the dark at 70 rpm and 37°C for four hours to allow for cellular binding of the nanodroplets. This incubation period was selected based on previous work indicating a 4 hour incubation of cancer cells with RGD-labeled nanoparticles is sufficient to afford integrin-mediated cell surface binding.[2] After this time, the cells were washed with warm HBSS to remove unbound peptisomes and 500 µL of fresh HBSS added. As a control, a volume of Phalloidin-PFP, similar to that used in formulation of Phalloidin-loaded peptisomes, was diluted in HBSS at a total volume of 500 µL and added to separate plates containing A549 cells. Insonation of the samples was performed using a Vevo® Sonigene™ low frequency ultrasound device (Visualsonics, Toronto, ON, Canada), equipped with a 1 MHz, 10 mm diameter transducer with an unfocused beam. The transducer was fixed to a ring stand, and lowered until it touched the surface of the sample solution (~0.5cm from the bottom of the plate). Ultrasound was applied for 90 sec at a duty cycle of 10% – 20% with intensity varied between 0.1 – 1.0 W/cm^2, corresponding to a peak negative pressure of 0.054 – 0.172 mPa, respectively. Samples were then placed in the incubator for 15 min. before addition of 500 µL of fresh HBSS. Cells were then collected and placed on ice, before analysis using a Beckman Coulter FACsCalibur flow cytometer (488 nm excitation laser). Gating was based on normalized fluorescence of untreated cells to evaluate the fluorescence increase of cells treated under the different conditions. Studies were performed in triplicate for each experimental condition.

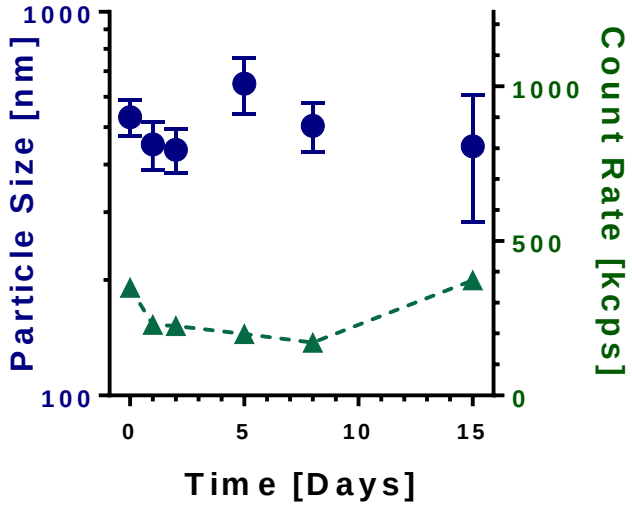
In separate experiments, A549 cells were seeded onto 6 well plates at 5 x 10^5 cells/well and allowed to adhere overnight. Cells were washed with fresh HBSS, followed by addition of 3 mL of HBSS containing Phalloidin-loaded peptisomes at a concentration of 2.24 x 10^7 particles/mL. After a four hour incubation period the cells were washed with HBSS and 3 mL of fresh buffer

added to each well. Ultrasound was applied at 0.5 W/cm^2, 20% duty cycle for 90 sec. to each well, and plates incubated for 15 min. During this time 2 µg/mL Hoechst 33342 dye was added to the solution to stain cell nuclei. To evaluate co-localization of delivered phalloidin and endosomes, a 30 µL aliquot of 5 mg/mL Texas Red-Transferrin (ThermoFisher Scientific) in milliQ water was added to treated cells to achieve a final concentration of 50 µg/mL, and plates incubated for 30 min. Following removal of the supernatant, washing with warm HBSS and addition of 3mL fresh buffer, plates were mounted onto an EVOS FL Auto fluorescent microscope (Life Technologies, Grand Island, NY) equipped with an environmental chamber to maintain 37°C and 5% CO_2 during experiments. Cells were imaged at 10X or 20X magnification using the manufacturer LED light cubes for DAPI (357/44 nm excitation, 447/60 nm emission), GFP (470/22 nm excitation, 510/42 nm emission) and Texas Red (585/29 nm excitation, 628/32 nm emission).

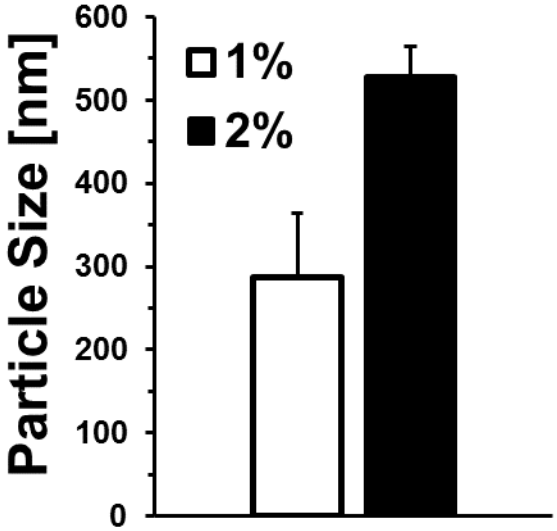
Cell Viability Assay. A549 cells were seeded onto 6 well plates at 5 x 10^5 cells/well and allowed to adhere overnight. Cells were then washed and 3 mL of warm HBSS added to each well. Ultrasound was applied for 90 sec at a duty cycle of 10% – 20% with intensity varied between 0.1 – 1.0 W/cm^2, corresponding to a peak negative pressure of 0.054 – 0.172 mPa, respectively. Wells not subjected to US, or cells incubated with 25% DMSO in HBSS for 1 hour, were used as negative and positive controls, respectively. Following US insonation, cells were incubated for 30 min. to recover and then washed with warm HBSS. 3mL of a 0.5 mg/mL solution of MTT reagent in HBSS was added to each well and incubated for 2 hours. The supernatant was removed and replaced with 3 mL of DMSO to dissolve the formazan product, followed by transfer of a 100 µL aliquot of the colored solution to a 96 well plate. Absorbance was then read at 540 nm using a UV plate reader (Biotek, Winooski, VT). The absorbance of negative controls was subtracted from each sample as a blank, and percent viability calculated using the equation: $(\text{Absorbance}_{\text{US-treated cells}}/\text{Absorbance}_{\text{untreated cells}}) \times 100$. Results shown represent the average of three independent experiments ± standard deviation.



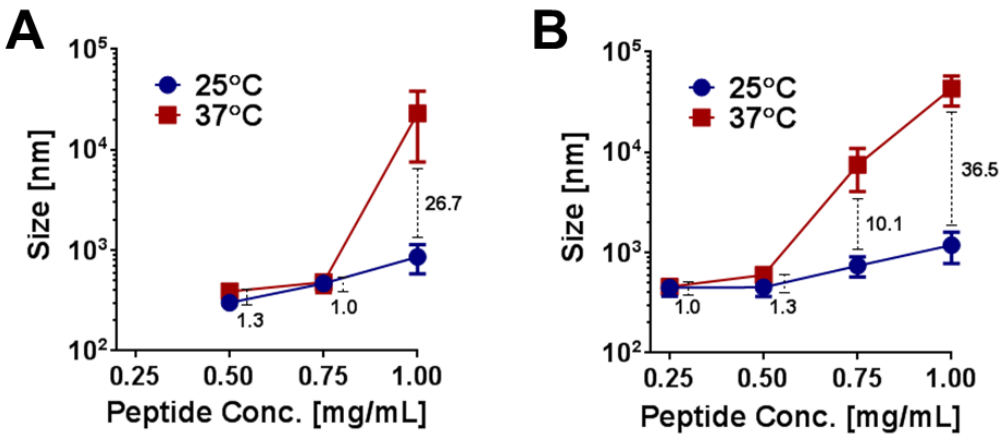
Supporting Figure 1: (**A**) Analytical HPLC (FluoroFlash $Si(CH_2)_2C_8F_{17}$; 0% - 100% B over 100 min. at 25°C) and (**B**) ESI (+) mass spectrum of purified $F_FF_FF_FGGGCCGGKGRGD\text{-}NH_2$ peptide.



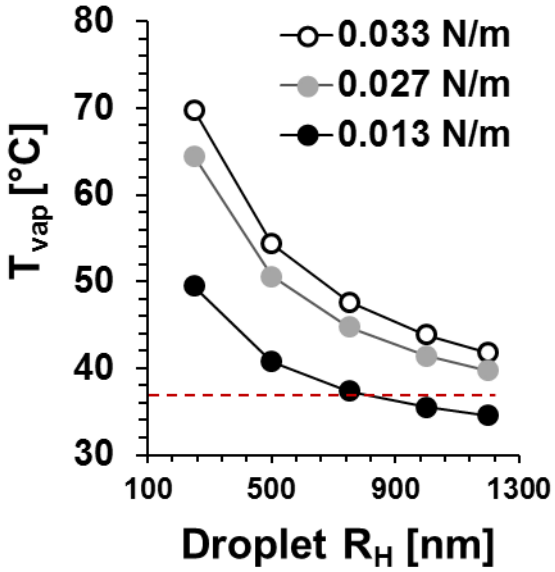
Supporting Figure 2: Stability of nano-peptisomes (formulation #5) during long-term storage in water at room temperature. Particle stability was measured via DLS over 15 days, and reported as particle size (left, blue circles) and count rate (right, green triangles).



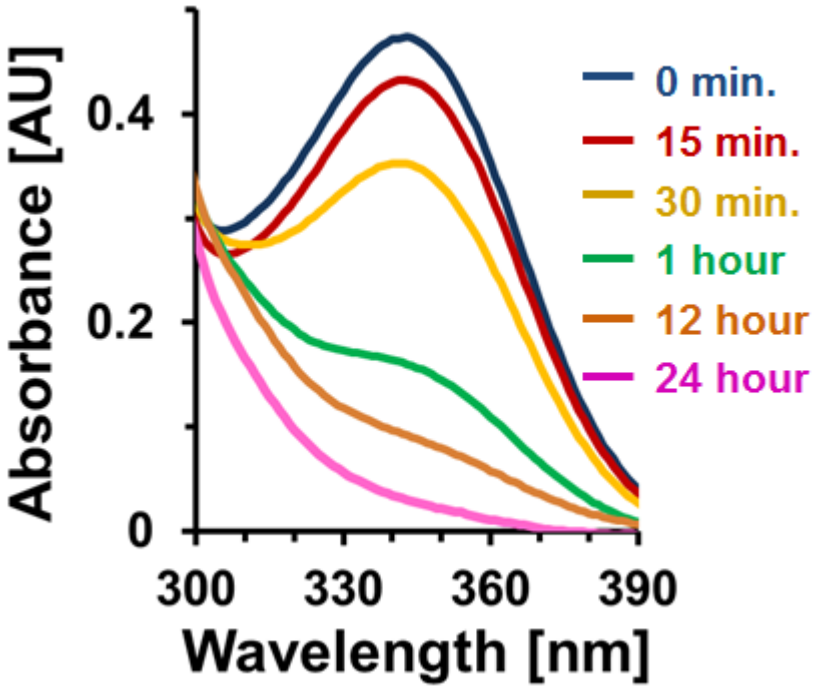
Supporting Figure 3: Particle size of 1 and 2 vol% PFP immediately after emulsion formation in the nano-peptisome pre-assembly solution (1:1 DMF:ACN, 1% TFA).



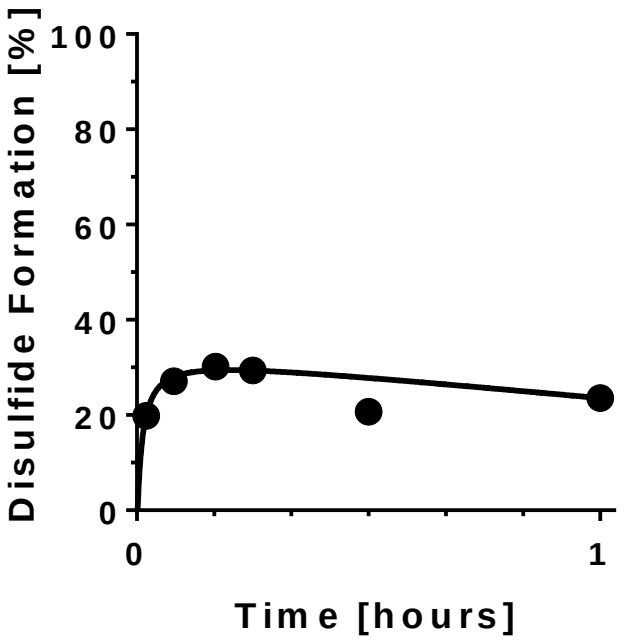
Supporting Figure 4: Temperature and formulation dependent size of nano-peptisomes prepared at 0.25 – 1.00 mg/mL peptide concentration and (**A**) 1% or (**B**) 2% v/v of PFP. Folds change in particle size between 25°C (blue) and 37°C (red) is represented next to the dashed line. Trendlines are included to guide the eye.



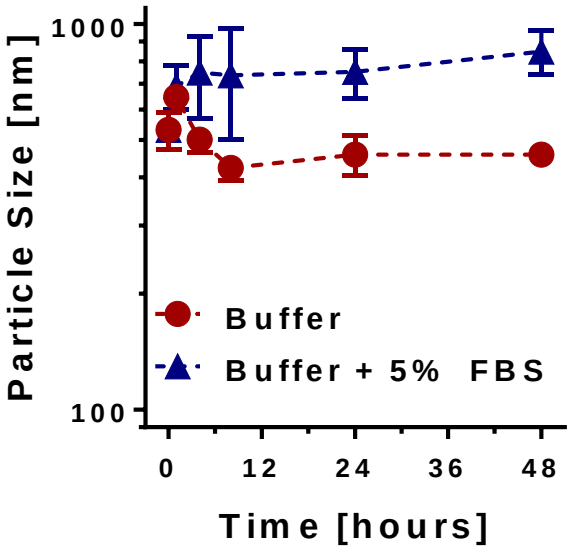
Supporting Figure 5: Relationship between PFP vaporization temperature (T_{vap}) and nanodroplet size (R_H, hydrodynamic radius), modeled at three different reported surface tension values for PFP emulsions formulated with the BSA protein (open circles), PEO-PLA polymer (gray circles) or CTAB surfactant (filled circles). Dashed red line indicates physiologic temperature (37°C). Of note, CTAB most closely resembles the physicochemical properties of the peptide surfactant utilized in this work.



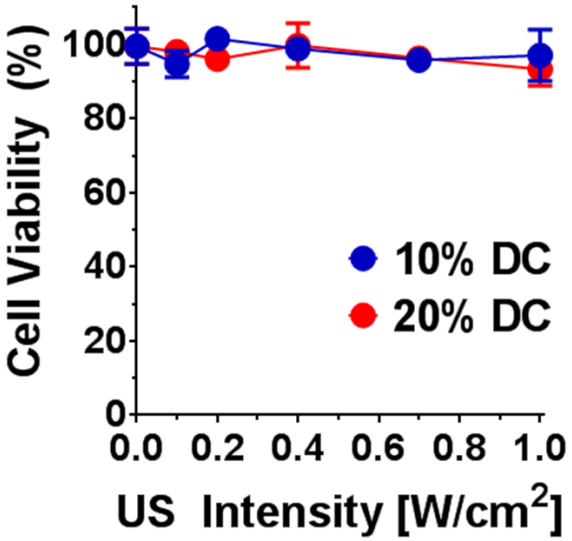
Supporting Figure 6: Time-dependent reduction of UV absorbance of the 2-mercaptopyridine reporter during disulfide exchange with 2,2'-dithiopyridine, monitoring the nano-peptisome disulfide bond formation.



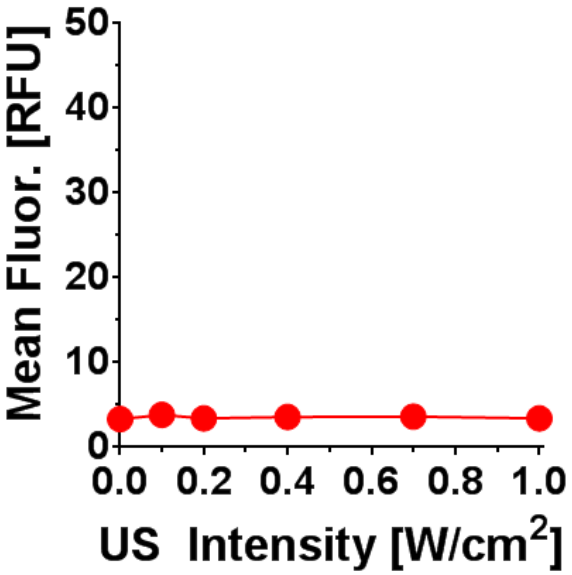
Supporting Figure 7: Percentage of disulfide cross-linking as a function of time following suspension of freshly prepared nano-peptisomes (formulation #5) in pure water. After 1 hour, particles could not be measured by dynamic light scattering under these conditions.



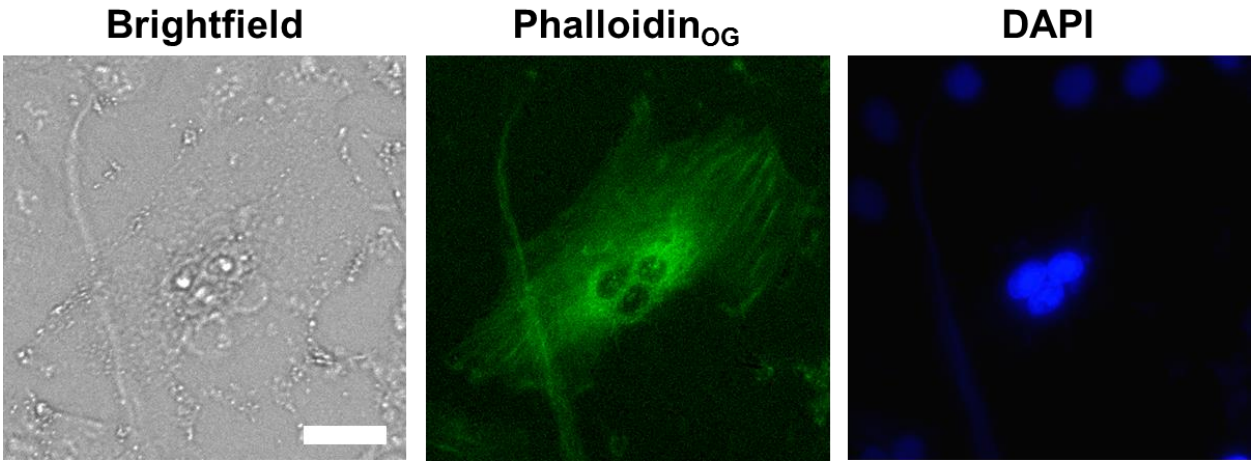
Supporting Figure 8: Stability of nano-peptisomes (formulation #5) suspended in blank characterization buffer (25mM Tris-HCl, 150mM NaCl, pH 7.4; red), or the same buffer supplemented with 5% fetal bovine serum (blue), and incubated at 37°C for 48 hours. Particles incubated with 5% FBS showed a small increase in size to approximately 700nm, most likely due to physical adsorption of serum proteins to the cationic particle surface. Particle count rates were 150 - 200 kcps for both conditions during the incubation period, suggesting nano-peptisomes remained stable in physiologic milieu.



Supporting Figure 9: Viability of A549 cells subjected to insonation at 0.0 – 1.0 W/cm^2 intensities, using either a 10% or 20% US duty cycle (DC).



Supporting Figure 10: Mean fluorescence (in relative fluorescence units; RFU) of A549 cells treated with Oregon Green 514-labeled phalloidin dispersed in PFP, as a function of applied ultrasound intensity (20% duty cycle).



Supporting Figure 11: Brightfield and fluorescence channels of a live A549 cell following US-mediated delivery of oregon green$_{514}$-phalloidin (Phalloidin$_{OG}$) from nano-peptisomes. Successful delivery of bioactive phalloidin is confirmed by its ability to stain intracellular filamentous actin. Scale bar = 30 µm.

Supporting References

1. Haines, L. A.; Rajagopal, K.; Ozbas, B.; Salick, D. A.; Pochan, D. J.; Schneider, J. P., Light-Activated Hydrogel Formation via the Triggered Folding and Self-Assembly of a Designed Peptide. *Journal of the American Chemical Society* **2005,** *127* (48), 17025-17029.
2. Majumder, P.; Bhunia, S.; Bhattacharyya, J.; Chaudhuri, A., Inhibiting tumor growth by targeting liposomally encapsulated CDC20siRNA to tumor vasculature: Therapeutic RNA interference. *Journal of Controlled Release* **2014,** *180*, 100-108.