Supporting information

Nanoparticle-Mediated Internalisation and Release of a Calcium Channel Blocker

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Experimental

Materials: The following materials were purchased from Aldrich unless stated otherwise: benzyl ether (99%), iron(III) acetylacetonate (97%), lomerizine dihydrochloride (LKT Laboratories, 98%), oleic acid (BDH, 92%), oleyl amine (70%), Pluronic F-108, polyethylenimine (50% solution, M_n 1200, M_w 1300), and 1,2-tetradecanediol (90%) were used as received. All tissue culture reagents were purchased from Invitrogen unless stated otherwise: fetal bovine serum, L-glutamine (200 mM), horse serum, non-essential amino acids (NEAA) 100×, penicillin G/streptomycin sulfate (50 U mL^{-1}, 50 µg mL^{-1}), poly(L-lysine) (Aldrich), RPMI1640, sodium pyruvate 100×, and trypsin/EDTA. Poly(glycidyl methacrylate) (PGMA) (M_w 200,000 g mol^{-1}) was provided by Bogdan Zdyrko and Igor Luzinov of Clemson University, SC.

Preparation of iron oxide nanoparticles: Fe_3O_4 was synthesised by the organic decomposition of $Fe(acac)_3$ in benzyl ether at 300 °C, in the presence of oleic acid, oleyl amine, and 1,2-tetradecanediol, as previously described.[1, 2]

Polymer nanosphere preparation: Nanoparticles were prepared using a non-spontaneous emulsification route. The organic phase was a prepared by dispersing iron oxide nanoparticles (15 mg) and dissolving PGMA (75 mg) and lomerizine dihydrochloride (10 mg) in a 1:3 mixture of $CHCl_3$ and MEK (6 mL). The organic phase was added dropwise, with rapid stirring, to an aqueous solution of Pluronic F-108 (1.25% w/v, 30 mL) in Tris buffer (10 mM) at pH 9.0. The resulting emulsion was homogenised with a probe-type ultrasonicator at low power for 1 min. The organic solvents were allowed to evaporate overnight under a slow flow of N_2. Centrifugation (3000*g,* 45 min)

removed large aggregates of iron oxide and excess polymer. For samples with PEI modification, the supernatant was removed to a 50 mL flask containing PEI (50 wt% solution, 100 mg) and heated to 80 °C for 18 h. The magnetic polymeric nanospheres were collected on a magnetic separation column (LS, Miltenyi Biotec), washed with Tris buffer (2 × 1.5 mL), and then flushed with Tris buffer until the filtrate ran clear. The resulting concentrated particle suspension was aliquoted for quantification by lyophilisation and subsequent use. Nanospheres were centrifuged, resuspended in PBS, and sterilised by UV irradiation immediately before use in tissue culture experiments.

Drug release: Experiments were performed at 37.0 ± 0.1 °C in phosphate buffered saline (PBS) at pH 5.0, 6.0 and 7.4. Nanospheres (10 mg) were dispersed in PBS (10 mL) and the sinks were sampled in duplicate over 10 h as follows. Aliquots of 150 µL were transferred to filter tubes (Millipore, Amicon Ultra-0.5, 50 kDa cutoff), centrifuged (17,000*g,* 5 min), and analysed by RP-HPLC using a similar procedure to Waki and Ando.[3] The measurements were run on a Waters 2695 separations module coupled with Waters 2489 UV/Vis detector and C_{18} column (150 × 4.60 mm, 5 µm, 25 ± 5 °C), using isocratic elution with 69:31 acetonitrile/0.1% potassium phosphate buffer (pH 6) at 10.0 mL min^{-1}, monitoring the eluent at 210 nm. Each sample was run for 13 min and the integrated area of the largest peak between the retention time 9–10 min was used to calculate the lomerizine concentration. No fresh PBS was introduced into the sinks. Lomerizine concentrations were determined from a standard curve and reported as mean values ± SE. The limit of detection for lomerizine in water at 210 nm was 0.1 µg mL^{-1}.

Cell culture: Rat pheochromocytoma cells (PC12) were obtained from the Mississippi Medical Center (Jackson, MS), cultured in poly(L-lysine)-coated polystyrene flasks in a humidified atmosphere containing 5% CO_2 at 37 °C, and maintained in RPMI1640 medium containing horse serum (10%), fetal bovine serum (5%), penicillin/streptomycin (100 U mL^{-1}, 100 µg mL^{-1}), L-glutamine (2 mM), non-essential amino acids (100 µM) and sodium pyruvate (1 mM). Cells were not differentiated with NGF.

Intracellular calcium quantification: For experiments, cells were seeded in 24-well plates containing 10 mm glass coverslips coated with poly(L-lysine) at a cell density of 5.0 × 10^4 mL^{-1} . Cells were

allowed to attach for 12 h and then growth media was replaced with treatments in media (lomerizine, 1 μM; empty PGMA nanoparticles + PEI (ENP), 20 μg mL^{-1}; nanoparticles containing lomerizine ± PEI (LNP±PEI), 20 μg mL^{-1}). Lomerizine was dissolved in DMSO at a non-toxic final concentration of 0.01%.[4] After 24 h incubation, glutamate was added to a final concentration of 10 mM. After a further 24 h, $[Ca^{2+}]_i$ was quantified as follows. Coverslips were transferred into HBS solution containing (in mM) NaCl 140, KCl 5.4, $CaCl_2$ 2.5, $MgCl_2$ 0.5, HEPES 5.5 and glucose 11, pH adjusted to 7.4, supplemented with Fura-2 AM (1 μM) and incubated at 37°C for 2 h. Cells were then imaged (ex 340/380 nm, em 510 nm). The ratio of the emissions at the two wavelengths is denoted $R = F_{340}/F_{380}$. Fluorescent data were measured on a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope, and analysed using Metamorph v6.3. Calibrations were performed in 8 cells. Cells were exposed to 5 μM ionomycin to achieve a steady state maximum (R_{max}). Media was then replaced with Ca^{2+} free HBS containing 3 mM EGTA to achieve a steady state minimum (R_{min}). $[Ca^{2+}]_i$ was calculated according to the equation:

$$[Ca^{2+}]_i = K_d \cdot b \cdot \frac{R - R_{min}}{R_{max} - R}$$

where R_{min} = 0.61 ± 0.02, R_{max} = 1.57 ± 0.21, b = 1.09 is the ratio of fluorescent intensities during illumination at 380 nm with 0 mM Ca^{2+} and 2.5 mM Ca^{2+}, and K_d = 224 nM is the dissociation constant as determined previously.[5]

References

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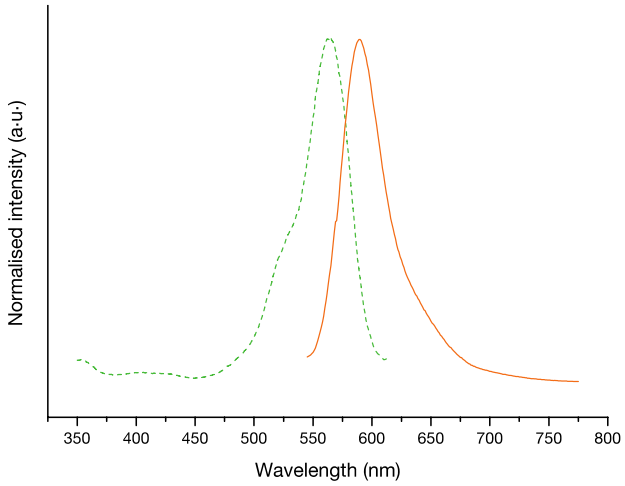

Figure S1. Fluorescence spectrophotometry displays the spectral characteristics of PEI-modified nanospheres; uncorrected excitation (dashed line) and emission (solid line) spectra were measured in water.

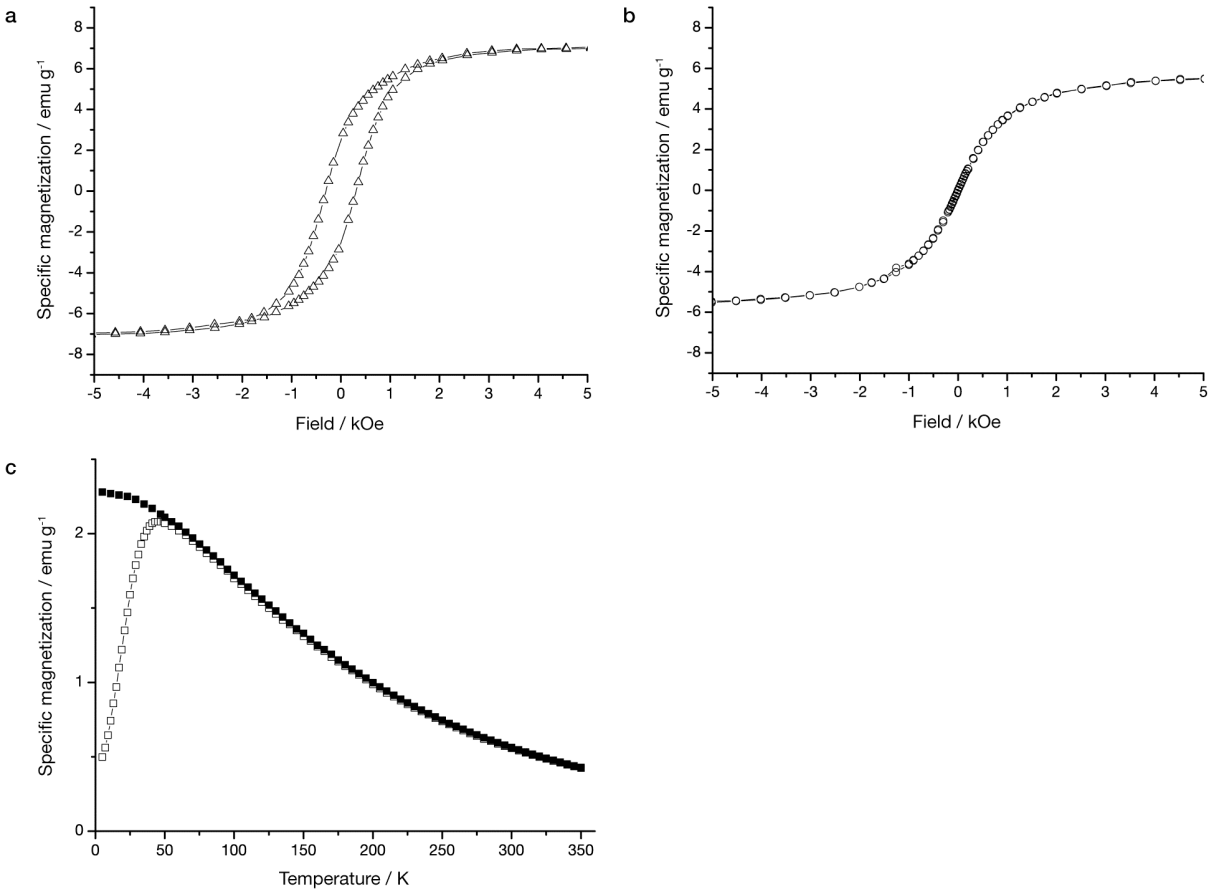

Figure S2. SQUID magnetometry of PEI-modified nanospheres reveals superparamagnetic behavior. (a) Hysteresis loop at 5 K. (b) The magnetization curve at 300 K displays no hysteresis and shows a specific saturation magnetization of 5.7 emu g^{-1}. (c) Zero-field-cooled (open points) and field-cooled (solid points) curves are coincident above *ca.* 50 K.