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Manipulating Directional Cell Motility Using Intracellular Superparamagnetic Nanoparticles

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Supporting Information

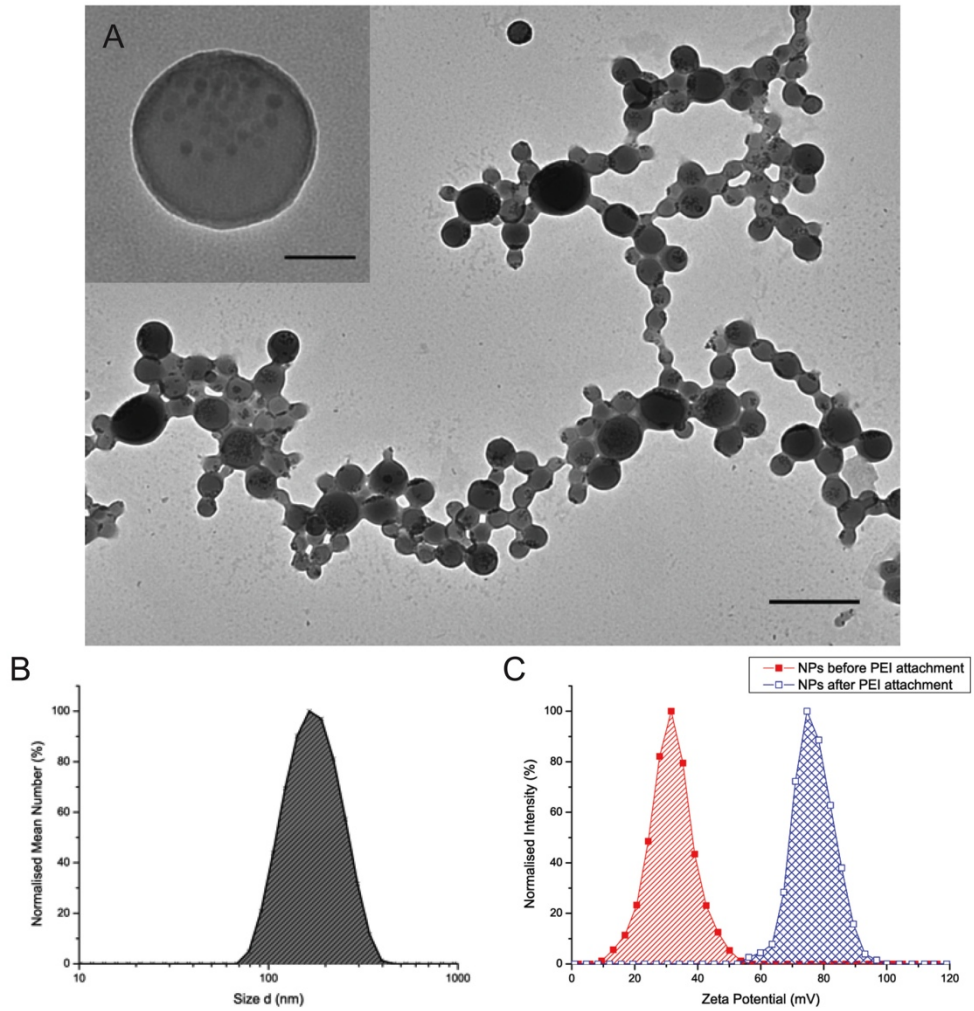
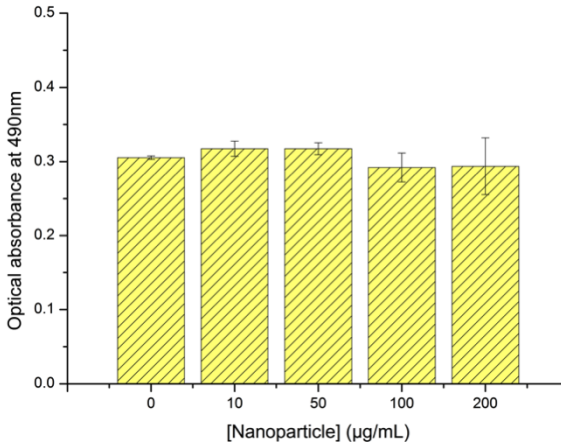


Fig. S1 A) Transmission electron microscopy images of the multicore PGMA-RhB-magnetite-PEI composite nanoparticles. The inset is a single particle of ≈100nm diameter showing several magnetic cores inside. Scale bars are 500nm in the main image and 50nm in the inset. B) The dynamic light scattering results show a mean hydrodynamic diameter of 160 nm. C) The zeta potential graph shows that the modification with PEI resulted in a more positive surface charge, which allows the particles to strongly interact with the cell membrane and be endocytosed.

A



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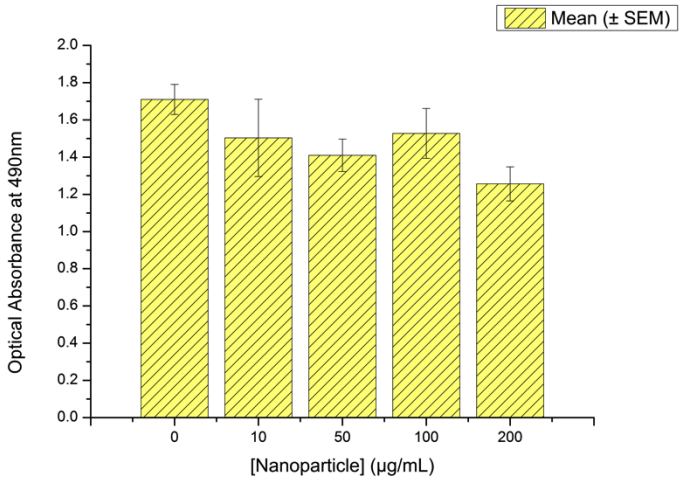

Fig. S2 A) HaCaT MTS cellular proliferation assay with different concentrations of nanoparticles. B) NIH-3T3 cell proliferation assay with different concentrations of nanoparticles. For both 24 hour assays, no significant inhibition of cellular proliferation was observed, even at 200 µg/ml nanoparticle concentration. Error bars show standard error of the mean.

Videos - Live cell time lapse confocal analysis of nanoparticle uptake

Live cell confocal movies at 10 frames per second of (a) NIH-3T3 cells and (b) HaCaT cells during magnetic nanoparticle uptake. Frames were captured every 15 seconds for the first 15 minutes after adding the nanoparticles and then every 10 minutes for 16 hours. In each case, the nanoparticles can be seen accumulating inside the cells.

(a) NIH-3T3

https://drive.google.com/file/d/0Bx9Rh6TIEXqnRnphN3FCMktGcFU/edit?usp=sharing

(b) HaCaT

https://drive.google.com/file/d/0Bx9Rh6TIEXqnSmFIRGNTejlXUVk/edit?usp=sharing

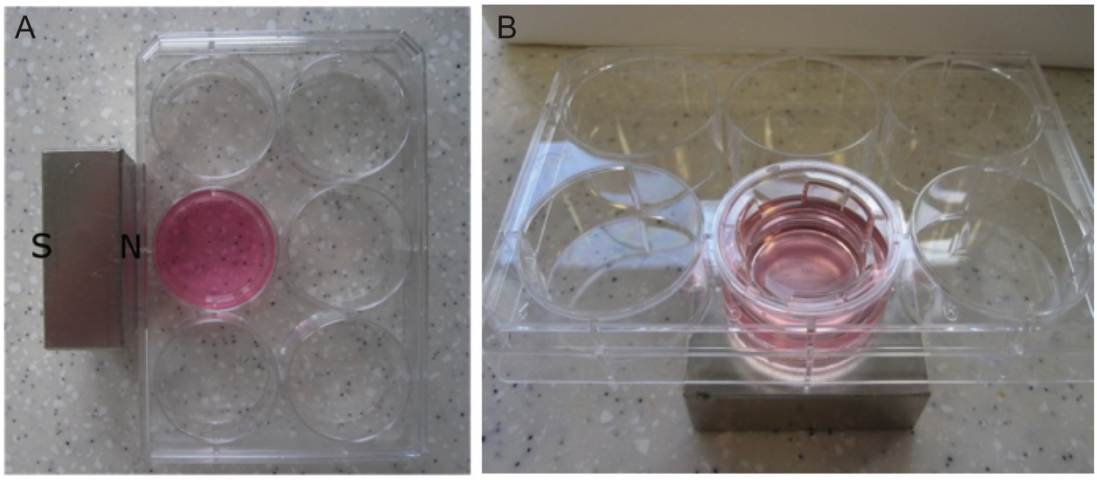

Fig. S3 A) 2D migration setup for measuring HaCaT cell migration with the permanent magnet to the side of the well plate. B) is the 3D migration setup for the NIH-3T3, with the magnet directly underneath the well, making use of the semi permeable Transwell membrane.

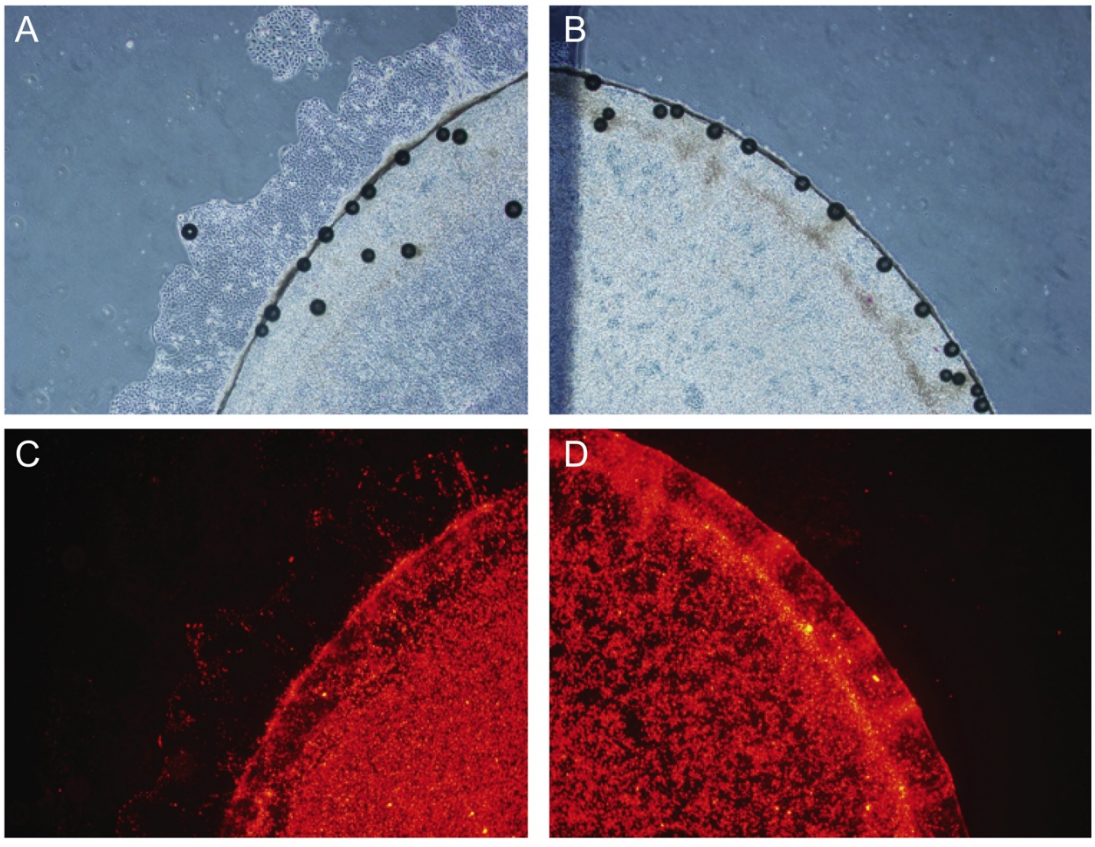

Fig. S4 Magnified images of the upper left and right side of the coverslip following 4 days of incubation of HaCaTs with nanoparticles in the presence of a magnetic field (note permanent magnet was located to the left of the coverslip). A) Brightfield image

of coverslips left edge, B) brightfield image of coverslips upper right edge, C) fluorescent image of rhodamine B signal (from nanoparticles), upper left edge of coverslip, and D) corresponding fluorescent image of rhodamine B signal (from nanoparticles) upper right edge of coverslip.

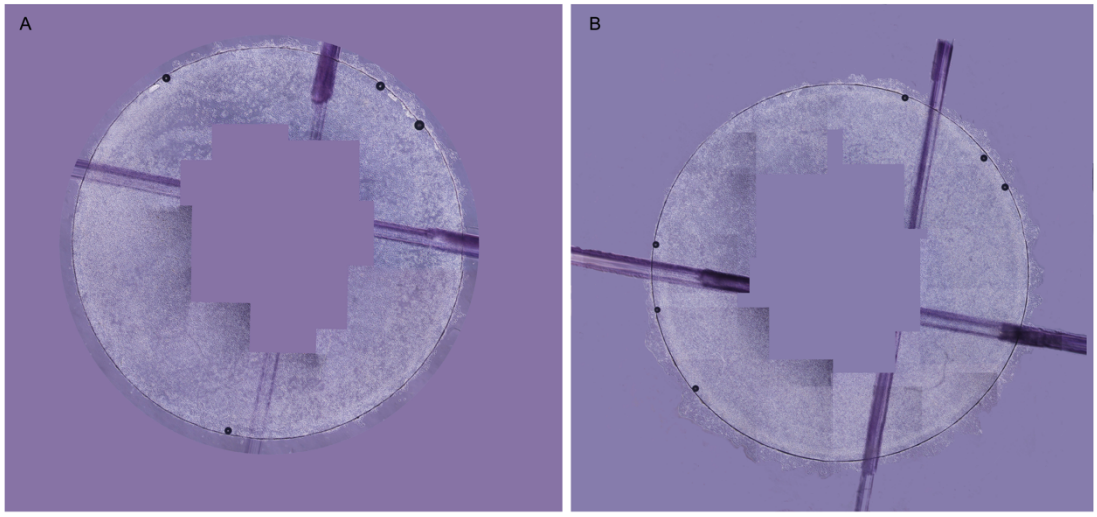

Fig. S5 Control experiments for 2D HaCaT cell migration. A) Stitched brightfield image of cells without nanoparticles or magnetic field present. B) Stitched brightfield image of cells without nanoparticles but incubated with the magnetic field (0.6T permanent magnet situated to the left of the coverslip).

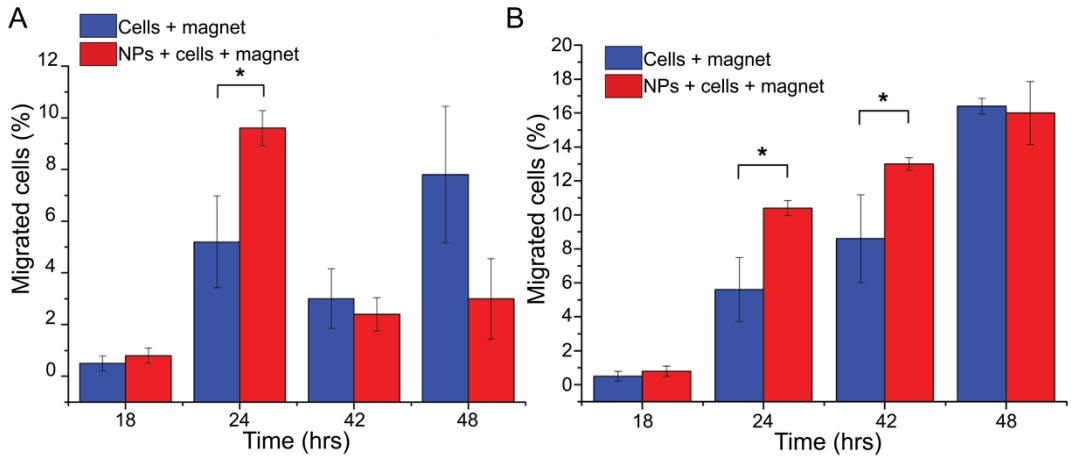

Fig. S6 Comparison of z-directed magnetically enhanced migration of cells with the magnetic field versus cells with internalised nanoparticles and the magnetic field. NIH-3T3 cells were grown to 70-80% confluence in 6 well plates and then incubated for 24 hours with 50 μg/mL nanoparticles. These cells were then seeded on a

Transwell with polycarbonate membrane insert. A 0.6T permanent magnet was placed directly underneath the well containing the membrane. The cells that migrated through the membrane were counted at different time points over a 48 hour period. A) Counted NIH-3T3 cells at the independent time points and B) the cumulative cell numbers across the experiment time course. Data displayed as mean ± standard error from triplicate measurements at each time point. Significance was determined with ANOVA and Bonferroni–Dunn post hoc tests (significance of $P \leq 0.05$).

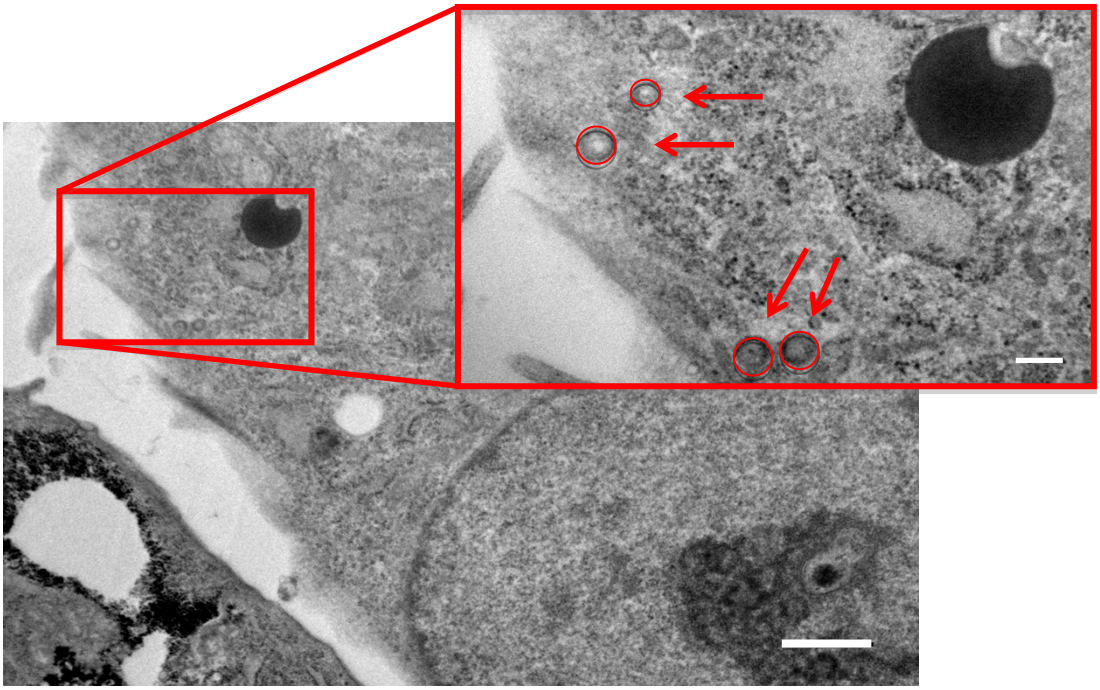

Fig. S7 Transmission electron microscopy (TEM) of primary dermal fibroblasts after 12 hr incubation with nanoparticles with red arrows indicating nanoparticles (circled) within the cell. Scale bar of 1 μm with high magnification inset scale of 200 nm.

Experimental methods

Materials

Chemicals were purchased from Sigma-Aldrich unless otherwise stated: benzyl ether (99%), ethyl methyl ketone (MEK), iron(III) acetylacetonate (97%), oleic acid (BDH, 92%), oleyl amine (70%), Pluronic® F108, polyethylenimine (PEI 50% solution, *M*n 1200, *M*w 1300), poly(glycidyl methacrylate) (PGMA, M_n=150,539 kDa and

polydispersity index 2.3, was a generous gift from Prof. Igor Luzinov, School of Materials Science and Engineering, Clemson University, Clemson, South Carolina, USA) Rhodamine B (RhB, Kodak, 95%), and 1,2-tetradecanediol (90%) were used as received.

Magnetite synthesis

Magnetite was prepared in accordance with the method described by Sun *et al*. Briefly, iron(III) acetylacetonate (2 mM), 1,2-tetradecanediol (10 mM), oleic acid (6 mM), oleylamine (6 mM), and benzyl ether (20 mL) were mixed with a magnetic stirrer and gradually heated under a constant flow of N_2. The mixture was held at 100 °C for ≈1h before being ramped to 200 °C, held for 2 h and finally heated to reflux (≈300 °C) and held for 1 h under a blanket of N_2, The sample was allowed to cool to room temperature overnight under N_2flow. The sample was collected and purified through a series of precipitations with ethanol, collection via centrifugation and then resuspension in hexane to be stored until use under Argon.

PGMA modification with Rhodamine B

PGMA (100 mg) and Rhodamine B (20 mg) were dissolved in ethyl methyl ketone (MEK) (30 mL) and heated to reflux under N_2 atmosphere for 18 h. The PGMA-Rhodamine modified polymer was precipitated with diethyl ether and dried before use in nanoparticle production.

Multimodal polymeric nanoparticle synthesis

Nanoparticles were prepared by an ‘oil in water’ emulsion process. The organic phase contained magnetite nanoparticles (18 mg), dissolved PGMA-Rhodamine B (90 mg) in a 1:3 mixture of $CHCl_3$ and MEK (6 mL). The organic phase was added drop wise to a vortexing aqueous solution of Pluronic® F108 (1.25% w/v, 30 ml) with the resulting microemulsion homogenized with a probe-type ultrasonicator for 1 min on low power. Organic solvents were allowed to evaporate under moderate stirring and

N_2 flow overnight. Magnetite aggregates and unreacted polymer were removed via centrifugation (3000g, 45 min), with the supernatant being collected and incubated with PEI (50 wt% solution, 100 mg) at 70 ˚C for 20 h. The PEI modified magnetic polymeric nanoparticles were collected on a magnetic separation column (LS, Miltenyi Biotec), washed with milli-Q water to remove excess Pluronic® and unattached dye before being collected, aliquoted and stored. The equivalent dry mass of samples was determined by freeze-drying.

Transmission Electron Microscopy (TEM)

Nanoparticle samples were prepared by deposition from water onto carbon-coated copper grids and imaged at 120kV on a JEOL JEM-2100.

Live cell confocal time lapse imaging

Cells were prepared in the wells of specialized cell culture dishes (MatTek, glass bottom dish; 35 mm dish diameter, 10 mm glass; bottom, No. 1.5 glass thickness). These specialized dishes were manufactured with a glass coverslip at the bottom, enabling high-resolution confocal microscopy images of cells to be taken. NIH-3T3s and HaCaTs were passaged and diluted to a cell density of 3.0×10^4 cells mL^{-1}. 200 μL of the cells were plated in the recess of the dish and incubated for 60 min to allow the cells to settle on the glass coverslip. The dishes were then topped up with an additional 3 mL of DMEM/F12 GlutaMAX mixture. The dishes were placed in a microscope incubator (37°C / 5% CO_2; Tokai Hit, Stage Top Incubator) and imaged with a confocal microscope (Nikon A1RMP) with a Plan Apo VC 20x DIC N2 objective. Images were taken every 15 seconds for the first 15min and then every 10min for 16 hours. Differential interference contrast optics were used for the brightfield images and an excitation/emission wavelength of 561, 595nm was used to detect the fluorescence from the magnetic nanoparticles.

Cell culture

HaCaT (immortalized human keratinocytes) and NIH-3T3 (mouse embryonic fibroblasts) cell lines were used. Both cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM/F12 - GlutaMAX; Invitrogen Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS; Invitrogen Gibco) and 1% penicillin/streptomycin (P/S; Invitrogen Gibco). This media concoction will be referred to as simply, "media" throughout the paper. The cells were incubated at 37°C and in an atmosphere of 5% CO_2. Cells were passaged at ~80% confluency and seeded in 75 cm^2 tissue culture flasks (Greiner Bio-One, cat. No. 658175). All procedures involving cell-handling were performed in a sterile laminar flow hood.

Cell proliferation assays (MTS assays)

Separate 96-well plates (Greiner Bio-One) were prepared for each sampling time point. Cells were seeded in the wells and after 24 hours, the nanoparticle-media solutions were added (t=0 timepoint). Control wells without the nanoparticles had 200 µL of DMEM mixture added. 100µL of HaCaT and NIH-3T3 cells (1.5 × 104 cells mL^{-1}) in DMEM mixture were seeded in each well at the t= -24 hr timepoint. The plates were left to incubate overnight (37°C / 5% CO_2). Three hours before each timepoint, 40µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS; Promega) solution was added to every well of the plate. The plate was further incubated (37°C / 5% CO2) for three hours and the absorbance for the plate was read at 490 nm (BMG Labtech, FLUOstar OPTIMA).

Magnet

The magnet used was a 50mm x 50mm x 20mm rectangular block, rare-earth neodymium, nickel coated permanent magnet purchased from http://aussiemagnets.com.au/. The stated surface field strength is 6386 Gauss (0.6386T) and the direction of magnetism is along the 20mm thickness.

Magnetic measurements

Quantitative analysis of the nanoparticle uptake was measured by AC magnetic susceptibility. Cell suspensions of two cell lines, NIH-3T3 and HACAT were incubated with nanoparticles (plus control cells with no nanoparticles) and freeze-dried overnight, resulting in dry powders with weights in the range between 6 and 11 mg. Dry samples were transferred into gelatine capsules for the magnetic measurements.

Low temperature gelling agar (Sigma 39346-81-1) suspensions in distilled water (2% w/v) of the nanoparticles at different concentrations were also prepared to be used as standards for the magnetic quantification. The suspensions were sonicated for 30 s with an ultrasonic probe, to disperse the particles and heat the agar, and frozen immediately after the sonication to obtain a homogeneous distribution of the particles in the gel. The frozen gels were also freeze-dried overnight and transferred to gelatin capsules.

Magnetic measurements were performed in a Quantum Design MPMS-XL SQUID susceptometer with an AC option. Measurements were carried out in between 1.8 and 310 K, at an AC frequency of 10 Hz and with field amplitude of 0.41 mT.

2D migration

HaCaTs were grown to confluence on 10mm glass coverslips, incubated with (50 μg/mL) nanoparticles for 24 hours and then transferred to a 6 well plate with 3 mL of DMEM/F12 media. The permanent magnet was placed to the side of the plate and placed in an incubator (37°C, 5% CO_2) for five days, after which the coverslips were washed with PBS and imaged with an Olympus IX71 optical microscope in brightfield and fluorescence mode.

3D migration

3T3 cells were grown to 70-80% confluence in 6 well plates and then incubated for 24 hours with 50 μg/mL nanoparticles. The cells were then washed to remove any excess

nanoparticles and trypsinised from the plates. The cells without nanoparticles went through the same washing procedure to replicate the conditions before trypsinisation. The cells were counted using a haemocytometer/light microscope and duplicate readings were taken, the concentration was then made up to $3x10^5$ cells/mL in 1.5mL (Total of 450,000 cells). These cells were seeded on a Corning 24mm Transwell with polycarbonate membrane insert (Product #3428). The 10 μm thick membrane has 8 μm pores with a nominal pore density of $1x10^5$ pores per cm^2 and a cell growth area of 4.67 cm^2. The membrane was sterilized with gamma radiation and treated for optimal cell attachment.

The 1.5mL of cells in media was added to the inside of the transwell insert and 2.6mL of media was added to the outside of the membrane. At each timepoint, the excess media on the top of the insert was poured off and the insert was transplanted to a new plate with pre-warmed media (2.6mL). An additional 1.5mL of top-up media was added and the plate was then returned to the incubator. After each timepoint, the cells that had migrated through the membrane were counted by trypsinising, centrifuging them down, re-suspending in 50μL of media and then taking duplicate counts with a haemocytometer/light microscope. Trypan blue was used to stain for live/dead cells. All measurements were completed in triplicate with values provided as the mean ± standard error. Significance was determined with ANOVA and Bonferroni–Dunn post hoc tests (significance of $P \leq 0.05$).