Supplementary Materials for

Intracellular Aggregation of Multimodal Silica Nanoparticles for Ultrasound-Guided Stem Cell Implantation

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Other Supplementary Material for this manuscript includes the following:
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Supplementary Methods

Optical equipment

Flow cytometry was performed on a FACSCalibur (Becton Dickinson) with 5000 – 10,000 cells collected per analysis. A Synergy 4 (Biotek) microplate reader was used for SiNP fluorescence characterization and cell proliferation/toxicity studies. For SiNP fluorescence, FITC filters cubes were used (485/20 nm excitation; 528/20 emission) with 35% detector sensitivity, and Xenon flash source.

Microscopy

All transmission electron microscopy (TEM) and energy-dispersive x-ray spectroscopy (EDS) was performed with a Tecnai G2 X-Twin (FEI Co.) instrument operating at 200 kV. After loading with SiNPs, MSCs were washed three times with PBS, removed from the flask with trypsin, washed with media and saline using 5 min of 1000 x g centrifugation to create the pellet and prepared for TEM.

Inductively coupled plasma (ICP) emission spectroscopy

Three studies were performed using ICP emission spectroscopy. The first measured the amount of Gd^{3+} per SiNP via three runs each of 50, 100, and 200 µl of SiNPs. The second examined the stability of the Gd^{3+}/Si system. Here, SiNPs (200 µl at 10 mg/ml) were added to 200 µL of mouse serum or water at 37^{o}C for 2 or 24 hours in triplicate. In both cases, the SiNPs were isolated by centrifugation and supernatant retained for analysis of Gd^{3+} content. The SiNP pellet was dissolved with 1 ml 10 N NaOH with 40 minutes sonication, neutralized with 1 ml concentrated nitric acid, and diluted to 5 ml with 5% nitric acid. The supernatant was similarly dissolved in 5% nitric acid. The samples were analyzed with an ICAP 6300 system (Thermo Scientific) using 10 and 100 ppm solutions of Gd^{3+} (ICP standard grade; Fluka) in nitric acid as calibrations and standards. The number of SiNPs per volume was determined using the density of silica assuming a sphere with a size of 300 nm and the reported density of silica for a molecular weight of 1.7×10^{10}. The third study determined the number of SiNPs per MSC. The number of SiNPs per cell was determined by measuring cellular Gd^{3+} and converting via the ratio of Gd^{3+}:SiNP determined above. Cells were prepared similarly, using strong base to dissolve SiNPs. ICP analysis of cell culture media employed centrifugation to remove denatured proteins after adjusting to low pH.

Differentiation experiments

Low passage number (<6) MSCs were used for differentiation experiments and done at least in duplicate. Cells were loaded with SiNPs as described above and the labeled cells were counted and plated as described below. Stained cells were imaged with a Leica light microscope.

The osteogenic protocol used 35 mm collagen-coated culture plates (World Precision Instruments) and 30,000 cells (loaded and unloaded with SiNPs) per plate. The next day, standard media was replaced with osteogenic media (Lonza PT-3002) supplemented with dexamethasone, ascorbate, and b-glycophophate. Control cells used standard media. The media for both control and labeled cells was changed every 2-3 days. After 24 days, cells were fixed with 70% ethanol on ice for one hour and then stained with 2% Alizarin Red in water (pH 4.2; freshly filtered) for 7 minutes followed by water washes until no excess stain was removed. Dissolving the colored complex in 10% acetic acid and measuring the optical density at 402 nm quantitated the degree of osteogenesis.

In the adipogenic protocol, 80,000 SiNP-loaded and unloaded cells were seeded in a 12 well plate and grown for 7 days until they were over-confluent. Cells in the induced population were subjected to three rounds of three-day growth in induction media (Lonza PT-3004) followed by 1-3 intervals in maintenance media. Adipogenic induction media contained recombinant insulin, dexamethasone,

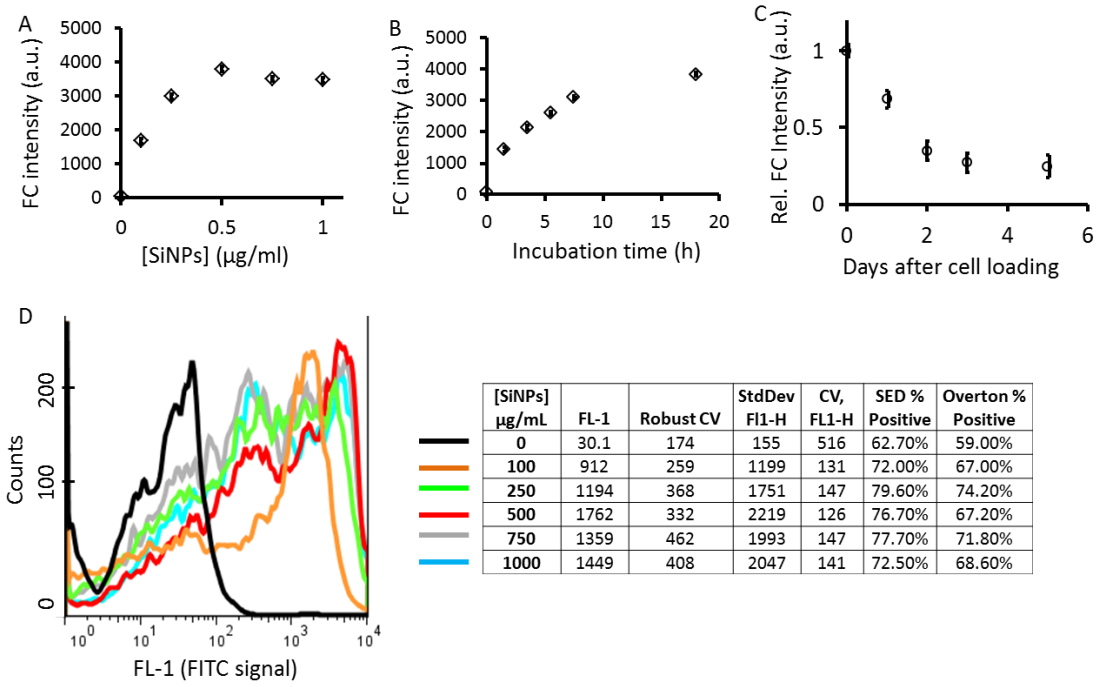
indomethacin, 3-isobutyl-1-methyl-xanthine, and gentamicin. Adipogenic maintenance media contained only insulin and gentamicin. Control cells were incubated only in maintenance media. One week after the final round of induction, cells contained a large number of microscopic lipid vacuoles. The MSCs were fixed in 10% formalin for 45 minutes and washed with water and then 60% isopropyl alcohol. Oil red O (Sigma Aldrich) was used to stain the adipogenic cells. To prepare this stain, 18 ml water was added to 27 ml of 3 mg/ml Oil red O in isopropyl alcohol. After ten minutes the solution was filtered and added to the fixed cells for five minutes followed by water wash. Cells were counterstained with hematoxylin for 2 minutes.

Induction of chondrogenesis again used MSCs loaded and unloaded with SiNPs. Control media (Lonza) was supplemented with dexamethasone, ascorbate, gentamicin, sodium pyruvate, proline, and L-glutamine per the manufacturer's instructions. Induction media contained the same as well as 10 ng/ml transforming growth factor beta (TGF-β). Cell pellets containing 250,000 MSCs were created in 15 ml polypropylene tubes and induced for 3 weeks. Media was changed every 2-3 days. Pellets were fixed with 4% glutaraldehyde, 3% acetic acid, and stained with 1% Alcian blue and then suspended in 2% agarose that was embedded in paraffin after cooling. Sections 5 μm thick were sliced with a microtome and immobilized on positively charged slides.

Chondrogenic transformation was confirmed by assaying for glycosaminoglycans with the dimethylmethylene blue (DMMB) reagent as described previously. Briefly, 5 ml of 1 mg/ml DMMB in ethanol was dissolved to 500 ml total volume of water and adjusted to pH 1.5 solution. Cell pellets and cartilage controls were dissolved with 1 mg Proteinase K per mg of sample at 60 ^{o}C for 16 hours in 100 mM ammonium acetate. Then 80 μL of the digest was added to 200 μL of the DMMB solution and analyzed at 595 nm on the plate reader. Chondroitin-6-sulfate was used for a calibration curve and pellets were analyzed in at least duplicate.

The potential for differentiation into a cardiac-like lineage was investigated with 5-azacytadine. MSCs on their second passage were plated at 5000 cells/cm^2 and allowed to grow for 2 days. Then media was supplemented with 10 μM 5- azacytidine and allowed to proceed for 24 hours. The media was then changed to fresh normal media after three washes with PBS. This treatment was repeated again after 2 weeks. Three weeks from the initial treatment, cells were evaluated with cytometry and fluorescence microscopy. For flow cytometry cells were removed from the plate with TripleLE express and stained with mouse antibodies specific to human alpha-actinin and desmin (200 μg/ml; Santa Cruz Biotech) at 1:50 dilution for 30 minutes on ice. After washing, they were counter-stained with goat anti-mouse IgG-Alexa Fluor 488 at 20 μg/ml, washed, and analyzed. For microscopy, cells were formalin fixed on the plate for 10 minutes and then permeabilized with 0.2% Triton 100X in 3% bovine serum albumin/PBS for 30 minutes. Primary antibody staining at the concentrations above were used in the cold room overnight followed by 90 minutes of secondary antibody staining at room temperature.

SUPPLEMENTARY FIGURES



[SiNPs] µg/mL	FL-1	Robust CV	StdDev Fl1-H	CV, FL1-H	SED % Positive	Overton % Positive
0	30.1	174	155	516	62.70%	59.00%
100	912	259	1199	131	72.00%	67.00%
250	1194	368	1751	147	79.60%	74.20%
500	1762	332	2219	126	76.70%	67.20%
750	1359	462	1993	147	77.70%	71.80%
1000	1449	408	2047	141	72.50%	68.60%

Figure S1. Optimization and characterization of SiNP cell loading. (**A**) MSCs were incubated with increasing concentrations of SiNPs and [what type of] signal monitored by flow cytometry (FC). (**B**) Incubation time of MSCs with SiNPs was optimized by monitoring the fluorescence signal over time. Data in (A and B) are means of gated signals and error bars represent the coefficient of variation (CV) of the FC histograms for cells in that gate. (**C**) The stability of cell loading was probed by serially imaging cells post loading. Data are means of signal and error bars represent the CV of the FC histograms for cells in that gate (n = 10,000 cells repeated twice). (**D**) The percentage of cells that contain the contrast agent is shown for the different loading levels. The black curve is cells without contrast agent, and the colored curves are at increasing concentrations of SiNPs (0 to 1000 µg/ml). A table shows the mean, CV, and SD of the flow data as well as the Overton and Super-Enhanced Dmax Subtraction (SED) methods to determine the percent positive cells.

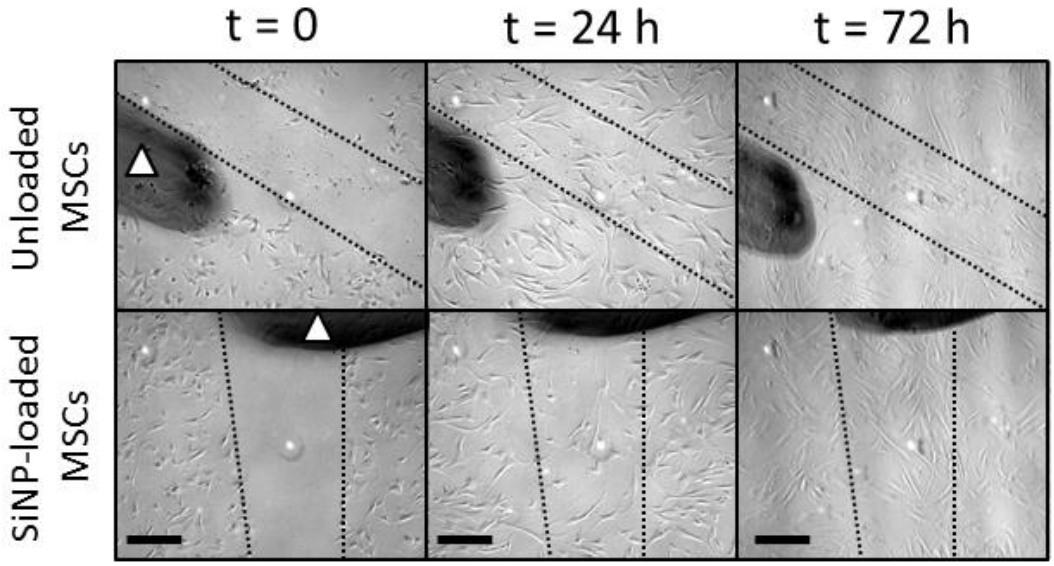


Figure S2. Cell migration assay. MSCs loaded with SiNPs and control cells without SiNPs were studied by light microscopy at 0, 24, and 72 hours to determine migration capacity. Dashed lines indicate the area from which cells were removed. Dark areas highlighted by triangle are fiducial markers used to orient the plate prior to imaging. Scale bars, 200 µm.

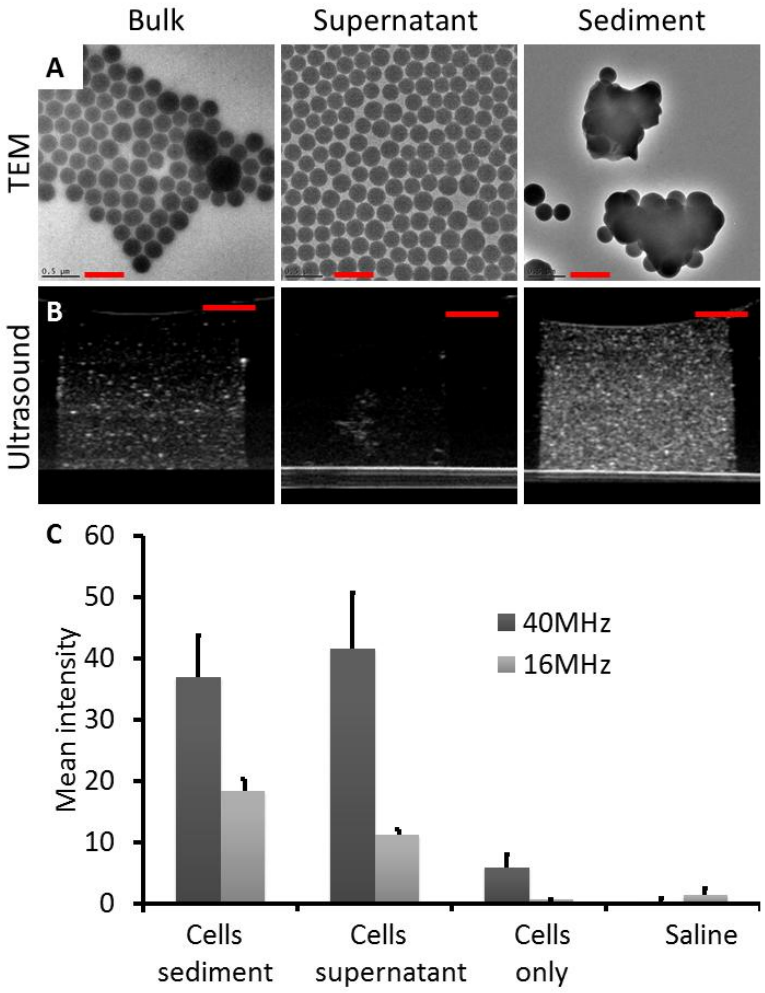


Figure S3. Impact of SiNP fraction on ultrasound intensity. **(A**) TEM images of a freshly mixed batch of SiNPs showing a distribution of sizes. The bulk batch was allowed to settle overnight, then the supernatant and sediment were collected and imaged. Scale bars, 500 nm. (**B**) Ultrasound contrast of the three fractions corresponding to the particle fractions in (A). Most of the US signal is generated by the larger aggregates. Scale bars, 2 mm. (**C**) Both sediment and supernatant generate ultrasound signal in hMSCs similar to the bulk sample used for in vivo work. Data are means ± SD (*n* = 5 FOVs, for each of 2 trials.)

Table S1. Serum toxicity tests. Data are means ± SD measured for mice in different payload groups: SiNP-MSC, MSCs only, sham injection, and uninjected controls 4 days after implantation (n = 4 animals per group). P-values were determined using a two-tailed t-test versus control (non-injected) animals. Values that are statistically significant (P<0.05) are highlighted in yellow. AST, aspartate aminotransferase; ALT, alanine transaminase; AlKPhos, alkaline phosphatase; GGT, γ-glutamyltransferase; Tbili, total bilirubin; CKMB, creatine kinase myocardial band; anion gap, [cations – antions]; TropI, troponin I.

		SiNP-MSC			**MSC**			**Sham injection**			**Uninjected control**	
Analyte	**Unit**	**Mean**	**SD**	***P***	**Mean**	**SD**	***P***	**Mean**	**SD**	***P***	**Mean**	**SD**
AST	U/l	80.5	33.83	0.34	91.6	68.06	0.655	79.6	29.88	0.268	108.6	45.57
ALT	U/l	34.5	5.802	0.23	38.2	10.21	0.308	41.8	13.16	0.477	51	24.26
AlkPhos	U/l	79.5	8.021	0.028	111.8	7.563	0.073	98.2	20.14	0.956	98.8	11.86
GGT	IU/l	0	0	N/A	1	1.732	0.233	0	0	N/A	0	0
Tbili	mg/dl	0.068	0.059	0.034	0	0	N/A	0	0	N/A	0	0
CKMB	U/l	172	39.1	0.391	149.4	66.73	0.221	133.3	6.702	0.133	222.2	103
Na^+	mM	151.5	3.109	0.853	151	1	0.347	151.6	1.14	0.817	151.8	1.483
K^+	mM	3.4	0.258	0.405	3.48	0.13	0.345	3.84	0.261	0.347	89.64	191.9
Cl^-	mM	113.8	1.708	0.971	114	0.707	0.849	114.2	1.304	0.733	113.8	2.168
CO_2	mM	17.18	1.164	0.004	14.32	2.199	0.319	14.78	2.022	0.168	13.02	1.627
Anion gap	mM	23.98	1.282	0.007	26.16	2.528	0.114	26.46	2.046	0.118	28.88	2.317
TropI	ng/ml	0.018	0.029	0.208	0.008	0.011	0.141	0	0	N/A	0	0

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SUPPLEMENTARY VIDEOS

Movie S1. Injection of hMSCs into the mouse left ventricle wall. A 27-gauge catheter was inserted into the mouse LV and positioned via ultrasound. The injection of MSCs begins near the 0:11 time point. The area of therapy is highlighted with a red circle.

Movie S2. Integration of hMSC bolus with the left ventricle. A 3-second clip of is repeated four times at reduced speed. The movement of the hMSCs correlated with the heart rhythm detected by ECG, suggesting integration into the cardiac tissue.