A luminescent probe containing a tuftsin targeting vector coupled to a terbium complex

Rebecca J. Aarons,[a] Jatinder K. Notta,[b] Marco M. Meloni,[a] Jianghua Feng,[c] Rishma Vidyasagar,[c] Johanna Narvainen,[c] Stuart Allan,[c] Neil Spencer,[b] Risto A. Kauppinen,[d] John S. Snaith*[b] and Stephen Faulkner*[a]

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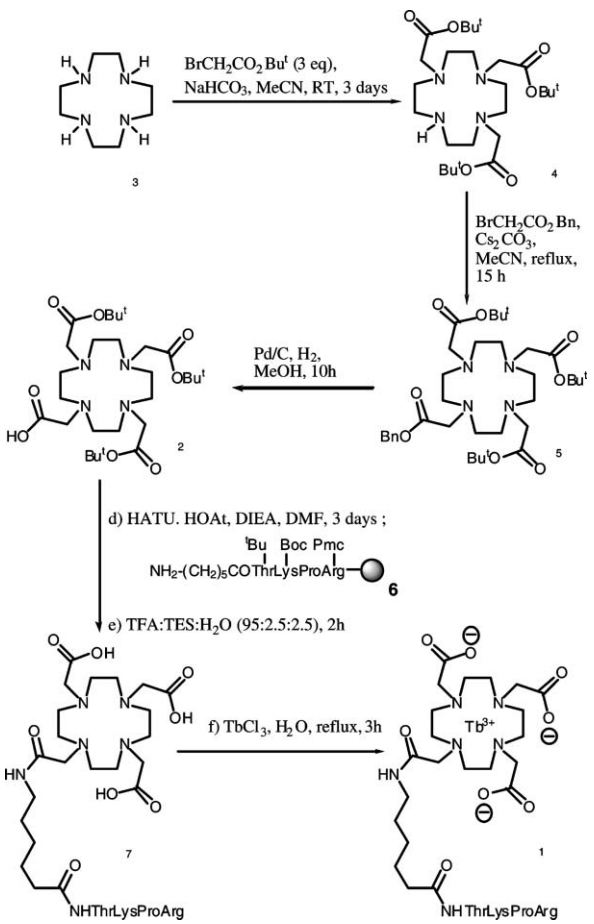
Orthogonal protection strategies have been used to prepare a series of luminescent and MRI active lanthanide complexes containing a tuftsin targeting vector that are internalised by macrophage cells.

In recent years, considerable interest has focused on the application of kinetically stable lanthanide complexes as probes for biological imaging and assaying.[1,2] While many have concentrated their efforts on the development of gadolinium contrast agents,[1,3,4] luminescent lanthanide ions such as terbium, europium, ytterbium and neodymium also have much to offer.[2,5–7] The large Stokes shifts and long-lived emission commonly associated with sensitised emission from these ions mean that it is easy to use time-gated spectroscopy to separate such signals from the short-lived signals that arise from scattered light and biological fluorescence.[8] As a result, considerable interest has centred on the use of luminescent lanthanides in bioassays and commercial assays have been developed to the point where sub-femtomolar detection limits are not uncommon.[9–11] More recently, we and others have developed methods for spatially resolved time-gated imaging and lifetime based imaging microscopy using lanthanide ions.[12–15]

We now report the synthesis and luminescence properties of a terbium complex that is targeted towards macrophage-monocyte lineage cells. The tetrapeptide tuftsin is known to be responsible for activation of macrophage cell lines and is internalised by macrophages.[16] This internalisation process is not disrupted by the presence of N-terminal substituents such as fluorescein,[17] suggesting that such conjugates can be used to image the immune response. However, the luminescence from fluorescein is short lived, and cannot be separated easily from background signals. We reasoned that the main emission line of the terbium ion (545 nm) would be compatible with the optics used for imaging fluorescein-containing probes, and that facile separation would offer the advantage of much lower detection limits.

[a]*School of Chemistry, University of Manchester, Oxford Road, Manchester, UK M13 9PL. E-mail: Stephen.Faulkner@manchester.ac.uk; Fax: +44 (0)161 275 4616; Tel: +44 (0)161 275 4616*
[b]*School of Chemistry, University of Birmingham, Edgbaston, Birmingham, UK B15 2TT. E-mail: J.S.Snaith@bham.ac.uk; Fax: +44 (0)121 414 4403; Tel: +44 (0)121 414 4363*
[c]*Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester, UK M13 9PT*
[d]*School of Sport and Exercise Science, University of Birmingham, Edgbaston, Birmingham, UK B15 2TT*

The synthesis of the tuftsin appended terbium complex **1** is outlined in Scheme 1. Our procedure involved coupling tris-*t*-butyl DOTA **2** to the protected tetrapeptide on a solid support, followed by deprotection and complexation with terbium. A variety of routes to triester monoacid derivatives of DOTA are known,[18,19] but all involve monoalkylation of cyclen **3** and this reaction is known to be capricious. Instead of using the established methods, we resolved to use the well known triester **4** as our basic building block.[20] This is prepared by reaction of cyclen with *t*-butyl bromoacetate at room temperature, and is readily purified by crystallisation from toluene. Reaction of the triester with benzyl bromoacetate yielded the orthogonally protected DOTA derivative



Scheme 1 Synthesis of the terbium complex.

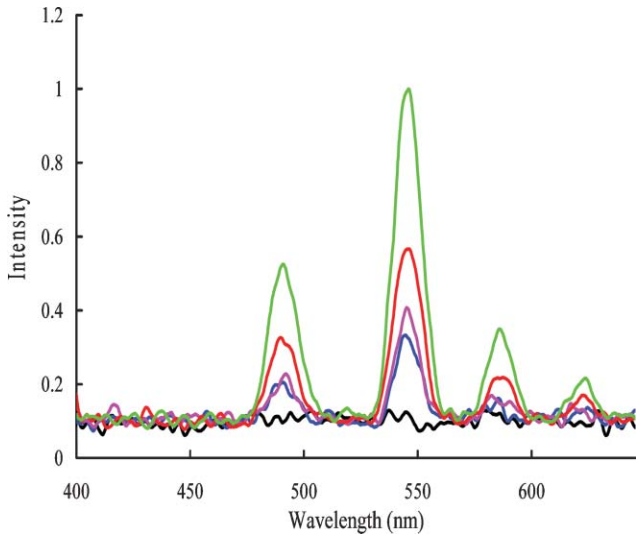


Fig. 1 Luminescence spectra of macrophages incubated with varying concentrations of the terbium complex (λ_{ex} = 366 nm). Spectra are shown for five different concentrations: 0 μM (black line), 10 μM (blue line), 20 μM (pink line), 50 μM (red line), 100 μM (green line) and were recorded using a time delay of 0.1 ms and a gate time of 10 ms

5, which was converted to the triester monoacid **2** by hydrogenolysis of the benzyl group. Tuftsin was synthesised on Wang resin in an automated peptide synthesiser using a standard Fmoc solid-phase synthesis protocol, and the N-terminal threonine was derivatized with an aminocaproic acid spacer unit.† Attachment of **2** to the protected peptide **6** was carried out on the solid phase using HATU. Cleavage from the support and deprotection under acid conditions was followed by reverse-phase HPLC purification to yield **7**, which was reacted with terbium chloride in aqueous solution to give the conjugate **1**. Terbium was chosen as the luminescent ion of choice as terbium complexes tend to be more emissive than europium complexes and can be used in conjunction with standard fluorescence microscopy filter sets.

The luminescence properties of the conjugate were initially investigated in isolation. Upon direct excitation of the metal ion (λ_{ex} = 366 nm, corresponding to the $^7F_6 \rightarrow {}^5G_6$ transition), the luminescence spectrum obtained showed transitions corresponding to emission from the $^5D_4$ state to the $^7F_6$, $^7F_5$, $^7F_4$, and $^7F_3$ states, at 488, 545, 590 and 620 nm respectively, with the most intense transition at 545 nm, as expected for a terbium complex.

The time-resolved emission spectra were considerably more informative. Luminescence lifetimes were measured in H_2O and D_2O. As expected, the lifetime obtained in D_2O (3.25 ms) was significantly longer than that obtained in H_2O (1.67 ms). These were then used to calculate q, the number of bound solvent molecules, using the relation:

$$q = 5(1/\tau_{H_2O} - 1/\tau_{D_2O} - 0.06)$$

where τ_{H_2O} and τ_{D_2O} are the luminescence lifetimes (in ms) in H_2O and D_2O respectively,[21] giving a calculated inner sphere hydration number of 1.2. This indicates that one water molecule is bound in the inner sphere, as would be expected for a DOTA derived complex, and confirms that the metal ion is bound by the macrocycle binding site rather than by the peptide.

Having established the properties of the complex in isolation, its uptake by macrophages was investigated. Macrophages were isolated from the peritoneal fluid of C57BL/6J mice (Harlan Olac, UK) and incubated in RPMI 1640 medium (Gibco) supplemented with 25 mM HEPES (pH 7.3), 2 mM glutamine, 5% foetal calf serum and penicillin/streptomysin overnight in an atmosphere containing 5% CO_2 at 37 °C. After incubation with the conjugate in phosphate buffered saline (PBS) supplemented with 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 5 mM KCl and 10 mM D-glucose for 30 min, the cells were spun down using an Eppendorf table centrifuge and the supernatant removed. Following this, the cells were then washed with PBS and centrifuged to remove any unbound terbium conjugate, then re-suspended in PBS, and their luminescence spectra obtained. The variation in the luminescence spectrum with concentration of conjugate is shown in Fig. 1. It can be seen that there is a linear increase in intensity over a wide concentration range, suggesting that the conjugate is internalised/bound through the tuftsin receptor mechanism of macrophages.[16] It should be noted that the application of a short delay time after excitation results in the spectrum being uncontaminated by background fluorescence or scatter (*cf.* the fluorescein conjugate mentioned above). Our results suggest that such conjugates are likely to be very useful as probes of the immune response with involvement of macrophage-monocyte cells in a variety of disease states.

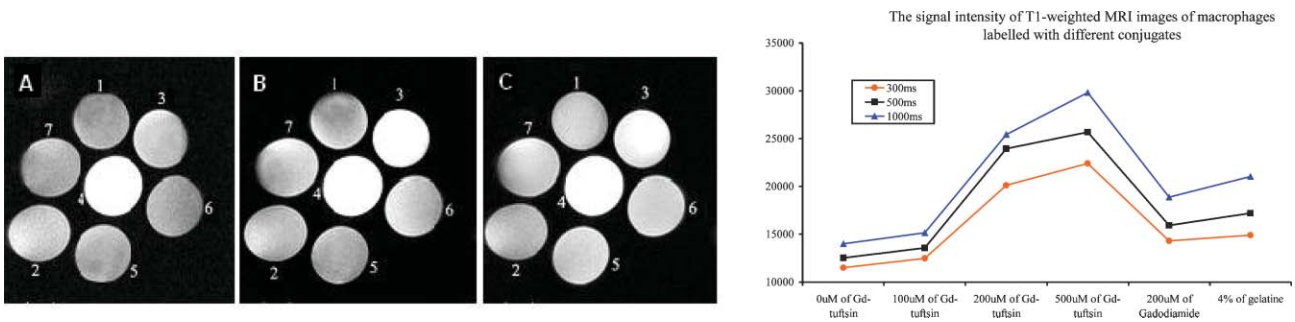


Fig. 2 T_1-weighted MRI of macrophage cell pellets suspended in gelatin. Cells were labelled with different concentrations of Gd-containing probes prior to suspending to 4% gelatin. Incubations were as follows: 1: 0 μM of Gd–tuftsin; 2: 100 μM of Gd–tuftsin; 3: 200 μM of Gd–tuftsin; 4: 500 μM of Gd–tuftsin; 5: 200 μM of Gadodiamide®; 6 and 7: 4% plain gelatine gel. (A: TR = 300 ms, TE = 10 ms; B: TR = 500 ms, TE = 10 ms; C: TR = 1000 ms, TE = 15 ms; TR = time to repetition, TE = time to echo.) Normalised signal intensity of each sample plotted as a function sample type (panel on the right).

The gadolinium complex was prepared in the same way as the terbium complex, and the product characterised by mass spectrometry and relaxivity measurements. The relaxivity at 400 MHz was found to be 2.87 $mmol^{-1}$ s^{-1} in phosphate buffered saline solution. This is comparable with that observed for other DOTA-monoamide complexes of gadolinium. Emboldened by this result we investigated the uptake of the gadolinium complex by cells. Fig. 2 shows the MR images at 300 MHz obtained from macrophages incubated with the gadolinium tuftsin conjugate, then pelleted and compared with a range of pelleted control samples. It may be seen that the intensity of the pellets containing the tuftsin conjugate is significantly greater than those of the other pellets; this confirms localisation of the targeting vector conjugate.

Further *in vivo* studies are currently in progress, using time-gated microscopy to explore the cellular localisation of the terbium complex and MRI with the gadolinium complexes.

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Notes and references

† *Synthetic procedure for preparation of the tuftsin conjugate*. The following protected amino acids (1 mmol) were loaded into an Applied Biosystems 433A peptide synthesiser: N^{α}–Fmoc–Pro–OH (337 mg); N^{α}–Fmoc–Lys(Boc)–OH (468 mg); N^{α}–Fmoc–Thr(^{t}Bu)–OH (397 mg); Fmoc–$NHC_5H_{10}COOH$ (353 mg). Synthesis was carried out according to the standard *FastMoc* procedure using N^{α}–Fmoc–Arg(Pmc) Wang resin (167 mg, 0.1 mmol, substitution 0.6 mmol g^{-1}). A double coupling protocol was used for all amino acids. Crude resin-bound peptide (223 mg) was transferred to a reaction vessel where it was washed with DMF (4 × 5 mL agitated for 5 min). Preactivation of **2** was achieved in a separate round bottomed flask. A mixture of **2** (172 mg, 0.30 mmol), HATU (114 mg, 0.30 mmol), HOAt (46 mg, 0.30 mmol), DIEA (38 μg, 52 μL, 0.30 mmol) in DMF (3 mL) was stirred for 10 min at room temperature and added to the resin *via* a syringe. The resin was agitated for three days using N_2 then washed thoroughly. A cleavage time of 2 h, using TFA : TES : H_2O (95 : 2.5 : 2.5, 5 mL), gave crude peptide (123 mg) containing 3 species, detected by analytical HPLC (H_2O : CH_3CN 100 : 1 to 0 : 100 over 60 min) with fraction 1 (14.77 min) being identified as peptide **7** using MALDI TOF mass spectrometry, *m/z* (MALDI) 1023.0 ($[M + Na]^+$, 44), 1001.0 ($[M + H]^+$, 100%, exact mass calc. 1000 for $[M + H]^+$). Preparative HPLC and subsequent lyophilisation gave the peptide **7** as a colourless solid (36 mg, 36 %); δ_H (500 MHz, 9 : 1 H_2O : D_2O) 1.24 (3H, d, *J* 6.4 Hz, C*H*$_3$), 1.33–1.43 (2H, m, CH_2), 1.48–1.60 (4H, env, 2 × CH_2), 1.62–1.68 (2H, m, CH_2), 1.70–1.79 (6H, env, 2 × CH*H* & 2 × CH_2), 1.82–1.89 (1H, m, CH*H*), 1.95–2.01 (2H, env, 2 × CH*H*), 2.04 (2H, m, CH_2), 2.31–2.45 (3H, env, CH*H* & CH_2), 3.00–3.08 (2H, m, CH_2), 3.20–3.24 (2H, m, CH_2), 3.24–3.30 (2H, m, CH_2), 3.30–3.38 (16H, br s, 8 × CH_2), 3.66–3.72 (1H, m, CH*H*), 3.78–3.88 (8H, env, 4 × CH_2), 3.88–3.93 (1H, m, CH*H*), 4.14–4.21 (1H, m, C*H*), 4.30–4.34 (1H, m, C*H*), 4.36–4.41 (1H, m, C*H*), 4.44–4.49 (1H, m, C*H*), 4.66–4.70 (1H, m, C*H*), 6.60–6.80 (1H, br s, N*H*), 7.20–7.27 (1H, br s, N*H*), 7.50–7.62 (2H, br s, NH_2), 8.01–8.08 (1H, br s, N*H*), 8.16 (1H, d, *J* 7.5, N*H*), 8.37 (1H, d, *J* 6.8, N*H*), 8.53 (1H, d, *J* 7.3, N*H*); δ_C (125 MHz, 9 : 1 H_2O : D_2O) 21.53, 24.91, 26.92, 27.13, 27.39, 28.13, 28.99, 30.22, 30.44, 31.85, 32.47, 37.81, 41.99, 43.12, 50.54, 52.07, 52.16, 52.24, 52.88, 53.98, 55.14, 56.84, 56.94, 57.80, 61.72, 62.86, 69.64, 159.47, 174.16, 174.48, 174.08, 174.28, 176.60, 177.99, 179.99.

1 E. Toth, L. Helm and A. E. Merbach, in *Comprehensive Coordination Chemistry II*, ed. M. D. Ward, Elsevier, Oxford, UK, 2004, pp. 841–882.
2 S. Faulkner and J. L. Matthews, in *Comprehensive Coordination Chemistry II*, ed. M. D. Ward, Elsevier, Oxford, UK, 2004, pp .913–944.
3 P. Caravan, J. J Ellison, T. McMurry and R. B. Lauffer, *Chem. Rev.*, 1999, **99**, 2293–2352.
4 D. Parker, R. S. Dickins, H. Puschmann, C. Crossland and J. A. K. Howard, *Chem. Rev.*, 2002, **102**, 1977–2010.
5 P. R. Selvin, *Annu. Rev. Biophys. Biomol. Struct.*, 2002, **31**, 275.
6 D. Parker, *Coord. Chem. Rev.*, 2000, **205**, 109–130.
7 S. I. Klink, H. Keizer and F. C. J. M. Van Veggel, *Angew. Chem., Int. Ed.*, 2000, **39**, 4319–4321.
8 B. Alpha, J. M. Lehn and G. Mathis, *Angew. Chem., Int. Ed. Engl.*, 1987, **26**, 266.
9 P. G. Sammes and G. Yahogliou, *Nat. Prod. Rep.*, 1996, 1–28.
10 G. Mathis, *Clin. Chem.*, 1995, **41**, 1391–1397.
11 I. Hemmila and V. M. Mukkala, *Crit. Rev. Clin. Lab. Sci.*, 2001, **38**, 441–519.
12 A. Beeby, S. W. Botchway, I. M. Clarkson, S. Faulkner, A. W. Parker, D. Parker and J. A. G. Williams, *J. Photochem. Photobiol. B: Biol.*, 2000, **57**, 83–89.
13 L. J. Charbonnière, R. Ziessel, M. Montalti, L. Prodi, N. Zaccheroni, C. Boehme and G. Wipff, *J. Am. Chem. Soc.*, 2002, **124**, 7779–7788.
14 L. J. Charbonniere, N. Weibel, C. Estournes, C. Leuvrey and R. Ziessel, *New J. Chem.*, 2004, **28**, 777–781.
15 N. Weibel, L. J. Charbonniere, M. Guardigli, A. Roda and R. Ziessel, *J. Am. Chem. Soc.*, 2004, **126**, 4888–4896.
16 I. Z. Siemion and A. Kluczyk, *Peptides*, 1999, **20**, 645–674.
17 A. A. Amoscato, P. J. A. Davies, G. F. Babcock and K. Nishioka, *Ann. N. Y. Acad. Sci.*, 1983, **419**, 114–134.
18 P. L. Anelli, L. Lattuada, M. Gabellini and P. Recanati, *Bioconjugate Chem.*, 2001, **12**, 1081–1084.
19 A. Heppeler, S. Froidevaux, H. R. Maecke, M. Behe, P. Powell and M Hennig, *Chem.–Eur. J.*, 1999, **5**, 1974–1981.
20 A. Dadabhoy, S. Faulkner and P. G. Sammes, *J. Chem. Soc., Perkin Trans. 2*, 2002, 348–357.
21 A. Beeby, I. M. Clarkson, R. S. Dickins, S. Faulkner, D. Parker, L. Royle, A. S. de Sousa, J. A. G. Williams and M. Woods, *J. Chem. Soc., Perkin Trans. 2*, 1999, 493–503.