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Supporting Information

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Supporting Information

for

Microsphere-Mediated Protein Delivery into Cells

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S1. General protocol for protein loading of microspheres.

0.5 and 2.0 μm aminomethyl functionalized microspheres were washed with PBS buffer (pH 7.4), NaOH (500 mM) and finally PBS (pH 7.4). After washing and centrifugation, microspheres were re-suspended in a 25 % (*w/v*) glutaraldehyde solution in PBS buffer and the mixture was shaken for 15 h at room temperature. After this time the microspheres were washed with PBS (pH 7.4), or a specific protein buffer, before addition of protein (5 μg/mL in PBS, pH 7.4). The mixture was shaken for 15 h at room temperature prior to washing with PBS (pH 7.4) and treatment with a sodium cyanoborohydride solution (20 mM) in PBS:EtOH (3:1) for 2 h. Finally the protein-loaded microspheres were washed with PBS and treated with a quenching solution (40 mM ethanolamine with 1% (*w/v*) BSA in PBS). The protein-loaded microspheres were washed and stored in PBS (pH 7.4) or a specific protein buffer.

β-galactosidase from E.coli was purchased from Biochemika and eGFP was a gift from Dr. P. Roch (University of Southampton, United Kingdom).

S2. Efficiency of GFP coupling.

The fluorescence intensity at 508 nm, after excitation at 475 nm, of a series of EGFP dilution solutions in PBS buffer, pH 7.4, was measured by spectrofluorometry to give the table below which was used for the construction of a reference curve.

Table S1. Fluorescence intensity of eGFP solutions of different concentrations.

eGFP concentration (μg/L)	Fluorescence intensity at 508 nm
5	4562190
0.5	789120
0.05	145470
0.005	68550
0.0005	38340
0.00005	30070

500 nm and 2 μm PEG-microspheres (100 μL, 2% sc, 0.26 mmol/g, 0.52 μmol, 1 equiv) were washed with PBS buffer pH 7.4 (200 μL), NaOH 500mM (200 μL) and finally PBS pH 7.4 (200 μL). After washing and centrifugation, microspheres were resuspended in a glutaraldehyde solution (400 μL, 25 % (*w/v*)) in PBS buffer (400 μL) and the mixture was shaken for 15 h after which time the microspheres were washed with PBS pH 7.4 (800 μL) before addition of an EGFP solution (400 μL, 5 μg/mL in PBS, pH 7.4) and the mixture was shaken overnight to give microspheres. A reference experiment was carried out in parallel where solutions of EGPF (400 μL, 5 μg/mL) in PBS buffer were added to amino-methyl polystyrene microspheres before being washed as described above. After coupling EGFP, microspheres were washed with PBS buffer (2 x 200 μL) then with a BSA solution (200 μL, 5 μg/mL) and then with PBS buffer again (200 μL). The supernatants were combined and the fluorescence at 508 nm (I_{Fluo}) was recorded.

TableS2. Efficiency of eGFP coupling to microspheres.

Samples	**I_{Fluo} (cps) (475 nm)**	**Conc. (μg/mL)**	**Volume (mL)**	**GFP released (μg)**	**Coupling efficiency (%)**
eGFP + microspheres	1506441	1.56	1.1	1.72	16
0.5 μm eGFP-Glut-PEG-microspheres	147240	0.016	0.85	0.013	99
2 μm eGFP-Glut-PEG-microspheres	349460	0.045	0.81	0.036	98

S3. Cell culture conditions and general protocol for cellular uptake experiment

Cells were cultured in RPMI or DMEM containing high glucose (4.5 mg/mL) and supplemented with 100 units/mL penicillin, 50 mg/mL streptomycin, and 10% FBS. The cells were maintained in a humid chamber at 37◦C in an atmosphere of 5% CO_2.

Cells were grown in DMEM or RPMI medium supplemented with 4 mM glutamine, 10 % FCS and 100 units/mL of penicillin/streptomycin in T75 cell culture flasks until 70 % confluency. Cells were suspended using trypsin/EDTA and plated in a 24 well-plate at a density of 4 x 10^4 cells per well. After overnight incubation, cells were incubated with 500 nm and 2 μm protein-labelled microspheres at different concentrations and for several incubation times at 37 ℃ with 5% CO_2. After the incubation time, cells were washed twice with PBS, harvested with trypsin/EDTA, and resuspended in 350 μL of 0.04% Trypan Blue (Sigma) solution diluted in Hank's Balanced Salt Solution (Bioclear). The internalization of labeled-microspheres was analyzed by flow cytometry analysis using a FACSAria flow cytometer (Becton Dickinson). A total of 10,000 events per sample were analyzed. Cell samples were excited with a 488-nm (Coherent® Sapphire™ solid state) laser and 530/30 nm (Fluorescein) band pass filter was used for fluorescence analysis of the cellular uptake.

Table S3. Percentage of cellular uptake for different cell lines. 2 µm GFP-labeled PEG-microspheres (0.2 mg/mL), 24 h incubation (at 37 ℃ and 5 % CO_2 atmosphere).

Cell Type	% Cellular uptake
HEK 293T	82
ND7	60
B16F10	46
HeLa	26

S4. FDG staining

For FDG staining cells were harvested with trypsin/EDTA, washed with fresh medium and resuspended in 2% FCS in PBS buffer. The cell suspension was then incubated at 37 ℃ for 5 min and 50 µL of fluorescein-β-D-galactopyranoside (FDG) (1 µg/µL dissolved in 98:1:1 H_2O/ethanol/DMSO) was added. After a further 5 min incubation at 37 ℃ in the dark and the addition of 200 µL of chilled cell medium and 100 µL of 0.4% Trypan Blue Solution (Sigma), the cells were analyzed by flow cytometry.

S5. Toxicity test of betagalactosidase-loaded microspheres. Cell viability assay (MTT assay).

Cells were seeded in a 96-well microplate (2x 10^4 cells/well) and allowed to attach for 15 h. Each well was incubated with microspheres in Dulbecco's complete medium at several concentrations and incubation times. Measurements were performed 4 to 8 times and experiments, where various parameters were compared, were run in the same 96-well plate, using the same batch of cells. At the end of the incubation time, cells were washed with PBS buffer pH 7.4 and incubated for further 24 h in serum-free, phenol-red-free DMEM. After this time 10 µL MTT solution in PBS buffer (1 mg/mL) were added and plates were incubated at 37℃ for 3 h. Formazan was then dissolved by adding 100 µL of a 10% TRITON X-100 in acidic isopropanol (0.1 N HCl) solution and thoroughly mixed.

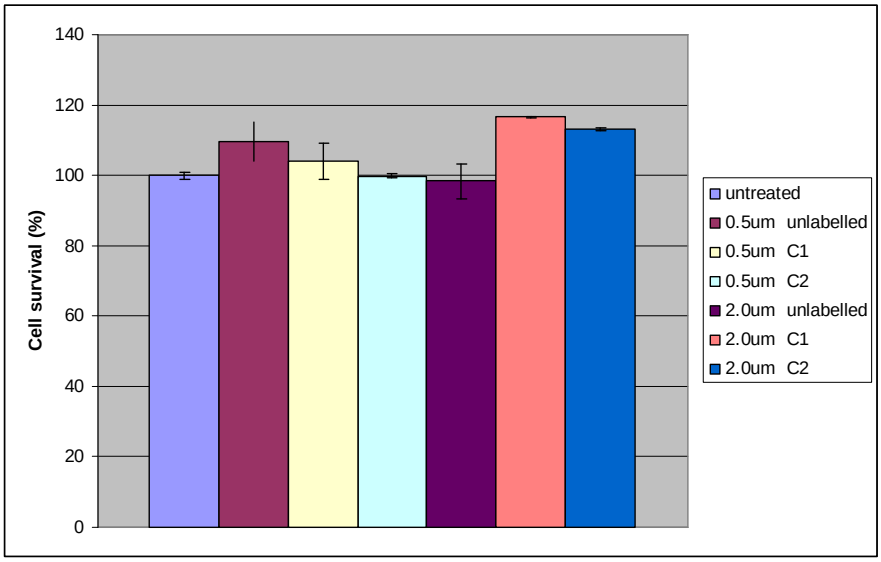

Figure S1. Results of toxicity test for β-galactosidase-loaded microspheres (C1 and C2 are 0.1 and 0.2 µg/µL, respectively).