Electronic Supplementary Material (ESI) for Chemical Communications

Targeting of anionic membrane species by lanthanide(III) complexes: towards improved MRI contrast agents for apoptosis.

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Supplementary information

1. Synthetic Details

Materials. [Eu(**L**)Zn_2]$(NO_3)_4$ solution in 10 mM HEPES buffer was prepared from the [Eu(**L**)] complex and $Zn(NO_3)_2 \cdot 6H_2O$, as described in previous publications.[1] [Gd(**L**)Zn_2]$(NO_3)_4$ solution for use with cells was prepared by first producing [Gd(**L**)Zn_2]$(NO_3)_4$ *in situ* in unbuffered aqueous solution from [Gd(**L**)] and $Zn(NO_3)_2 \cdot 6H_2O$; this was then lyophilised, and rehydrated in a sterile environment to yield a stock solution for imaging experiments.

Preparation of vesicles. Lipids DOPS (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine, sodium salt) and DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc.(Alabaster, AL). Nucleopore track-etched membranes and polycarbonate drain discs (Whatman, now GE Healthcare) were purchased from VWR Int, U.K.

Stock solutions of DOPS (25 mg/mL), DOPC (25 mg/mL) and the hydrophobic chromophore 2,3-naphthalimide, **2,3-Nap**, (5 mg/mL) in $CHCl_3$ were prepared. Three different sets of solutions namely DOPS:DOPC (**1**), DOPS:DOPC:2,3-Nap (**1-S**), and DOPC:2,3-Nap (**2-S**) were prepared by mixing the above solutions (see Table S1 for quantities of each solution and molar composition). The solvent was evaporated and the mixtures were dried under vacuum overnight resulting in a thin film. Stock solutions of the corresponding vesicles were made by rehydration of these thin films at room temperature in 5 mL HEPES buffer (10 mM HEPES, pH 7.4). The resulting multilamellar vesicles were extruded to form unilamellar vesicles with a LIPEX™ Extruder purchased from Northern Lipids, Canada. The samples were extruded 12 times through 25-mm Nucleopore track-etched membranes (200-nm diameter pores) and a polycarbonate drain disc.

Table S1.

Solution	**Volume of DOPC from stock solution (mmol in brackets)**	**Volume of DOPS from stock solution (mmol in brackets)**	**Volume of 2.3-Nap from stock solution (mmol in brackets)**
DOPS:DOPC:2,3-Nap (**1-S**)	0.393 mL (0.012 mmol)	0.405 mL (0.013 mmol)	0.099 mL (0.003 mmol)
DOPS:DOPC (**1**)	0.393 mL (0.012 mmol)	0.405 mL (0.013 mmol)	0 mL (0 mmol)
DOPC: 2,3-Nap (**2-S**)	0.786 mL (0.024 mmol)	0 mL (0 mmol)	0.099 mL (0.003 mmol)

2. Spectrofluorimetric Studies.

General. Emission spectra were obtained using a Horiba Jobin Yvon, Fluorolog-2 spectrofluorimeter using 10 x 10 mm quartz cuvettes. In all cases, the excitation wavelength of **2,3-Nap** (λ_{ex} = 335 nm) was used the temperature was 25 °C; no samples were degassed.

Job's Plot. A Job's plot was produced for the binding of the DOPS head group by [Eu(**L**)Zn_2]$(NO_3)_4$. The 1:1:0.1 DOPS:DOPC:**2,3-Nap** liposomes and [Eu(**L**)Zn_2]$(NO_3)_4$ were mixed together to prepare a series of solutions wherein the combined concentration of 'available' DOPS (presuming 50% of total DOPS head groups are displayed on the outer membrane surface) and [Eu(**L**)Zn_2]$(NO_3)_4$ was kept constant (100 μM) and the mole fractions of [Eu(**L**)Zn_2]$(NO_3)_4$ varied. Emission intensities at 614 nm were monitored for each, and a 'Job's plot' of intensity vs. mole fraction of [Eu(L)Zn_2]$(NO_3)_4$ obtained (see, inset in Figure 3 in main text).

Titrations. [Eu(**L**)Zn_2]$(NO_3)_4$ solutions of varying concentrations (0-400 μM) were made up in HEPES buffer (10 mM) along with a fixed concentration of liposome (**1-S**, 200 μM total lipid, 50 μM PS); each sample was allowed to equilibrate for 15 minutes before recording the spectra (emission shown to be stable from 10 mins to several hours). See Figure S1 for spectra.

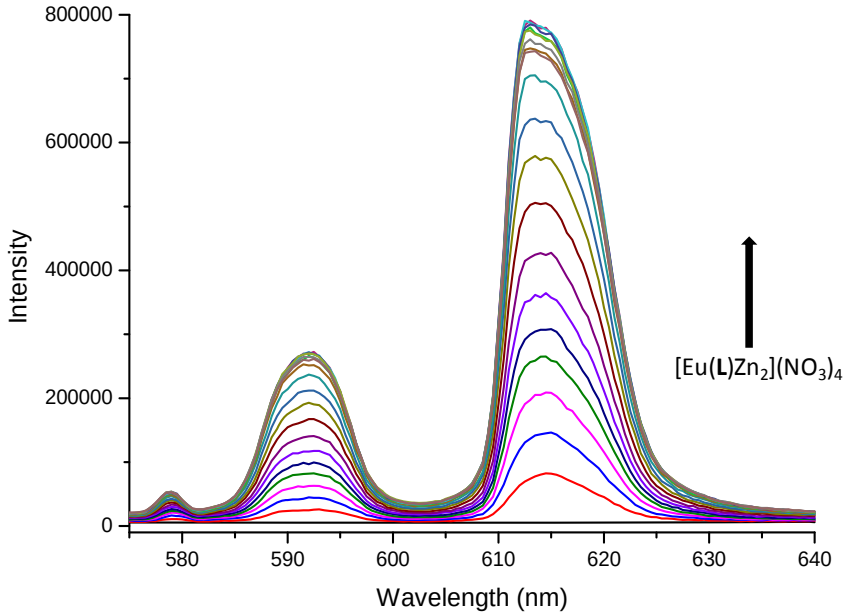

Figure S1: Emission spectra of DOPS:DOPC:**2,3-Nap** (**1-S**) vesicle (200 μM) in HEPES buffer on addition of [Eu(**L**)Zn_2]$(NO_3)_4$ in varying concentrations [10-300 μM), (λ_{ex} = 335 nm, excitation and emission slit width are 5 nm and 1 nm respectively)].

Binding constant:

An association constant for the binding of the DOPS head group was estimated by least square fitting of the titration data to a 1:1 model.

A standard expression[2] for simple 1:1 binding (Eq 1) was used to for host/guest complex formation, [HG], from which concentrations of free host and guest, [H] and [G], could be obtained by mass balance (Eqs 2+ 3). The molar free guest response (a_g, molar intensity per free DOPS head group) was obtained from the experimental data (Eq 4, where [H] = 0). The association constant, K_a, along with the other molar response factors, a_H, a_{HG}, were then obtained from least squares fitting of the experimental data (I_{exp}, a_g) to Equations 1, 2, 3 and 5, using Microsoft Excel Solver.

$$[HG] = \frac{1}{2}\left(G_T + H_T + \frac{1}{K_a}\right) - \sqrt{\left(G_T + H_T + \frac{1}{K_a}\right)^2 + 4[H_T][G_T]} \quad (1)$$

$$[H] - [H_T] - [HG] \quad (2)$$

$$[G] = [G_T] - [3G] \quad (3)$$

$$a_G = \frac{I_{exp}}{[G]} \quad (4)$$

$$I_{calc} = a_H[H] + a_G[G] + a_{HG}[HG] \quad (5)$$

Where:

$[H_T]$ = total concentration of host ($[Eu(\mathbf{L})Zn_2](NO_3)_4$) present (M)

$[G_T]$ = total concentration of guest (available DOPS head group) present (M)

$[HG]$ = concentration of complex present (M)

$[H]$ = concentration of free host present (M)

$[G]$ = concentration of free guest present (M)

K_a = association constant between H and G (M^{-1})

I_{calc} = calculated emission intensity

I_{exp} = experimentally-determined emission intensity

a_H = molar response (emission) factor for H

a_G = molar response (emission) factor for G

a_{HG} = molar response (emission) factor for HG

Confirmation of reversibility

The reversibility of the interaction between **1-S** and $[Eu(\mathbf{L})Zn_2]^{4+}$ was corroborated by the dramatic decrease of Eu-centred emission upon addition of 10 equivalents of pyrophosphate (which has previously shown is very strongly bound by $[Eu(\mathbf{L})Zn_2]^{4+}$)[1] to a 1:1 mixture of **1-S** and $[Eu(\mathbf{L})Zn_2]^{4+}$ (see Figure S2).

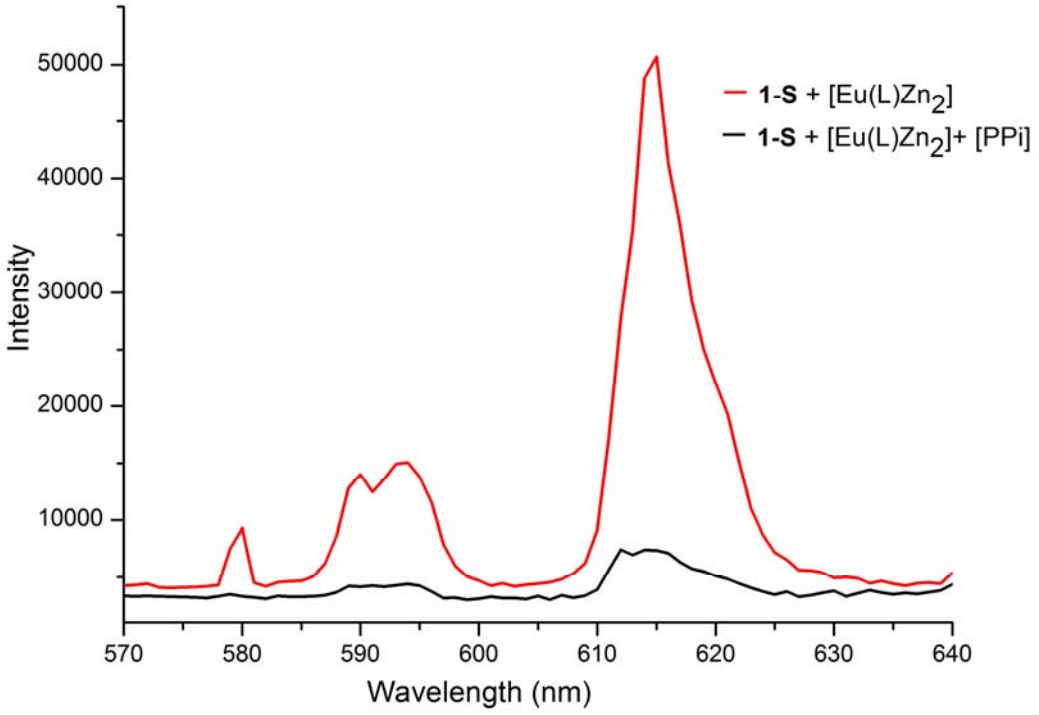

Figure S2: Emission spectrum of DOPS:DOPC:2,3-Nap (**1-S**) vesicle (50 μM) in HEPES buffer + $[Eu(\mathbf{L})Zn_2](NO_3)_4$ (50 μM) in HEPES buffer (red line), and the same after addition of 10 equivalents of pyrophosphate (PPi, black spectrum) (λ_{ex} = 335 nm, excitation and emission slit width are 5 nm and 1 nm respectively)

Comparative emission of DOPS:DOPC:2,3-Nap (1-S) vesicle exposed to $[Eu(L)Zn_2](NO_3)_4$ and the zinc-free [Eu(L)] complex.

DOPS:DOPC:2,3-Nap (**1-S**) vesicle were incubated not only with $[Eu(L)Zn_2](NO_3)_4$ but also with the zinc-free [Eu(L)] complex. The purposes of this experiment was to assess any non-specific binding effects that would lead to enhancement of the emission even if the compound was not directly binding to the PS on the vesicles' surface. As shown in Figure S3, the free-zinc complex displays a much lower intensity enhancement than $[Eu(L)Zn_2](NO_3)_4$ indicating that only minor contributions to the emission are due to non-specific binding (or small amounts of free Eu – see below).

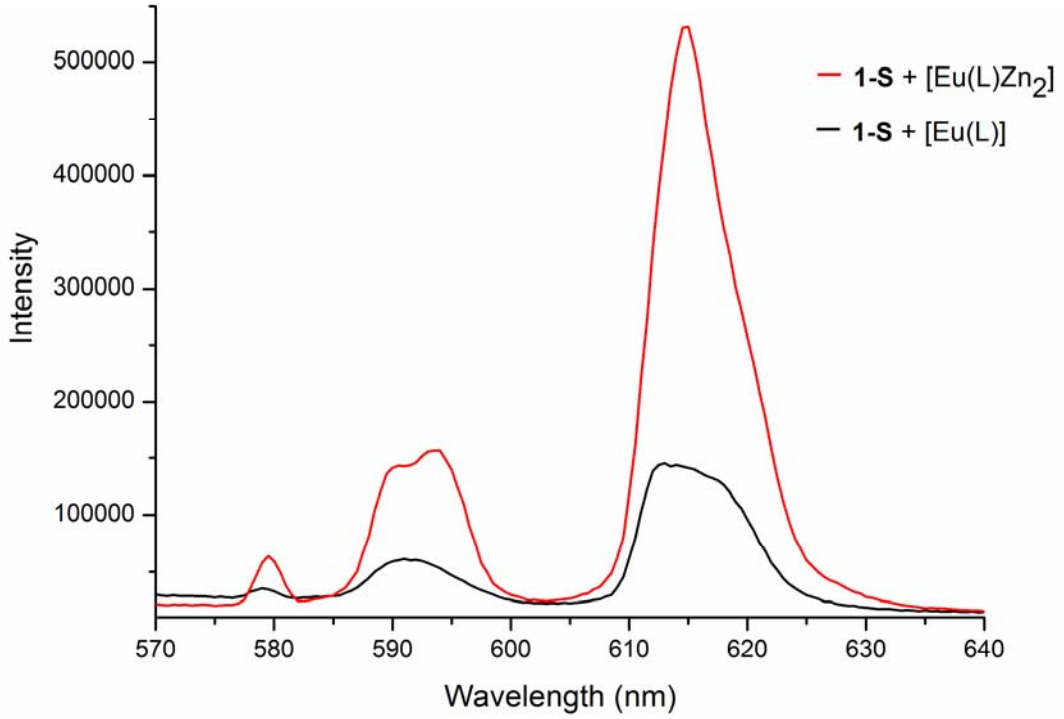

Figure S3: Emission spectrum of DOPS:DOPC:2,3-Nap (**1-S**) vesicle (200 µM) in HEPES buffer with $[Eu(\mathbf{L})Zn_2](NO_3)_4$ (50 µM, red line) and [Eu(**L**)] (50 µM, black line) in HEPES buffer (λ_{ex} = 335 nm, excitation and emission slit width are 5 nm and 2 nm respectively)

3. Cell Culture and Apoptosis Induction

Murine lymphoma cells (EL4) were grown in RPMI 1640 medium (Sigma-Aldrich, Dorset, UK) supplemented with 10% heat inactivated foetal bovine serum (Invitrogen, Paisley, UK). Once confluent the cell density was adjusted to $1x10^6$ cells/mL in a T75 flask and a further 15 mL of media was added and incubated at 37 ^oC in a humidified incubator with 5% CO_2 for 16 hours. In parallel, to induce apoptosis 15 μM Etoposide (Sigma-Aldrich, Dorset, UK) was added to $1x10^6$ cells/mL in a T75 flask and a further 15 mL of media was added and incubated at 37 ^oC in a humidified incubator with 5% CO_2 for 16 hours. To confirm and quantify the percentage of cells undergoing apoptosis, cells were collected by centrifugation and the density was adjusted to 1 $x10^6$ cells/mL in saline. A commercially available apoptosis kit was used with 10 μL of propidium iodide and 5 μL of Annexin V-FITC (Sigma-Aldrich, Dorset, UK) added to 1mL of the cell suspension and flow cytometry used for assessment, with 10,000 profiles recorded (see Figure S4).

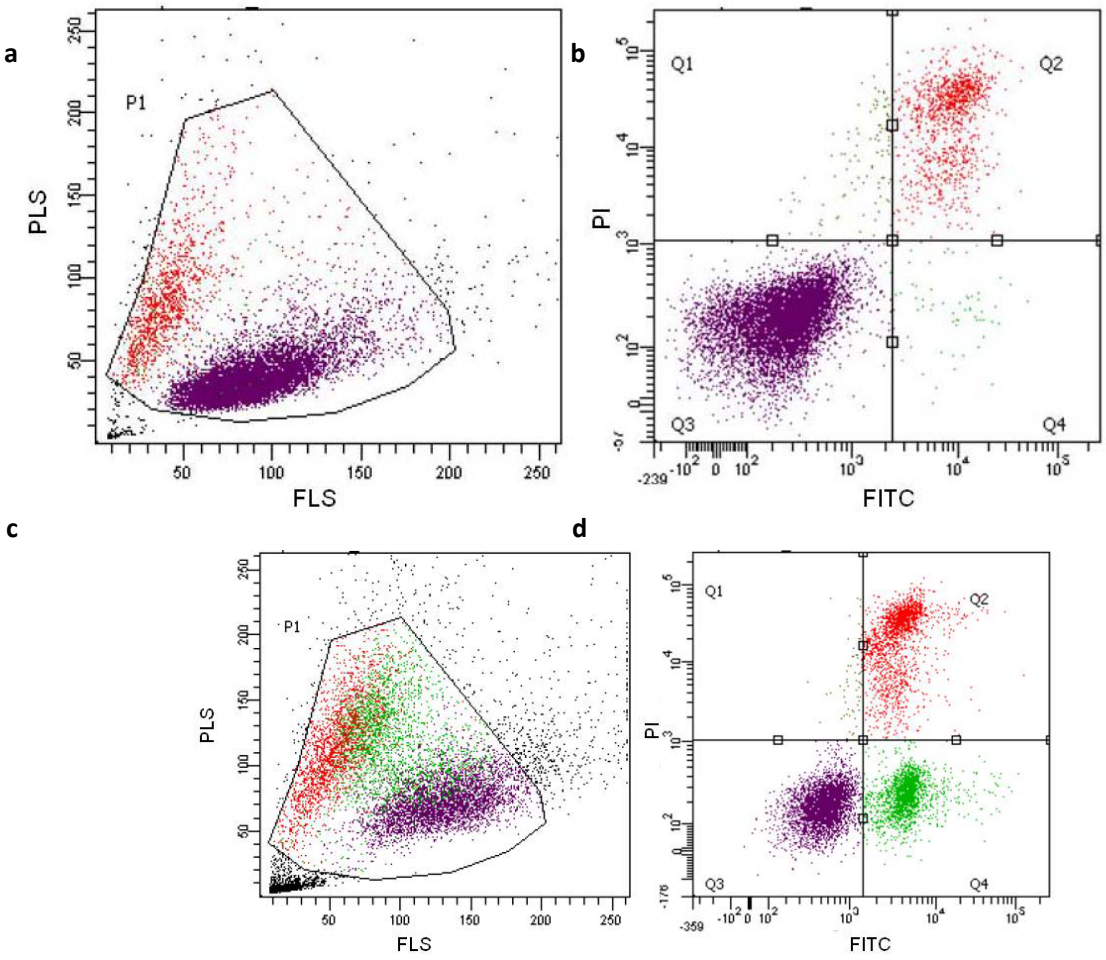

Figure S4: Untreated non-apoptotic **(a + b)** and Etoposide treated apoptotic **(c + d)** EL4 cells incubated with propidium iodide and annexin V-FITC and analysed using flow cytometry. **(a + c)** light scattering profile, the *x* and *y* axis represent the light intensity from forward light scattering (FLS) and perpendicular light scattering (PLS), respectively. **(b + d)** the *x* and *y* axis represent fluorescent intensity profiles from FITC and PI respectively. Profiles from 10,000 cells are presented. Untreated cells had 10.5% in Q2, 84.1% in Q3 and 0.6% in Q4 representing necrotic, viable and early apoptotic cell populations respectively and Etoposide treated cells had 19.2% in Q2, 38.1% in Q3 and 19.6% in Q4 representing necrotic, viable and early apoptotic cell populations, respectively.

4. Cell viability MTS assay

EL4 cells (grown as indicated above) were seeded into a 96-well plate at a concentration of $60x10^3$ cells per well and were treated with compound $[Gd(\mathbf{L})Zn_2](NO_3)_4$ at concentrations between 1 µM and 500 µM for 2 h at 37 °C (total volume of 100 µL). After 2 h, 20 µL of a 2 mg/mL MTS + 100 µL of a PMS 0.96 mg/mL solutions were added to each well and the mixture was incubated for a further 16 h at 37 °C. After this time, an ELISA reader was used to record the absorption at 490 nm and the cell viability (as % of the control, i.e. cells treated with MTS/PMS but without added $[Gd(\mathbf{L})Zn_2](NO_3)_4$). The results, in triplicate, are shown in Figure S5.

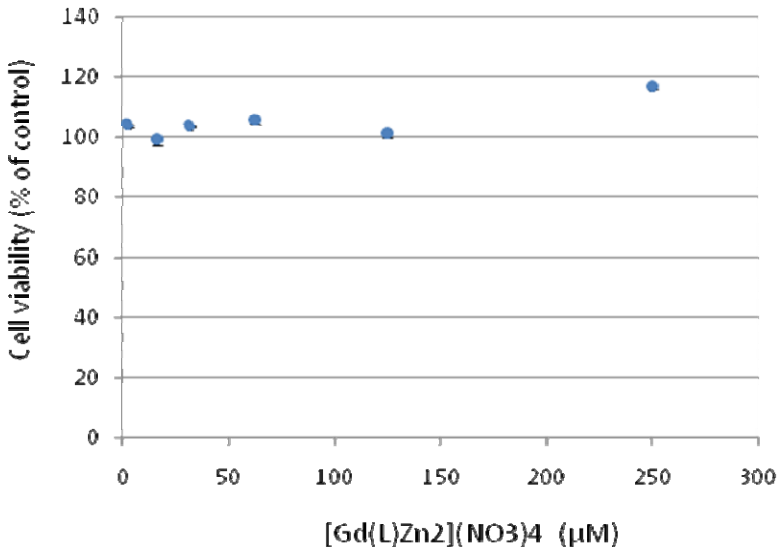

Figure S5. Cytotoxicity of compound $[Gd(\mathbf{L})Zn_2](NO_3)_4$. EL4 cells were treated with up to 500 µM of the $[Gd(\mathbf{L})Zn_2](NO_3)_4$. Data shown is average of three measurements (error bars have been included but they are not very clear since all standard deviations were below ± 2%)

5. MRI Detection of Apoptotic Cells

Cells were grown as above, collected by centrifugation and cell density adjusted to 3 x10^6 cells/mL in saline. In duplicate, 1mL of the untreated and Etoposide treated cell suspensions were incubated with different contrast agent concentrations (0, 100, 300 and 600 μM) at room temperature for half an hour. Cells were collected by centrifugation, the unbound contrast agent was removed and the cell pellet was resuspended in 100 μL of saline, mixed with 100 μL of 2% agarose, to stop cell sedimentation and loaded into 250 μL PCR tubes for MRI.

MRI was performed on a 4.7T Varian Direct Drive scanner, with the samples placed into a Perspex holder within the RF coil. T_1 values were measured from a series of spin echo multi slice scans with an echo time (TE) of 10.97 msec, a field of view (FOV) of 80 x 40 mm, a matrix size of 256 x 128, one average, ten dummy scans, ten 1 mm thick coronal slices and 8 repetition times (TR) ranging from 0.3 to 10 s.

Images were analysed using ImageJ software (National Institutes of Health, USA), with a region of interest (ROI) drawn to encompass as much of the sample as possible, whilst ensuring no air is included, for each sample. The T_1 values were calculated by plotting the mean signal intensity (M_z) over the ROI taken for each TR and each sample and fitted to the Equation 6 using Prism (GraphPad, San Diego, USA).

$$M_z = M_0(1-e^{-TR/T_1}) \qquad (6)$$

The T_1 weighted image with TR: 0.7 s was used to compare the signal intensities between the different concentrations and treatment groups. Students t-tests were performed to determine the effectiveness of the contrast agent at the various concentrations between apoptotic and non-apoptotic cells for both T_1 and signal intensities.

References

(1) A. J. Surman, C. S. Bonnet, M. P. Lowe, G. D. Kenny, J. D. Bell, E. Toth and R. Vilar, *Chem. Eur. J.*, 2011, ***17***, 223

(2) P. Thordarson, *Chem. Soc. Rev.*, 2010, ***6***, *1305*