ESI

The mechanism of cell uptake for luminescent lanthanide optical probes: the role of macropinocytosis and the effect of enhanced membrane permeability on compartmentalisation

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Figure 1

Fluorescence microscope images showing Tb luminescence of CHO cells treated with $[Tb.L^{5}]^{3+}$ (50 μM, 4h) following thirty minute incubation with inhibitor: (a) control, (b) chloroquine (10μM), (c) chlorpromazine (50 μM), (d) filipin (1 μg/mL), (e) wortmannin (300 nM), (f) 1,2-dipalmitoyl-*rac*-diacylglycerol (50 ng/mL). Scale bar represents 20 μm.

Table S1

Changes in the uptake of $[Eu.L^{1}]^{3+}$ (50 μM, 4h) in CHO cells following treatment with various inhibitors/promoters.

Table S2

Changes in the uptake of $[Eu.L^{4a}]^{3+}$ (50 μM, 4h) in CHO cells following various treatments.

Experimental details of cell culture, microscopy, and cytotoxicity and lipophilicity measurements

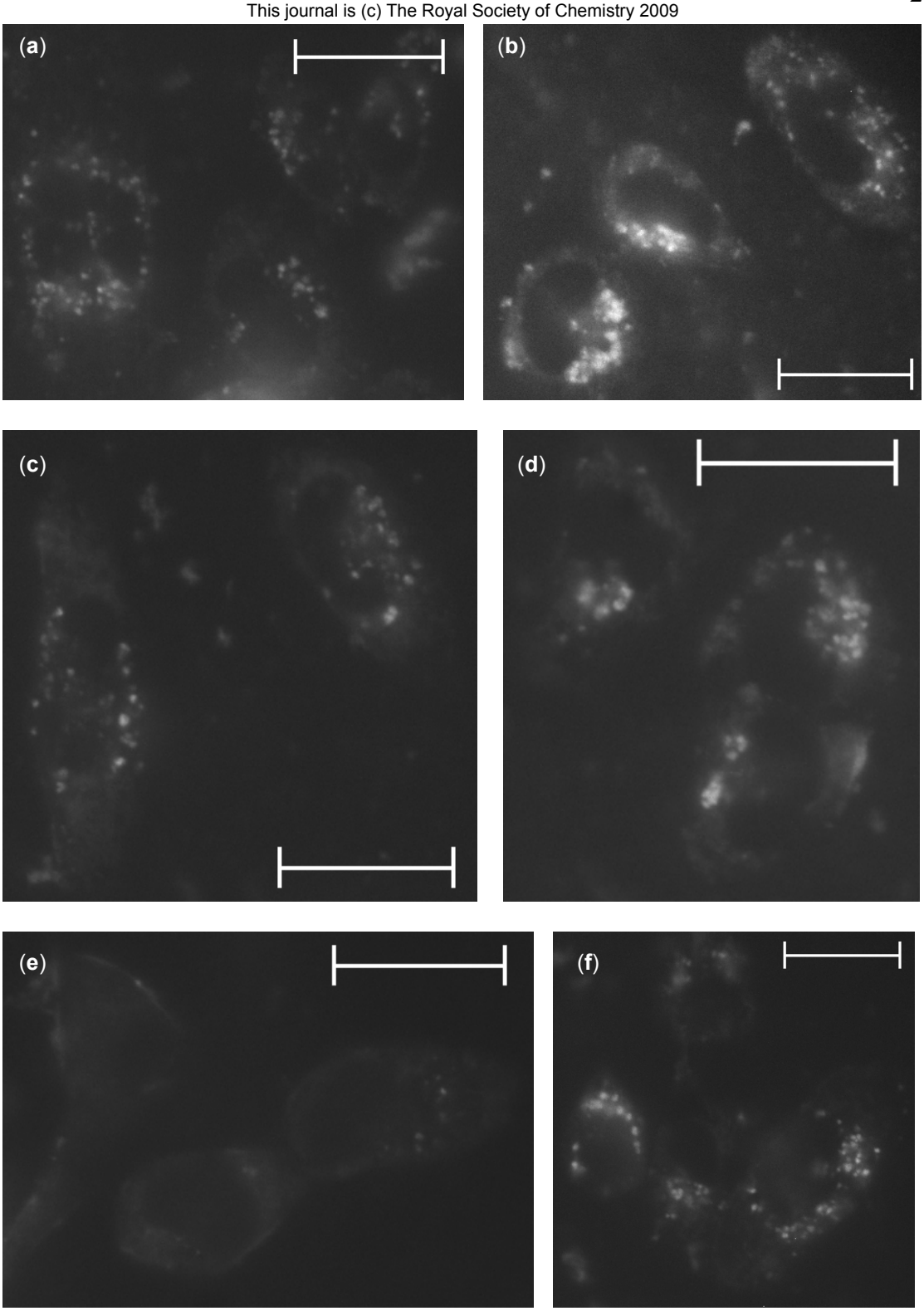

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Table S1

Changes in the uptake of $[Eu.L^{1}]^{3+}$ (50 μM, 4h) in CHO cells following various treatments.

	Uptake μmol Eu / g protein					
	replicate 1	replicate 2	replicate 3	average	% uptake	St Dev
Control	3.38	3.25	3.59	3.41	100.00	5.06
Chloroquine	2.93	3.30	3.40	3.21	94.18	7.44
Chlorpromazine	3.27	2.33	4.00	3.20	93.86	20.04
Filipin	3.08	2.30	4.35	3.25	95.22	25.35
Monensin	2.89	1.92	2.98	2.60	76.19	14.53
Poly-L-lysine	2.82	3.53	4.23	3.53	103.46	17.78
Sucrose	2.77	2.48	3.51	2.92	85.65	11.12
Wortmannin	2.06	1.81	2.85	2.24	65.76	12.48
Amiloride	2.03	2.45	2.69	2.39	70.13	8.73
Phorbol 12-myristate-13-acetate	4.16	6.15	4.30	4.87	142.86	39.19
1,2-dipalmytoylglycerol	6.36	7.84	6.01	6.74	197.64	38.01
5 ºC	1.50	1.35	1.44	1.43	41.97	2.19

Table S2

Changes in the uptake of $[Eu.L^{4a}]^{3+}$ (50 μM, 4h) in CHO cells following various treatments.

	Uptake μmol Eu / g protein					
	replicate 1	replicate 2	replicate 3	average	% uptake	St Dev
Control	5.21	5.01	5.49	5.23	100.00	4.61
Chloroquine	4.83	4.32	4.53	4.56	87.17	5.12
Chlorpromazine	6.26	4.04	4.89	5.07	96.78	20.75
Filipin	4.90	4.55	4.99	4.82	92.01	1.79
Monensin	4.61	4.18	4.39	4.39	83.94	4.19
Poly-L-lysine	6.30	5.21	6.02	5.84	111.62	8.48
Sucrose	-0.01	4.85	5.19	3.34	63.89	1.65
Wortmannin	3.01	3.15	3.10	3.09	59.00	3.40
Amiloride	3.84	3.73	3.75	3.77	72.12	3.36
Phorbol 12-myristate-13-acetate	7.29	6.43	6.19	6.64	126.80	13.61
1,2-dipalmytoylglycerol	8.56	6.95	6.68	7.40	141.34	21.39
5 ºC	1.20	1.97	0.76	1.31	25.04	12.96

Experimental Details

Cell culture

Three cell lines were selected for cellular studies: CHO (Chinese Hamster Ovary), NIH 3T3 (mouse skin fibroblast) and HeLa (human endothelial carcinoma cells). Cells were maintained in exponential growth as monolayers in F-12 (Ham) medium, DMEM (Dulbecco's Modified Eagle Medium) and RPMI 1640 medium respectively. For each cell line, the medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Cells were incubated at 37 °C, 20% average humidity and 5% (v/v) CO_2.

Microscopy

Cells were seeded in 12-well plates on glass cover slips and allowed to grow to 40% – 60% confluence, at 37 ºC in 5% CO_2. At this stage, the medium was replaced and cells were treated with drugs and complexes as described below. For NIH 3T3 and HeLa cells, the non-phenol red containing DMEM was used. Following incubation, the cover slips were washed with phosphate-buffered saline (PBS; pH 7.5), mounted on slides and sealed with nail varnish. Epifluorescence images were taken on a Zeiss Axiovert 200M epifluorescence microscope with a digital camera, and were processed using the Zeiss Axiovision software. G365 filters (Zeiss) were employed for excitation of the complexes, with 546/12 and 575-625 filters (Comar) used for emission of Tb and Eu respectively.

For the co-localisation study, cells were treated with 2.5 mg/mL FITC-Dextran 70000 conjugate (Invitrogen) for fifteen minutes. The medium was then removed and the cells washed three times with medium prior to fifteen minute incubation with 50 μM complex.

For membrane permeabilisation studies, cells were washed with ice-cold PBS and then treated with ice-cold solutions of ethanol (70%), saponin (0.5 mg/mL) or Triton X-100 (1%) for fifteen minutes. The medium was then removed and the cells washed in turn with ice-cold PBS, room temperature PBS, 37 °C PBS and finally 37 °C medium. The cells were then incubated with 50 μM complex for 4 hours.

Uptake studies

Cells were seeded in 6-well plates and allowed to grow to 80% – 100% confluence, at 37 ºC in 5% CO_2. At this stage, the medium was replaced and cells treated with drugs and complexes as described below. Following incubation, the medium was removed and the cells washed three times with PBS. 500 μL of lysis buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% protease inhibitor cocktail) was then added to each well, and the cells incubated at 5 °C for fifteen minutes. 3 x 25 μL aliquots of the supernatant were taken from each well for the BCA assay, as described below, and 400 μL was submitted for ICP-MS analysis.

BCA assay

Total protein content was determined in lysed cells using the bicinchoninic acid protein assay (BCA™ Protein Assay Kit). A BSA standard curve was constructed in the range 0.2 to 1 mg/mL. 25 μL of standards and samples were transferred into triplicate wells of a 96-well plate and 200 μL of BCA reagent mix was added to each well. Absorbance at 540 nm was measured after 10 h incubation at room temperature using a microplate reader. Protein concentration for each sample was determined against the standard curve.

ICP-MS

Inductively coupled plasma mass spectrometry determinations of europium concentrations were made by Dr. C. Ottley in the Department of Earth Sciences at Durham University.

Drug treatments

For the 5 °C treatment, cells were incubated in a refrigerator set at 5 °C. Concentrations of inhibiting or activating drugs used were 10 μM chloroquine, 50 μM chlorpromazine, 1 μg/mL filipin, 2 μM monensin, 0.01% poly-L-lysine, 50 mM sucrose, 300 nM wortmannin, 3 mM amiloride, 50 ng/mL phorbol 12-myristate-13-acetate and 500 ng/mL 1,2-dipalmitoyl-*rac*-diacylglycerol. Cells were subjected to appropriate treatments for 30 min. prior to treatment with 50 μM complexes for 4h.

Cytotoxicity

IC_{50} values were determined using the MTT assay, as described by Carmichael[1], which makes use of the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple formazan product by the mitochondrial dehydrogenase of viable cells. This insoluble formazan was quantified spectrophotometrically upon dissolution in DMSO. Approximately 1 x 10^4 NIH-3T3 cells in 100 μL DMEM were seeded into each well of flat-bottomed 96-well plates and allowed to attach overnight. Complex solutions were added to triplicate wells to give final concentrations over a 2-log range. Following 24 h incubation, MTT (1.0 mM) was added to each well, and the plates incubated for a further 4 h. The culture medium was removed, and DMSO (150 μL) was added. The plates were shaken for 20 seconds and the absorbance measured immediately at 540 nm in a microplate reader. IC_{50} values were determined as the drug concentration required to reduce the absorbance to 50% of that in the untreated, control wells, and represent the mean for data from at least three independent experiments.

Lipophilicity

Lipophilicity was measured as the relative partitioning of the complex between water and 1-octanol. Water was saturated with 1-octanol, and 1-octanol was saturated with water. Complexes were prepared as 100 μM solutions in water and mixed with 1-octanol in 1:2, 1:1 and 2:1 water:octanol ratios. Mixtures were agitated for twelve hours, after which time emission spectra of the water and octanol layers were collected. Emission spectra were measured on an Instruments SA Fluorolog 3-11 spectrometer and DataMax v2.1 for Windows. The complex concentration was calculated at the emission maximum with reference to calibration curves constructed using at least five concentrations between 0 and 100 μM for water and 0 and 40 μM for 1-octanol. For each mixture, the logP value was calculated according to the following equation:

$$\log P = \log_{10} \frac{[X]_{1-\text{octanol}}}{[X]_{\text{water}}}$$

Final logP values were calculated as the average of at least two replicates of the three solvent mixtures.

[1] J. Carmichael, W. G. DeGraff, A. F. Gazdar, J. D. Minna, and J. B. Mitchell, *Cancer Res.*, 1987, **47**, 936.