COMMUNICATION

Selective Delivery of Remarkably High Levels of Gadolinium to Tumour Cells Using an Arsonium Salt

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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The use of a triphenylarsonium vector for mitochondrial targeting leads to a dramatic increase in Gd^{3+} uptake in human glioblastoma multiforme cells by up to an order of magnitude over the isosteric triarylphosphonium analogue, with significant implications for 'theranostic' applications involving delivery of this important lanthanoid metal ion to tumour cells.

Introduction

Delocalised lipophilic cations (DLCs) such as triarylphosphonium salts[1] are effective vectors for the delivery of fluorophores,[2–11] chemotherapeutics,[12,13] boron clusters,[14–16] radiolabels,[17–19] and *f*-block elements such as Gd[20,21] to tumour and normal (e.g. myocardial) cell mitochondria. Far less utilised are the closely-related triarylarsonium salts for which only very limited biological data have been reported to date.[22–24] Arsenic can potentially offer distinct advantages over the lighter Group 15 congener phosphorus as it can act as a heavy atom label for synchrotron X-ray fluorescence microscopy (XRF/XFM) cell imaging studies, for example. Indeed, the use of arsenic instead of phosphorus has been shown to not significantly change the tissue distribution or intracellular uptake and retention of such agents in cancer cells or myocardial tissue and, for example, the delocalised lipophilic cation tetraphenylarsonium (TPA) has shown similar penetration into mitochondria to tetraphenylphosphonium (TPP).[25] Liu and co-workers have also reported a series of ^{64}Cu-labelled triphenyl-phosphonium and -arsonium cations as tumour-selective, positron-emission tomography (PET) imaging agents.[26] However, in general, the biodistribution properties of both of these ^{64}Cu-radiotracers were found to be almost identical in athymic nude mice bearing U87MG human glioma xenografts. Herein we present compelling evidence that the replacement of a phosphorus atom by arsenic in a Ph_3ER^+ salt (E = As, P) containing Gd(III) (**1** and **2**, Figure 1) leads to a significant enhancement of Gd uptake into tumour cells despite the two complexes being isosteric. For the first time, we demonstrate that altering the nature of the Group 15 atom in this class of DLCs, in place of altering the nature of the substituents at the phosphonium centre and/or bridging linker groups, offers a new pathway to dramatically enhancing the levels of Gd(III) within tumour cell mitochondria. This strategy has implications in the use of these types of compounds for 'theranostic'[27] applications such as photon activation therapy (PAT),[28–33] neutron capture therapy (NCT),[34–36] and magnetic resonance imaging (MRI),[37–39] where high levels of Gd localised within tumour cells are critical to the clinical success of these cutting-edge cancer therapies.

Results and discussion

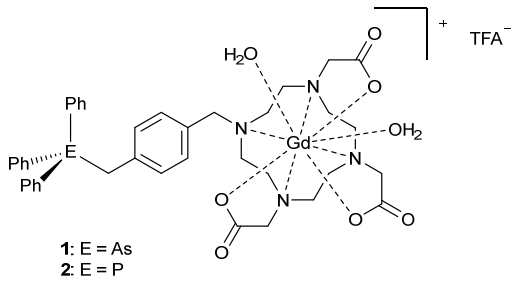

Figure 1 Structure of Gd(III)-containing Group 15 salts.

Syntheses

The synthesis of **1** followed a related synthetic route to the analogous and previously-reported triphenylphosphonium salt **2**.[20] The first step in the synthesis of the arsonium analogue involved the nucleophilic substitution of triphenylarsine with 1,4-dibromoxylene (Scheme 1). This reaction required the use of a polar solvent and toluene was replaced with the considerably more polar nitromethane. The arsonium salt **3** was successfully obtained in good yield (73%) and characterised by ^{1}H and ^{13}C{^{1}H} NMR spectroscopy and ESI-MS, the latter showing a characteristic molecular ion peak at *m/z* 488.87 (calc: *m/z* 489.02), corresponding to the $[M - Br]^+$ species. The triphenylarsonium ligand **4** was prepared in a similar manner to the previously-reported triphenylphosphonium analogue[20] by means of a nucleophilic substitution reaction involving **3** and

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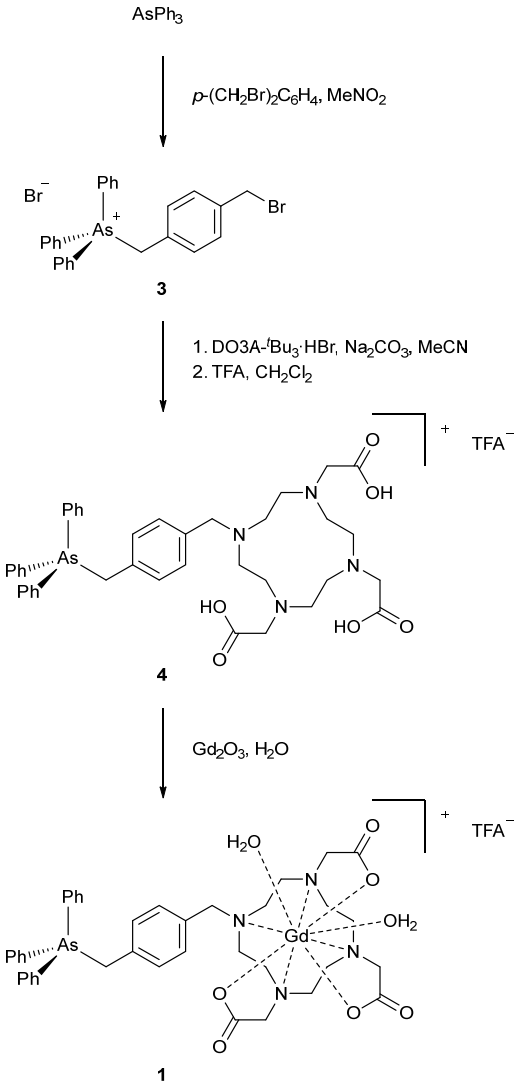
Electronic Supplementary Information (ESI) available: synthetic, computational and biological experimental methods. See DOI: 10.1039/x0xx00000x

the ^{t}Bu-protected macrocycle DO3A-^{t}Bu$_3$·HBr (Scheme 1) in the presence of Na_2CO_3, and was successfully purified by reverse-phase HPLC.[20] Characterisation of **4** was performed by means of ^{1}H and ^{13}C{^{1}H} NMR spectroscopy, and high-resolution ESI-FTICR-MS confirmed the formation of the target ligand with the major isotopic peak for $[M - TFA]^+$ observed at *m/z* 755.27819 (calc: *m/z* 755.27843). The target Gd(III)-arsonium complex **1** was prepared using the same protocol as that used for the related phosphonium complex (Scheme 1) involving treatment of **4** with a suspension of Gd_2O_3 in H_2O at 60°C for 16 h, followed by centrifugation to remove unreacted Gd_2O_3, and evaporation of the filtrate *in vacuo* to afford a colourless solid.[20] Analytical reverse-phase HPLC confirmed the purity of the Gd(III) complex such that no further purification was necessary. The structure of the target Gd(III)-arsonium complex **1** was confirmed by high resolution ESI-FTICR-MS with a molecular envelope located at *m/z* 910.17989 (calc: *m/z* 910.18037), corresponding to $[M - TFA]^+$, with a characteristic Gd isotopic pattern observed.

Biological studies

Complex **1** was evaluated for its stability in phosphate-buffered saline (pH 7.4), *in vitro* cytotoxicity, and cellular uptake in tumour and normal cell lines. It was found to be stable to hydrolysis and did not show any sign of degradation in aqueous solution at pH 7.4 after 24 h at RT. A MTT assay was performed on complex **1** using a human glioblastoma multiforme (T98G) cell line over a 0 – 4 mM concentration range and, like **2** (IC_{50} = 2.55 ± 0.33 mM),[20] the MTT assay confirmed the low cytotoxicity of the arsonium complex (IC_{50} = 2.07 ± 0.12 mM).

Complex **1** was analysed for its uptake into the T98G cell line and the non-cancerous human glial cell line SVG p12 by determining the Gd content in dosed cells by means of ICP-MS. Each complex was administered at three concentrations (10, 100, and 1000 μM), all of which were below the IC_{50} value for the complex (*vide supra*). The cellular Gd content was then normalised to cellular protein content and cell count such that Gd uptake was expressed as ng Gd/mg protein and Gd atoms/cell, respectively (Table 1). The *in vitro* tumour : normal (T/N) selectivity for tumour cells was calculated by determining the ratio of uptake into the tumour cell line *vs*. the healthy cell line (Table 1). Complex **1** was found to deliver remarkably high levels of Gd to tumour cells when compared to **2** at all assessed concentrations, in some cases exceeding the levels of **2** by an order of magnitude or greater. Complex **1** was also found to be selective for tumour cells at all assessed concentrations, with the T/N cell selectivity found to be highest (15.4-16.4) at 100 μM when compared to that of **2** (4.5-4.8) at the same dose, thus corresponding to an overall difference in selectivity of 3.2 - 3.6. Whilst, in general, higher doses of **1** or **2** lead to a higher cellular uptake, as expected, the relationship is clearly a non-linear one. Furthermore, the T/N ratios are also non-linear at different doses, with the lowest ratio found at the highest dose (1000 μM), most likely related to receptor saturation at this dose. We reported that a reduction in the degree of delocalisation around the phosphonium centre in complexes closely related to **2** (where Me groups replaced aryl groups at the phosphonium centre) also lowered the selectivity of such complexes for tumour cells and, like **1**, showed enhanced Gd uptake.[40] The log*P* values of **1** and **2** (1.25 ± 0.03 and 1.24 ± 0.02, respectively) are identical within experimental error and thus relative lipophilicities cannot account for the observed differences in either cell uptake or tumour cell selectivity.



Scheme 1 Synthesis of the Gd(III)-arsonium salt **1**.

Table 1 ICP-MS analysis of Gd content and tumour cell selectivity (with standard errors) in T98G and SVG p12 cells treated with **1** or **2** [c] for 48 h (N = 3).

Complex	Conc. (μM)	T98G (ng Gd/mg protein)	T98G (Gd atoms/cell)	SVG p12 (ng Gd/ mg protein)	SVG p12 (Gd atoms/cell)	T/N cell ratio [a]	T/N cell ratio [b]
1	10	548.7 ± 94.7	$1.1 \times 10^9 \pm 1.2 \times 10^8$	111.8 ± 21.8	$1.4 \times 10^8 \pm 3.4 \times 10^7$	4.9 ± 1.3	7.9 ± 2.2
1	100	10675.9 ± 2288.4	$2.3 \times 10^{10} \pm 5.4 \times 10^9$	693.7 ± 94.8	$1.4 \times 10^9 \pm 1.3 \times 10^8$	15.4 ± 4.0	16.4 ± 5.0
1	1000	14278.1 ± 1950.8	$4.3 \times 10^{10} \pm 5.1 \times 10^9$	8496.8 ± 1887.4	$8.4 \times 10^9 \pm 1.7 \times 10^9$	1.7 ± 0.4	5.1 ± 1.2
2	10	420.1 ± 5.4	$5.8 \times 10^8 \pm 6.8 \times 10^6$	31.8 ± 5.0	$3.8 \times 10^7 \pm 6.3 \times 10^6$	13.2 ± 2.1	15.2 ± 2.5
2	100	1487.0 ± 146.1	$1.9 \times 10^9 \pm 2.8 \times 10^8$	308.7 ± 18.4	$4.2 \times 10^8 \pm 1.7 \times 10^7$	4.8 ± 0.6	4.5 ± 0.7
2	1000	3000.3 ± 457.7	$4.0 \times 10^9 \pm 6.3 \times 10^8$	6440.5 ± 177.5	$8.4 \times 10^9 \pm 3.4 \times 10^8$	0.5 ± 0.1	0.5 ± 0.1

[a] Derived from ng Gd/mg protein. [b] Derived from Gd atoms/cell. [c] Data obtained from ref 40.

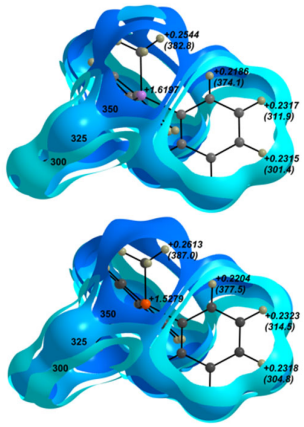


Figure 2 Electrostatic potential isosurfaces for the model cations Ph_3EMe^+ (**3**$^+$, E = As and **4**$^+$, E = P; top and bottom, respectively), as calculated by means of DFT. The displayed isosurfaces (values in k*J* mol^{-1}) have an electron density range of 0.0010-0.0025 ea_0^{-3}. NPA atomic charges (*e*) of the As and P atoms and selected H atoms are in italics; the values in parentheses (k*J* mol^{-1}) are the electrostatic potential energy at the point where the C–H bond axis intersects the 0.0010 ea_0^{-3} isosurface.

Notwithstanding the near-identical log*P* values of **1** and **2**, DFT calculations (B3LYP/def2-TZVPD//B3LYP-D3(0)/def2-SV(P)) on the model cations Ph_3EMe^+ (**3**$^+$, E = As) and (**4**$^+$, E = P) indicate differences in their charge distribution. Using the IEF-PCM solvation model,[41–43] the electrostatic free energy for the interaction of **3**$^+$ with water is 12.8 k*J* mol^{-1} *lower* than that of **4**$^+$. However, when a solvent probe radius of 1.54 Å is employed (the calculated atomic radius for a H-atom using a 0.001 ea_0^{-3} electron density fall-off criterion[44]), cation **3**$^+$ is now 0.5 k*J* mol^{-1} *higher* in energy than **4**$^+$. These changes can be rationalised by an examination of the electrostatic potential energy isosurfaces of **3**$^+$ and **4**$^+$. The higher polarisability of As compared to P leads to higher electrostatic potentials at the concave surfaces of **3**$^+$, hence the lower free energy of the entire surface. However, the convex surfaces of **3**$^+$ are of lower potential compared to **4**$^+$, leading to the reversal in stability with the 1.54 Å probe. These differences are substantially associated with higher partial charges of the surface H-atoms of **4**$^+$ when compared with those of **3**$^+$ (Fig. 2). The calculated natural population analysis (NPA) atomic charges[45] are consistent with inductive effects owing to the higher electronegativity of P compared to As being responsible for these differences. This feature alone is most likely to influence the levels of cellular uptake and selectivity for the two Gd(III) complexes. Such effects have not previously been reported for ^{64}Cu triaryl-phosphonium and -arsonium salts *in vitro* and *in vivo*,[26,46,47] and the observed differences found in this work may be related to the unique molecular structures of **1** and **2**. Delocalisation of DLCs having a direct effect upon the cellular uptake and tumour cell selectivity has recently been reported for a series of related Gd-arylphosphonium salts.[40] Of particular interest is the cellular uptake of **1**, which somewhat parallels that of the Ph_2MePR^+ salt rather than that of **2**[40] but, unlike **1** and **2**, the Ph_2MePR^+ complex displayed only limited tumour cell selectivity at all assessed Gd(III) concentrations (10 – 1000 μM). Indeed **1** shows a distinct advantage over both **2** and the Ph_2MePR^+ salt;[40] at 100 μM, **1** exhibits a much greater tumour cell selectivity than **2** and Ph_2MePR^+, and additionally, at both 100 and 1000 μM, **1** was still capable of delivering Gd atoms to the tumour cells at levels over an order of magnitude greater than that of **2** (Table 1).

Conclusions

Our work clearly demonstrates that a triphenylarsonium salt can act as a versatile vector for the delivery of significant levels of Gd(III) to tumour cells, and we report a new paradigm in the design of 'theranostic' agents containing this important lanthanoid metal ion.[27] This work has direct implications in the development of new Gd-based theranostics, for example in cutting-edge binary cancer therapies such as NCT and PAT, where the levels of lanthanoid element must exceed *ca*. 10^8 atoms per tumour cell in order to observe any significant cell kill. Furthermore, these high levels of lanthanoid accumulation would potentially permit *in vivo* cell tracking applications using MRI.[48] We are currently exploring the structure-activity relationships (SAR) of triarylarsonium salts related to **1**, and *in vivo* biodistribution studies of **1** and related complexes are planned.

We thank the Australian Research Council (Grant ID: DP190103461) for funding, Dr Ian Luck for advice regarding NMR spectra, and Dr Nick Proschogo for assistance with the MS.

Conflicts of interest

There are no conflicts to declare.

Notes and references

1 J. Zielonka, J. Joseph, A. Sikora, M. Hardy, O. Ouari, J.

Vasquez-Vivar, G. Cheng, M. Lopez and B. Kalyanaraman, *Chem. Rev.*, 2017, **117**, 10043–10120.
2 S. Davis, M. J. Weiss, J. R. Wong, T. J. Lampidis and L. B. Chen, *J. Biol. Chem.*, 1985, **260**, 13844–13850.
3 L. V. Johnson, M. L. Walsh, B. J. Bockus and L. B. Chen, *J. Cell Biol.*, 1981, **88**, 526–535.
4 R. K. Emaus, R. Grunwald and J. J. Lemasters, *BBA - Bioenerg.*, 1986, **850**, 436–448.
5 H. Rottenberg and S. Wu, *Biochim. Biophys. Acta - Mol. Cell Res.*, 1998, **1404**, 393–404.
6 G. Solaini, G. Sgarbi, G. Lenaz and A. Baracca, *Biosci. Rep.*, 2007, **27**, 11–21.
7 G. Serviddio and J. Sastre, *Methods Mol. Biol.*, 2010, **594**, 107–121.
8 S. W. Perry, J. P. Norman, J. Barbieri, E. B. Brown and H. A. Gelbard, *Biotechniques*, 2011, **50**, 98–115.
9 A. A. Gerencser, C. Chinopoulos, M. J. Birket, M. Jastroch, C. Vitelli, D. G. Nicholls and M. D. Brand, *J. Physiol.*, 2012, **590**, 2845–2871.
10 D. G. Nicholls, in *Methods in Molecular Biology*, 2018, vol. 1782, pp. 121–135.
11 S. Onoe, T. Temma, Y. Shimizu, M. Ono and H. Saji, *Cancer Med.*, 2014, **3**, 775–786.
12 C. J. Zhang, Q. Hu, G. Feng, R. Zhang, Y. Yuan, X. Lu and B. Liu, *Chem. Sci.*, 2015, **6**, 4580–4586.
13 W. S. Shin, M. G. Lee, P. Verwilst, J. H. Lee, S. G. Chi and J. S. Kim, *Chem. Sci.*, 2016, **7**, 6050–6059.
14 J. A. Ioppolo, J. K. Clegg and L. M. Rendina, *Dalton Trans.*, 2007, 1982.
15 J. A. Ioppolo, M. Kassiou and L. M. Rendina, *Tetrahedron Lett.*, 2009, **50**, 6457–6461.
16 D. E. Morrison, F. Issa, M. Bhadbhade, L. Groebler, P. K. Witting, M. Kassiou, P. J. Rutledge and L. M. Rendina, *JBIC J. Biol. Inorg. Chem.*, 2010, **15**, 1305–1318.
17 Z. Li, M. Lopez, M. Hardy, D. M. McAllister, B. Kalyanaraman and M. Zhao, *Cancer Biother. Radiopharm.*, 2009, **24**, 579–587.
18 H. Yuan, H. Cho, H. H. Chen, M. Panagia, D. E. Sosnovik and L. Josephson, *Chem. Commun.*, 2013, **49**, 10361.
19 D.-Y. Kim and J.-J. Min, *Nucl. Med. Mol. Imaging (2010).*, 2016, **50**, 185–195.
20 D. E. Morrison, J. B. Aitken, M. D. de Jonge, F. Issa, H. H. Harris and L. M. Rendina, *Chem. – A Eur. J.*, 2014, **20**, 16602–16612.
21 D. E. Morrison, J. B. Aitken, M. D. de Jonge, J. A. Ioppolo, H. H. Harris and L. M. Rendina, *Chem. Commun.*, 2014, **50**, 2252–2254.
22 P. C. Srivastava, H. G. Hay and F. F. Knapp, *J. Med. Chem.*, 1985, **28**, 901–904.
23 N. Lalwani, Y.-S. Chen, G. Brooke, N. A. Cross, D. W. Allen, A. Reynolds, J. Ojeda, G. J. Tizzard, S. J. Coles and N. Bricklebank, *Chem. Commun.*, 2015, **51**, 4109–4111.
24 Y. V. Bakhtiyarova, A. F. Aksunova, V. I. Galkin and I. V. Galkina, *Russ. J. Gen. Chem.*, 2015, **85**, 2058–2064.
25 P. Gros, F. Talbot, D. Tang-Wai, E. Bibi and H. R. Kaback, *Biochemistry*, 1992, **31**, 1992–1998.
26 J. Wang, C.-T. Yang, Y.-S. Kim, S. G. Sreerama, Q. Cao, Z.-B. Li, Z. He, X. Chen and S. Liu, *J. Med. Chem.*, 2007, **50**, 5057–5069.
27 A. G. Robertson and L. M. Rendina, *Chem. Soc. Rev.*, 2021, **50**, 4231–4244.
28 G. De Stasio, *Clin. Cancer Res.*, 2006, **12**, 206–213.
29 A. Forouzannia, G. M. Richards, D. Khuntia and M. P. Mehta, *Expert Rev. Anticancer Ther.*, 2007, **7**, 785–794.
30 M. P. Mehta, W. R. Shapiro, S. C. Phan, R. Gervais, C. Carrie, P. Chabot, R. A. Patchell, M. J. Glantz, L. Recht, C. Langer, R. K. Sur, W. H. Roa, M. A. Mahe, A. Fortin, C. Nieder, C. A. Meyers, J. A. Smith, R. A. Miller and M. F. Renschler, *Int. J. Radiat. Oncol.*, 2009, **73**, 1069–1076.
31 T. D. Mody and J. L. Sessler, *J. Porphyr. Phthalocyanines*, 2001, **05**, 134–142.
32 R. A. Miller, K. W. Woodburn, Q. Fan, I. Lee, D. Miles, G. Duran, B. Sikic and D. Magda, *Clin. Cancer Res.*, 2001, **7**, 3215–21.
33 W. N. William, R. G. Zinner, D. D. Karp, Y. W. Oh, B. S. Glisson, S.-C. Phan and D. J. Stewart, *J. Thorac. Oncol.*, 2007, **2**, 745–750.
34 N. S. Hosmane, J. A. Maguire, Y. Zhu and M. Takagaki, *Boron and Gadolinium Neutron Capture Therapy for Cancer Treatment*, WORLD SCIENTIFIC, 2012.
35 E. L. Crossley, H. Y. V. Ching, J. A. Ioppolo and L. M. Rendina, in *Bioinorganic Medicinal Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2011, pp. 283–305.
36 F. Issa, J. A. Ioppolo and L. M. Rendina, in *Comprehensive Inorganic Chemistry II*, Elsevier, 2013, pp. 877–900.
37 P. Caravan, *Chem. Soc. Rev.*, 2006, **35**, 512.
38 S. Faulkner and O. A. Blackburn, in *The Chemistry of Molecular Imaging*, John Wiley & Sons, Inc, Hoboken, NJ, 2014, pp. 179–197.
39 J. Wahsner, E. M. Gale, A. Rodríguez-Rodríguez and P. Caravan, *Chem. Rev.*, 2019, **119**, 957–1057.
40 M. Busse, M. S. A. Windsor, A. J. Tefay, M. Kardashinsky, J. M. Fenton, D. E. Morrison, H. H. Harris and L. M. Rendina, *J. Inorg. Biochem.*, 2017, **177**, 313–321.
41 E. Cancès, B. Mennucci and J. Tomasi, *J. Chem. Phys.*, 1997, **107**, 3032–3041.
42 M. Cossi, V. Barone, B. Mennucci and J. Tomasi, *Chem. Phys. Lett.*, 1998, **286**, 253–260.
43 B. Mennucci and J. Tomasi, *J. Chem. Phys.*, 1997, **106**, 5151–5158.
44 M. Rahm, R. Hoffmann and N. W. Ashcroft, *Chem. – A Eur. J.*, 2016, **22**, 14625–14632.
45 A. E. Reed, R. B. Weinstock and F. Weinhold, *J. Chem. Phys.*, 1985, **83**, 735–746.
46 Y.-S. Kim, C.-T. Yang, J. Wang, L. Wang, Z.-B. Li, X. Chen and S. Liu, *J. Med. Chem.*, 2008, **51**, 2971–2984.
47 C.-T. Yang, Y.-S. Kim, J. Wang, L. Wang, J. Shi, Z.-B. Li, X. Chen, M. Fan, J.-J. Li and S. Liu, *Bioconjug. Chem.*, 2008, **19**, 2008–2022.
48 A. K. Srivastava, D. K. Kadayakkara, A. Bar-Shir, A. A. Gilad, M. T. McMahon and J. W. M. Bulte, *Dis. Model. Mech.*, 2015, **8**, 323–336.

