Electronic Supplementary Material (ESI) for Nanoscale.

Electronic Supplementary Information

Promoting intracellular delivery of sub-25 nm nanoparticles via defined levels of compression

Hongrong Yang,[a] Yifei Yao,[a] Huize Li,[a] Lok Wai Cola Ho,[a] Bohan Yin,[a] Wing-Yin Yung,[b] Ken Cham-Fai Leung,[b] Arthur Fuk-Tat Mak,[a] and Chung Hang Jonathan Choi*[a]

[a] Department of Biomedical Engineering, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China.

[b] Department of Chemistry, Hong Kong Baptist University, Kowloon, Hong Kong, China

*To whom correspondence should be addressed. E-mail: jchchoi@cuhk.edu.hk.

Materials and Methods

Chemicals and reagents: Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$, ≥99.9%, Sigma); sodium citrate (anhydrous, 99%, Alfa Aesar); sodium borohydride ($NaBH_4$, 99.99%, Sigma); tannic acid (ACS reagent, Sigma); methoxy-poly(ethylene glycol)-thiol (mPEG-SH, Mw=1000, JenKem Technology); Cyanine 5-PEG-thiol (Cy5-PEG-SH, Mw=1000, Ponsure Biotechnology); agarose (Regular agarose G-10, Biowest); Ellman's reagent [5,5'-Dithio-bis-(2-nitrobenzoic acid), 99%, J&K Scientific]. 18.2 MΩ·cm Nanopure water obtained from the Barnstead™ Smart2Pure™ system (Thermo) was used in all steps. All glassware was cleaned by using *aqua regia* [3:1 volumetric ratio of 37% concentrated HCl (VWR chemicals) to 68% concentrated HNO_3 (VWR Chemicals)] followed by rinsing with copious amounts of Nanopure water.

Synthesis of gold nanospheres of different sizes (AuX NPs): To synthesize AuNPs of 3 nm in diameter (X = 3),[1] 100 µL of 50 mM $HAuCl_4$ and 129 µL of 38.8 mM sodium citrate (1% w/v) was added into 20 mL of Nanopure water. The mixture immediately turned brownish orange after injection of 0.6 mL of 0.1 M freshly prepared $NaBH_4$ under rapid stirring. The resultant solution was stirred for 30 min, followed by incubation at 60°C oven for another 30 min.

To synthesize AuNPs of 6 nm in diameter (X = 6),[2] 50 mL of 0.01% (w/v) $HAuCl_4$ was brought to boil in a 125 mL flask for 6 to 7 min. A reducing agent containing 1 mL of 38.8 mM sodium citrate (1% w/v) and 225 µL of freshly prepared 1% (w/v) tannic acid was injected into the boiling solution under vigorous stirring. The solution immediately changed to dark violet and then wine red in a few seconds. Boiling of the reaction mixture continued for another 5 min before cooling down to room temperature.

AuNPs of 13 nm in diameter (X = 13) was synthesized by following the classic Frens' method.[3] Briefly, 50 mL of 1 mM $HAuCl_4$ was brought to boil, followed by a rapid injection of 5 mL of 38.8 mM sodium citrate under vigorous stirring. The reaction was stopped after 15 min.

Synthesis of AuNPs of 20 nm in diameter (X = 20) followed the seed-mediated method.[4] 150 mL of 2.2 mM sodium citrate was heated to boil in a three-necked round bottom flask with rapid stirring, followed by an injection of 1 mL of 25 mM $HAuCl_4$. The solution became orange red in about 15 min. Later, the reaction was cooled down to 90°C and maintained at this temperature. Then, 1 mL of 60 mM sodium citrate and 1 mL of 25 mM $HAuCl_4$ was sequentially injected (with a time delay of ~2 min). After 30 min, 0.5 mL of AuNPs was extracted from the flask for UV-Vis measurement. Addition of sodium citrate and $HAuCl_4$ was repeated until the surface plasmon resonance (SPR) peak of the AuNP solution reached 522 nm.

Quantification of PEG strands on AuNPs: The density of PEG strands attached to the AuNP surface (in terms of number of PEG strands per NP or per nm^2) was determined based on the thiol depletion approach by using Ellman's assay.[5] After PEGylation of AuNPs of different sizes, the supernatant containing free mPEG-SH strands was obtained after centrifugation. Then, 20 µL of concentrated supernatant was added to 100 µL of assay buffer (1 mM EDTA and 0.1 M sodium phosphate dibasic; pH=8). 50 µL of detection buffer (0.5 mg/mL Ellman's reagent formulated in assay buffer) was added into the mixture. Known concentrations of mPEG-SH were used as standards. The reaction was allowed to proceed for 10 min and the absorbance at 412 nm was recorded on a UV-absorbance plate reader (Thermo Scientific Multiskan GO). The PEG amount on the AuNP surface was calculated by subtracting the PEG amount in the supernatant from the total PEG amount initially added. The PEG density was calculated by dividing the number of PEG strands by the surface area and number of AuNPs.

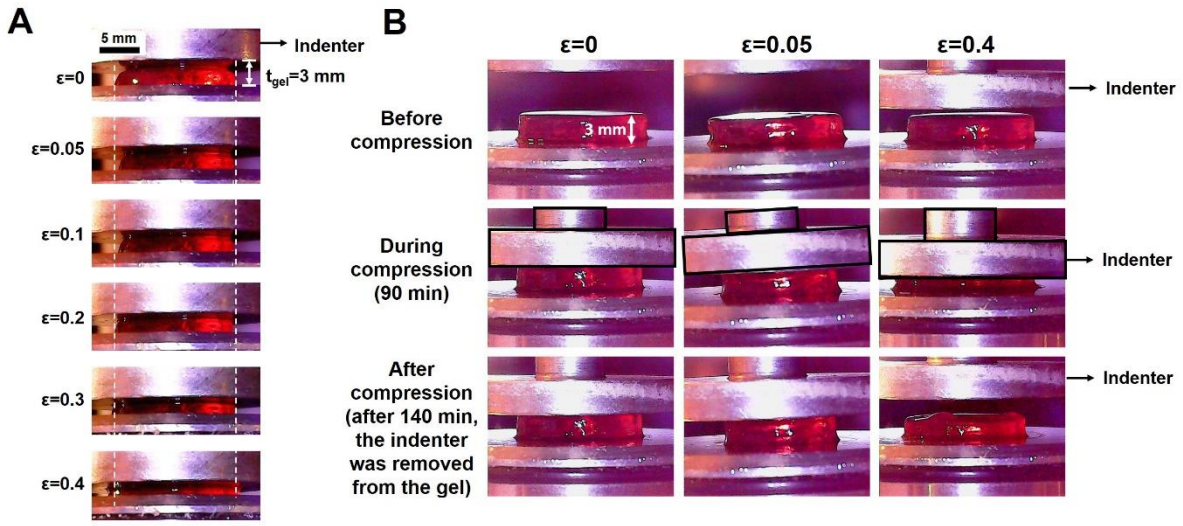

Figure S1. Deformation of a NP-containing agarose gel as a function of ε and compression time. The whole experimental set-up, including the automated micromechanical indenter, agarose gel, and camera, is placed inside the same incubator and maintained at 37°C and 5% CO_2. Note that this incubator is also used for our cellular uptake experiments. The 0.5% w/v agarose gel, with a diameter of 14.78 mm and a thickness of 3 mm, contains 5 nM Au13-PEG NPs (red). A GUOSUO digital microscope, fixed at the same location for all values of ε tested, was used to capture the images. (A) Lateral deformation of an agarose gel as a function of ε. These images were taken 10 min after the desired level of ε was reached, to allow the gel to reach mechanical equilibrium (see Figure S3). Dotted lines indicate the dimension of the agarose gel without compression ($\varepsilon = 0$). For the highest value of considered ($\varepsilon = 0.4$), the diameter of the gel increases by ~1 mm, indicating that the total area of the gel increases by ~12.8% when compared to the uncompressed gel. For other smaller values of ε considered, the increase in area of the gel is under 3%. (B) Distribution of NPs in and lateral deformation of the agarose gel after 140 min of compression. The distribution of the Au13-PEG NPs (as revealed by the characteristic red color of the Au NP cores) remained homogenous in both vertical and lateral directions, implying limited gravitational sedimentation of the NPs in the agarose gel upon compression. For the applied strains of $\varepsilon = 0$ (uncompressed control), $\varepsilon = 0.05$ (smallest strain), and $\varepsilon = 0.4$ (largest strain), the percentage of lateral shrinkage (as determined by ImageJ) is 4.1%, 9.5% and 4.1%, after 140 min of compression, respectively.

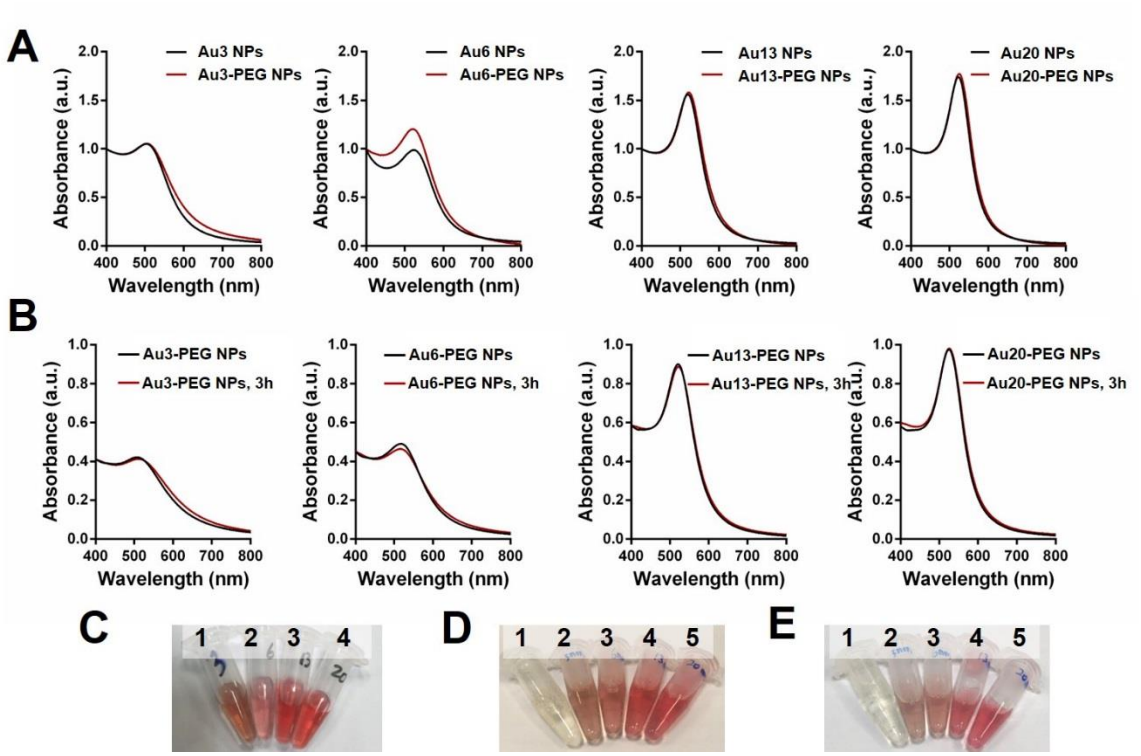

Figure S2. Characterization of AuX-PEG NPs of different sizes. (A) UV-Vis spectra of AuNPs with a core size of 3 nm (Au3 NPs), 6 nm (Au6 NPs), 13 nm (Au13 NPs) and 20 nm (Au20 NPs) in water before and after PEGylation. (B) UV-Vis spectra of AuX-PEG NPs after incubation in DMEM (without phenol red) supplemented with 10% FBS at 37°C for 3 h. (C) Colors of AuX-PEG NPs in water. From left to right are (1) Au3-PEG NPs, (2) Au6-PEG NPs, (3) Au13-PEG NPs, and (4) Au20-PEG NPs. Colors of AuX-PEG NPs before (D) and after (E) incubation in FBS-containing DMEM without phenol red at 37 ºC for 3 h. In Panels D and E, from left to right are (1) FBS-containing culture medium only, (2) Au3-PEG NPs in FBS-containing medium, (3) Au6-PEG NPs in FBS-containing medium, (4) Au13-PEG NPs in FBS-containing medium, and (5) Au20-PEG NPs in FBS-containing medium.

Table S1. Physical diameters and hydrodynamic diameters of AuX NPs

Sample name	Au3 NPs	Au6 NPs	Au13 NPs	Au20 NPs
Physical diameter* (nm)	3.6±0.6	5.4±0.8	12.9±1.3	19.9±2.5
Hydrodynamic diameter (nm)	7.0±0.0 (0.309±0.044)	9.4±0.2 (0.288±0.079)	16.0±0.3 (0.205±0.010)	20.5±0.2 (0.189±0.008)

*Measured by TEM imaging. At least 200 NPs were counted.

Table S2. ζ-potentials of AuX NPs, AuX-PEG NPs, and Cy5 labeled AuX-PEG NPs in 1 mM KCl (in mV)

Sample name	X = 3 nm	X = 6 nm	X = 13 nm	X = 20 nm
AuX NPs	-18.37±2.23	-6.63±2.00	-33.54±0.42	-20.22±0.74
AuX-PEG NPs	-1.57±0.48	-5.04±0.77	-4.80±0.64	-4.73±0.72
Cy5-labeled AuX-PEG NPs	-2.06±0.74	-3.90±1.27	-2.41±0.27	-8.45±0.23

Table S3. Hydrodynamic diameters of AuX-PEG NPs and Cy5-labeled AuX-PEG NPs under different conditions

Sample name		Au3-PEG NPs	Au6-PEG NPs	Au13-PEG NPs	Au20-PEG NPs
Hydrodynamic diameter (nm)* (mPEG only AuX-PEG NPs)	Water	8.7±0.2 (0.258±0.036)	12.5±0.1 (0.157±0.017)	18.6±0.1 (0.209±0.006)	24.0±0.3 (0.161±0.021)
	DMEM+10%FBS (RT, ~5 min)	11.2±0.2 (0.571±0.000)	13.6±0.6 (0.571±0.000)	18.7±0.2 (0.223±0.003)	24.4±0.2 (0.191±0.005)
	DMEM+10%FBS (37 ºC, 180 min)	12.1±0.3 (0.571±0.000)	12.8±0.3 (0.508±0.109)	18.9±0.1 (0.218±0.010)	25.0±0.2 (0.194±0.009)
Hydrodynamic diameter (nm)* (Cy5-labeled AuX-PEG NPs)	Water	8.7±0.1 (0.203±0.002)	13.1±0.2 (0.185±0.021)	18.7±0.1 (0.127±0.007)	25.0±0.2 (0.153±0.003)
	DMEM+10%FBS (RT, ~5 min)	11.8±1.1 (0.571±0.000)	12.8±1.7 (0.571±0.000)	19.0±0.1 (0.223±0.002)	24.2±0.1 (0.190±0.004)
	DMEM+10%FBS (37 ºC, 180 min)	11.4±2.5 (0.571±0.000)	14.5±0.2 (0.571±0.000)	18.8±0.1 (0.222±0.006)	25.1±0.3 (0.210±0.010)

*Numbers in the bracket denote polydispersity index (PDI). RT = room temperature.

Table S4. Amount of polyethylene glycol (PEG ;MW = 1000) attached to the AuNP surface of AuX-PEG NPs

Sample name	Au3-PEG NPs	Au6-PEG NPs	Au13-PEG NPs	Au20-PEG NPs
PEG density (#PEG strands/NP)	178.09±0.38	259.29±76.87	2057.74±2.66	4669.55±455.16
PEG density (#PEG strands/nm^2)	4.38±0.01	2.64±0.45	3.88±0.01	2.81±0.27

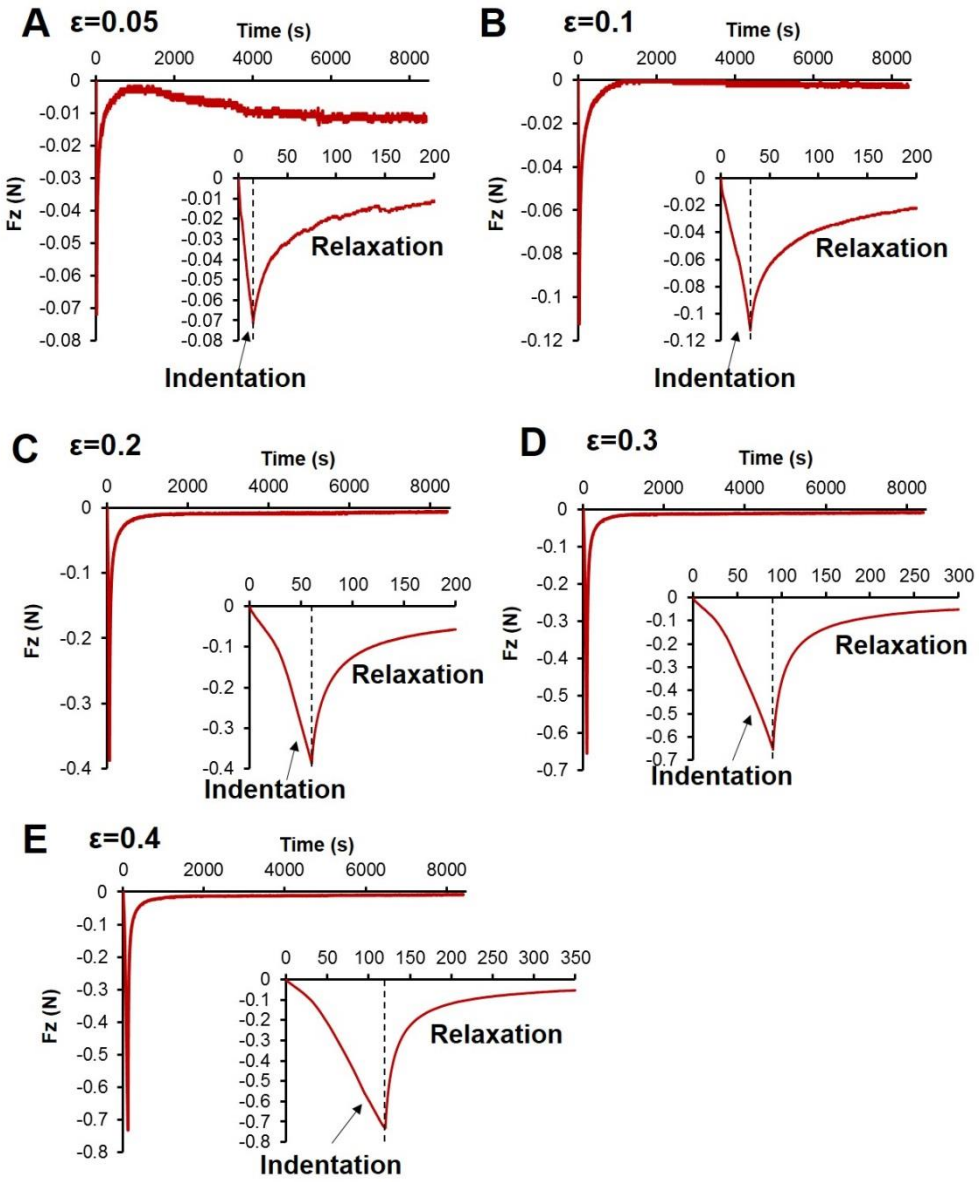

Figure S3. Stress relaxation curves of the 0.5% agarose gel under different levels of compressive strain (ε) during the 140 min of compression: (A) $\varepsilon = 0.05$, (B) $\varepsilon = 0.1$, (C) $\varepsilon = 0.2$, (D) $\varepsilon = 0.3$, and (E) $\varepsilon = 0.4$.

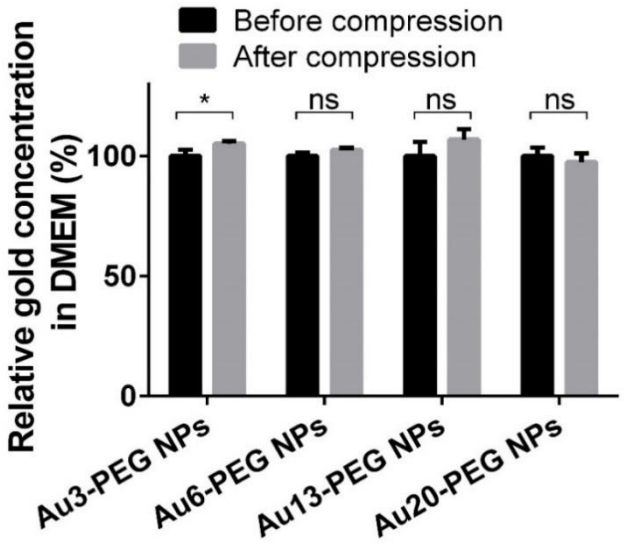

Figure S4. Concentration of AuX-PEG NPs in the culture medium before and after compressing the NP-containing agarose gel. In this experiment, we imposed compression to an agarose gel containing 5 nM AuX-PEG NPs surrounded by culture medium also containing 5 nM AuX-PEG NPs (as illustrated in Figure 1A), except that the cell-seeded culture dish is replaced by a blank culture dish (no cells seeded). The applied strain (ε) was kept at the maximal value of 0.4. After 140 min of compression, the culture medium was collected for ICP-MS analysis of its concentration for comparison against the concentration of the medium before compression. For AuX-PEG NPs with a core NP diameter of 6 nm or larger (X≥6), the change in mean NP concentration of the culture medium before and after compression is not statistically significant. For the smallest AuX-PEG NPs with a core NP diameter of 3 nm (Au3-PEG NPs), the change in mean NP concentration before and after compression is around 4%. For pairwise comparison of the NP concentration between the "before compression" and "after compression" cases within each NP size, the mean NP concentration of AuX-PEG NPs before compression was normalized to "100%". Two-tailed t-test was used for statistical analysis for each NP size. *$p < 0.05$, ns: not significant. Error bars denote SD resulting from triplicate experiments.

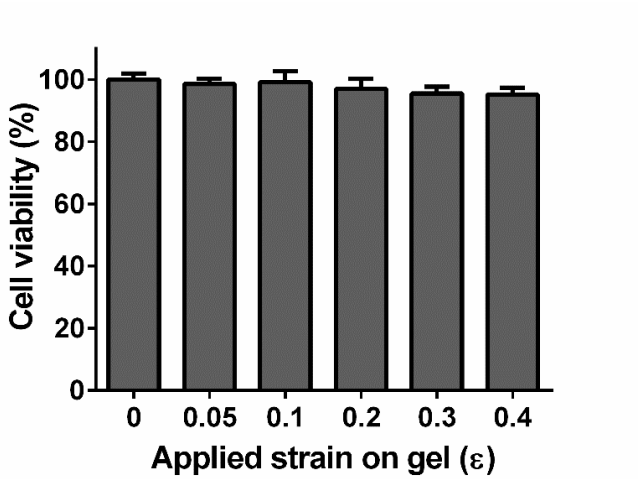

Figure S5. Viability of C2C12 cells upon compression *via* a 0.5% agarose gel at different levels of applied compressive strain (ε) for 140 min by the alamarBlue test. Cell viability remains above 95% for values of ε tested. Error bars denote SD resulting from triplicate experiments.

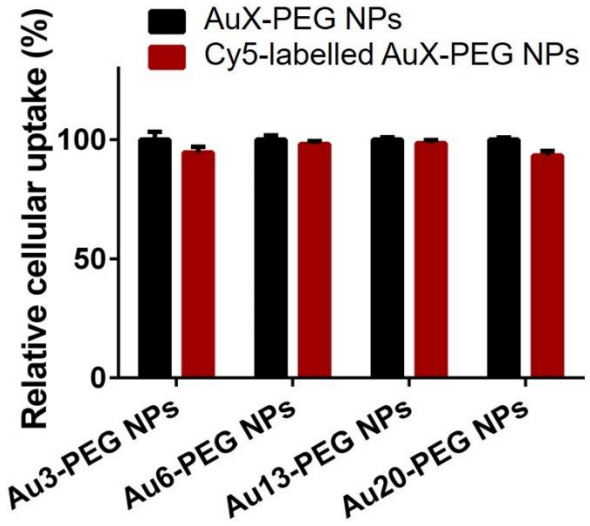

Figure S6. Uptake of AuX-PEG NPs and Cy5-labeled AuX-PEG NPs by C2C12 cells. Across all NP sizes, the difference in mean cellular uptake between AuX-PEG NPs (black) and Cy5-labeled AuX-PEG NPs (red) is less than 5%. For pairwise comparison of cellular uptake between "AuX-PEG NPs" and "Cy5-labeled AuX-PEG NPs" cases within each NP size, the mean uptake of non-fluorescent AuX-PEG NPs by C2C12 cells was normalized to "100%". Error bars denote SD resulting from triplicate experiments.

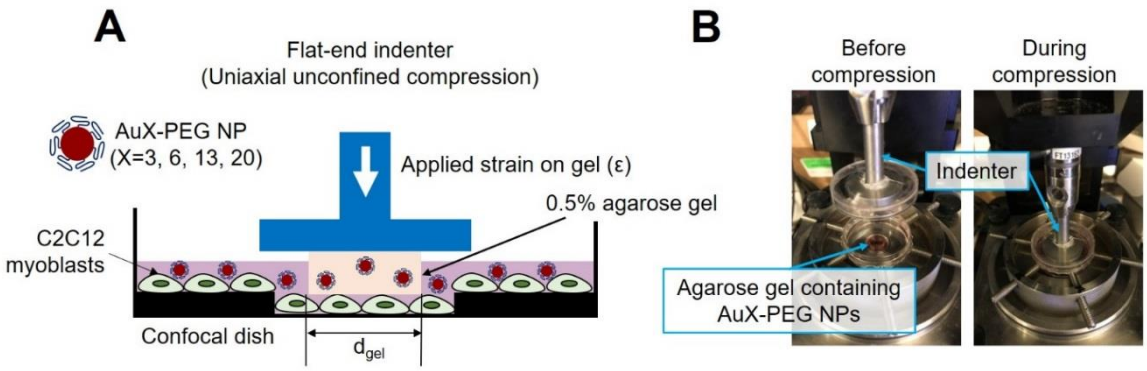

Figure S7. Experimental set-up for studying the cellular uptake of Cy5-labeled AuX-PEG NPs under defined levels of compression by confocal imaging. (A) Schematic illustration. This set-up is almost identical to that depicted in Figure 1A, except that the agarose gel was cut out by a smaller mold of 8.52 mm (d_{gel}) in diameter to cater for the size of the well inside the confocal dish. (B) Actual experimental set-up. The indenter was pressed on top of the agarose gel, which was placed on top of the C2C12 cells seeded in a confocal dish.

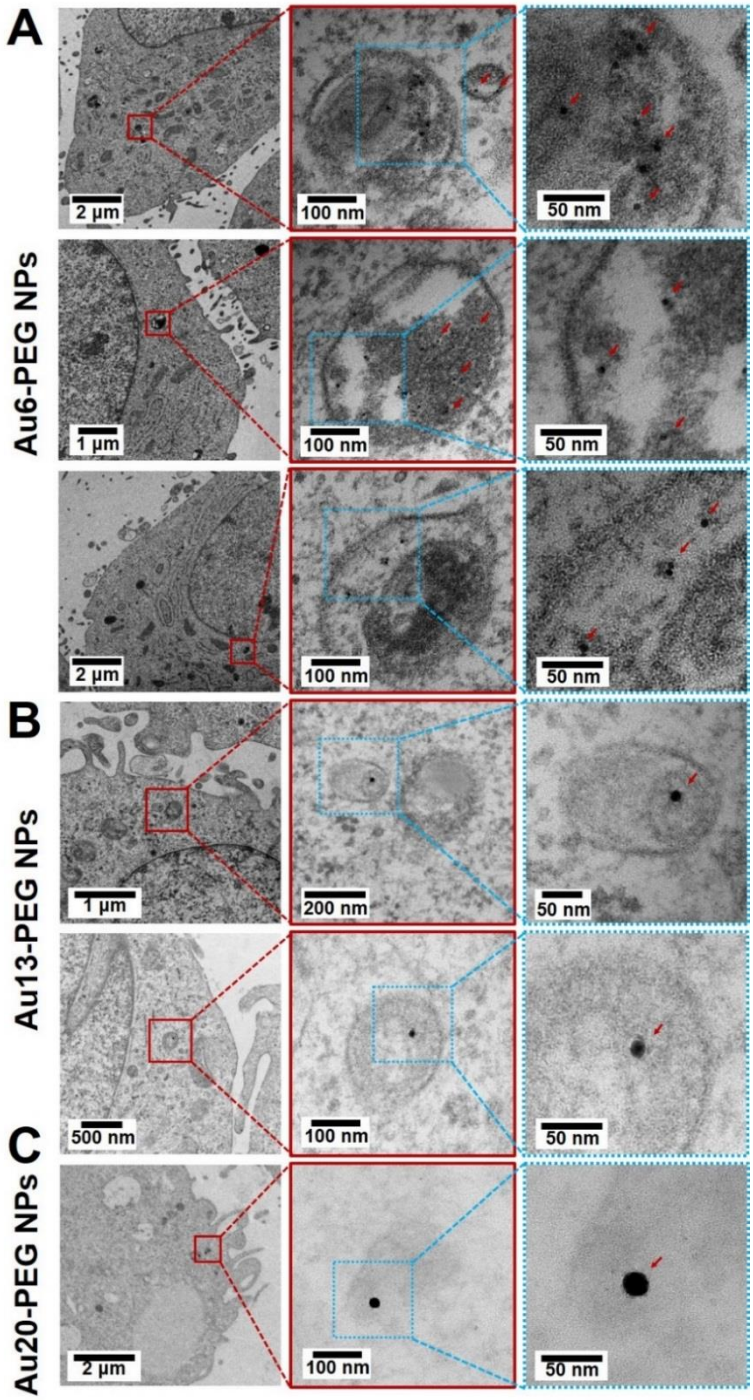

Figure S8. Additional representative TEM images of compressed C2C12 cells incubated with (A) Au6-PEG NPs, (B) Au13-PEG NPs and (C) Au20-PEG NPs under their respective optimal compressive stress. Images in middle column show the enlarged region boxed in red of the images in left column. Images in right column show the enlarged region boxed in blue of the images in middle column. (Legend: Nu = nucleus; Cy = cytosol; and Ex = extracellular space)

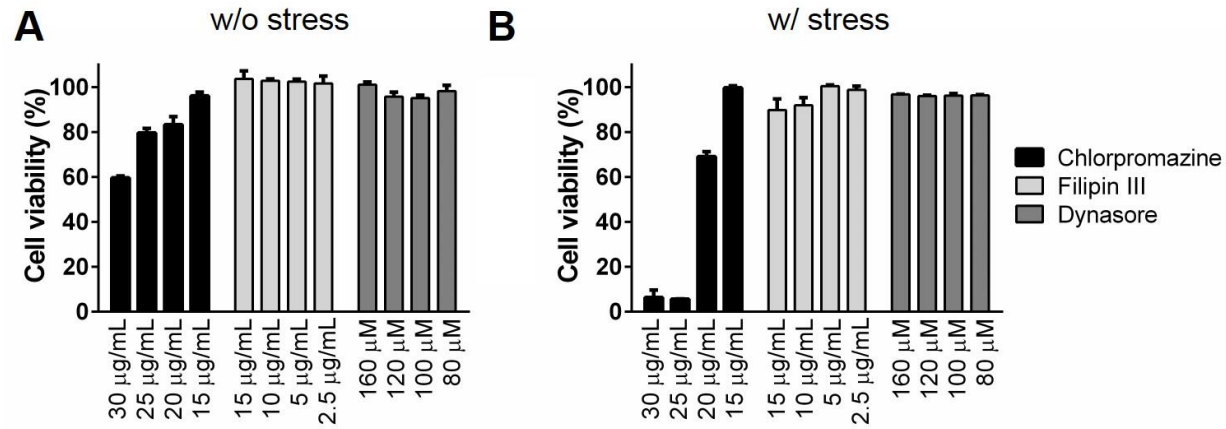

Figure S9. Viability of C2C12 cells treated with different pharmacological inhibitors without (A) or with stress (B). The three inhibitors include filipin III, chlorpromazine, and dynasore. Cells were pre-treated with different inhibitors at a series of concentrations for 1 h before compression. The alamarBlue reagent was used to evaluate the cell viability. Error bars denote SD resulting from triplicate experiments.

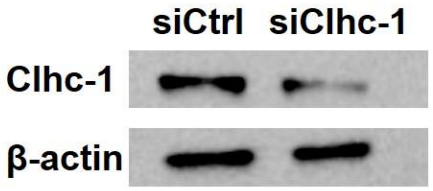

Figure S10. Western blotting confirms significant knockdown of the gene encoding the heavy chain 1 of clathrin (Clhc-1) in C2C12 cells by RNA interference. β-actin is the loading control.

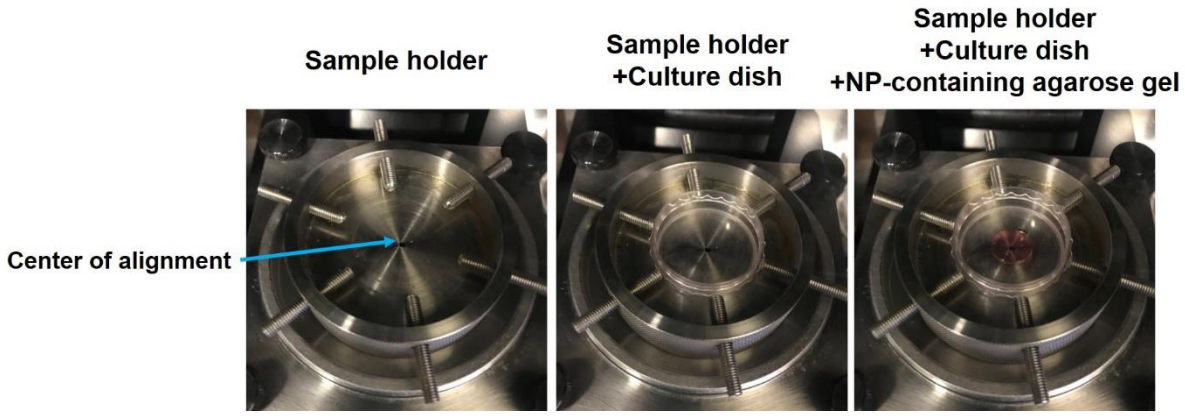

Figure S11. Step-by-step alignment of sample holder, culture dish, and agarose gel in our experimental set-up. (Left panel) Firstly, we used a black marker to delineate the center of the sample holder as the "center of alignment". (Middle panel) Then, we inserted the 35 mm culture dish to the sample holder and fixed its location by six screws, to accurately align the center of the dish to our "center of alignment" that we indicated in the sample holder. (Right panel) Finally, we placed the NP-containing gel to the center of the dish and used pipet tips to carefully adjust the location of the gel.

References:

1. Jana, N. R.; Gearheart, L.; Murphy, C. J., Seeding growth for size control of 5− 40 nm diameter gold nanoparticles. *Langmuir* **2001**, 17 (22), 6782-6786.
2. Mühlpfordt, H., The preparation of colloidal gold particles using tannic acid as an additional reducing agent. *Cell. Mol. Life Sci.* **1982**, 38 (9), 1127-1128.
3. Frens, G., Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. *Nature* **1973**, 241 (105), 20-22.
4. Bastús, N. G.; Comenge, J.; Puntes, V., Kinetically Controlled Seeded Growth Synthesis of Citrate-Stabilized Gold Nanoparticles of up to 200 nm: Size Focusing versus Ostwald Ripening. *Langmuir* **2011**, 27 (17), 11098-11105.
5. Chou, L. Y.; Chan, W. C., Fluorescence-tagged gold nanoparticles for rapidly characterizing the size-dependent biodistribution in tumor models. *Adv Healthc Mater* **2012**, 1 (6), 714-21.