ChemComm

A Switch-On MRI Contrast Agent for Noninvasive Visualization of Methylmercury

Journal:	*ChemComm*
Manuscript ID:	CC-COM-02-2015-001723.R1
Article Type:	Communication
Date Submitted by the Author:	26-May-2015

Complete List of Authors:	Wang, Yun-Ming; National Chiao Tung University, Department of Biological Science and Technology; National Chiao Tung University, Institute of Molecular Medicine and Bioengineering Singh, Gyan; National Chiao Tung University, Institute of Molecular Medicine and Bioengineering Hsu, Kuang-Mei; National Chiao Tung University, Institute of Molecular Medicine and Bioengineering; National Chiao Tung University, Biological Science and Technology Chen, Yu-Jen; National Chiao Tung University, Department of Biological Science and Technology Wu, Shou-Cheng; National Chiao Tung University, Department of Biological Science and Technology Chen, Chiao; Kaohsiung Medical University, cDepartment of Medical Imaging, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan

ChemComm RSCPublishing

COMMUNICATION

www.rsc.org/

Cite this: DOI: 10.1039/xoxxoooooox

A Switch-On MRI Contrast Agent for Noninvasive Visualization of Methylmercury

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/chemcomm

Gyan Singh,[a] Kuang-Mei Hsu,[a] Yu-Jen Chen,[a] Shou-Cheng Wu,[a] Chiao-Yun Chen,*[cd] Yun-Ming Wang*[ab]

This communication presents first Gd(III)-based T_1 MR contrast agent, *o*-MeHgGad, for noninvasive visualization of CH_3Hg^+. *o*-MeHgGad showed a relaxivity enhancement of 62% in the presence of 1 equiv. of CH_3Hg^+. Moreover, noticeable contrast enhancement was recorded in liver, kidney, and intestine of mice exposed to CH_3Hg^+. Thus, the newly designed contrast agent has potential to be used for *in vivo* bio-imaging of CH_3Hg^+ and could be useful for biomedical applications.

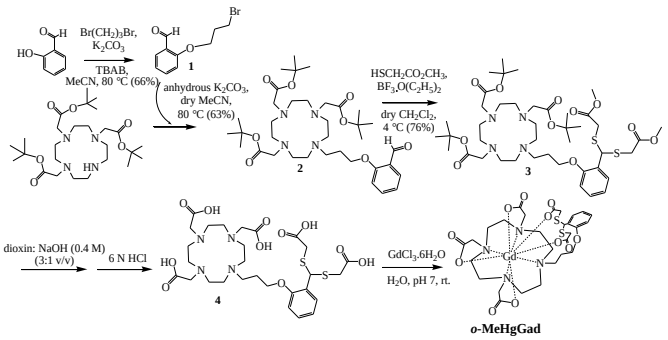
Methylmercury (CH_3Hg^+) is a ubiquitous environmental toxicant and a powerful neurotoxicant.[1] Because of lipid solubility CH_3Hg^+ can readily pass through biological membranes, including placental barrier during pregnancy.[2] Therefore, fetuses, infants, and young children are most susceptible to CH_3Hg^+ neurotoxicity with the likelihood of long-lasting neurological and developmental deficits upon exposed to CH_3Hg^+.[3] Consumption of fish and marine mammals is the major source of human exposure to CH_3Hg^+.[4] A report from the Food and Agriculture Organization of the United Nations (USFAO) suggests that about one billion people rely on seafood as their primary source of protein (FAO, 2000).[5] Hence, a large share of global population is exceedingly vulnerable to CH_3Hg^+ toxicity. Although an array of highly sensitive and specific fluorescent molecular probes has been developed for inorganic mercury (Hg^{2+})[6] only a few has been investigated as a potential CH_3Hg^+ sensor till date.[7]

[a]*Department of Biological Science and Technology, Institute of Molecular Medicine and Bioengineering, National Chiao Tung University, Hsinchu 300, Taiwan. E-mail: ymwang@mail.nctu.edu.tw; Fax:+886-3-5729288; Tel: +886-3-5712121 ext 56972.*
[b]*Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung 807, Taiwan*
[c]*Department of Radiology, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan.*
[d]*Department of Medical Imaging, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan. E-mail: ccy0103@hotmail.com; Fax: +886-7-3154208; Tel: +886-7-3208235*

More recently, state of the art molecular probe for the selective detection of CH_3Hg^+ in the presence of Hg^{2+} has been reported.[8] However, fluorescent probe has its own limitation on penetration depth of biological tissues when it comes to *in vivo* imaging.[9] *In vivo* detection of CH_3Hg^+ becomes even more important concerning the prolonged latency periods of CH_3Hg^+ poisoning symptoms after exposer.[10] It is therefore essential to develop an alternative method which can facilitate real-time *in vivo* detection of CH_3Hg^+ for instant diagnosis and for elucidation of CH_3Hg^+ toxicity. Magnetic Resonance Imaging (MRI) has been extensively used for *in vivo* study and considered to be clinically proven safest imaging modality for use on patients.[11] Gadolinium (III) complex as an extracellular MRI contrast agents has been widely adopted in clinical practice during MRI examinations to enhance the quality of the acquired image. Notably, in recent years, significant advancements have been made in the development of the functional MRI contrast agents for molecular imaging of biomolecules. MRI contrast agents for pH,[12] metal ions,[13] and enzyme activities,[14] have been developed. To our knowledge, no MRI contrast agents for sensing CH_3Hg^+ is reported.

In this study, we designed and synthesized a new Gd(III)-based turn-on MRI contrast agent, *o*-MeHgGad, for noninvasive visualization of CH_3Hg^+. The *o*-MeHgGad was obtained through straightforward and facile synthesis route as shown in Scheme 1. Briefly, the synthesis of *o*-MeHgGad was accomplished in 6 steps. 2-(3-Bromopropoxy)benzaldehyde (**1**) was obtained by reacting 2-hydroxybenzaldehyde with 1,3-dibromopropane. Alkylation of DO3A (tris-*tert*-butyl ester) with compound **1** generated benzaldehyde derivative of DO3A (tris-*tert*-butyl ester) (**2**). Thiolation of **2** in the presence of $BF_3{\bullet}O(C_2H_5)_2$ gave the compound **3**. Subsequent deprotection of the compound **3** first with a solution of dioxin and NaOH (3:1 v/v) and then with 6 N HCl gave the final ligand (**4**). Metalation of **4** with $GdCl_3.6H_2O$ in water at pH 7 followed by HPLC purification yielded *o*-MeHgGad. Additional details on the synthesis of *o*-MeHgGad are provided in the Electronic Supplementary Information (ESI†). In addition,

following the synthetic procedure of *o*-MeHgGad, *p*-MeHgGad (para derivative) was synthesized and details have been provided in ESI†.



Scheme 1 Synthesis of *o*-MeHgGad

The activation mechanism of *o*-MeHgGad is based on the previously reported Hg^{2+}-promoted elimination of dithioacetals groups[15] and CH_3Hg^+ is expected to show similar chemical reaction. However, we were little sceptical about the sensitivity of reaction due to the less thiophilic nature of CH_3Hg^+ than that of Hg^{2+}.[7a] Therefore, preliminary investigations towards proposed chemical reactions leading to activation of contrast agent were carried out by performing ^{1}H-NMR of *o*-MeHgGad ligand in the absence and presence of 3 equiv. of CH_3Hg^+, under two different solvent systems, dry DMSO-d_6 and D_2O. Noticeable difference in ^{1}H-NMR spectra were not observed in the absence or presence of CH_3Hg^+ in dry DMSO-d_6 (Fig. S1, ESI†). On the contrary, significant changes in the spectra were observed in D_2O (data not shown). This prompted us to carry out a concentration dependent ^{1}H-NMR titration and the titration spectra are shown in Fig. 1.

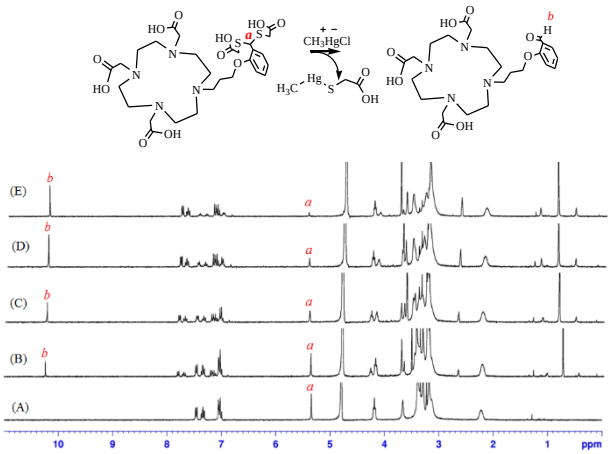


Fig. 1 ^{1}H NMR spectra (D_2O, 300 MHz): (A) *o*-MeHgGad ligand; (B) 1:0.5, (C) 1:1, (D)1:1.5, and (E)1:2 mixture of *o*-MeHgGad ligand and CH_3Hg^+.

As indicated in Fig. 1 a slender shift in aromatic proton along the downfield was observed in the presence of CH_3Hg^+. In addition, peak at 5.3 ppm was found to be gradually disappearing with the simultaneous appearance of a new singlet at 10.3 ppm with an increasing concentration of CH_3Hg^+ up to 2 equiv. The singlet at 10.3 ppm represents the proton on benzaldehyde formed as a result of acetylthio elimination in the presence of CH_3Hg^+. From this study, it can be concluded that CH_3Hg^+ can induce desulfurization elimination reaction and mechanism is similar to that of observed with inorganic mercury.[15]

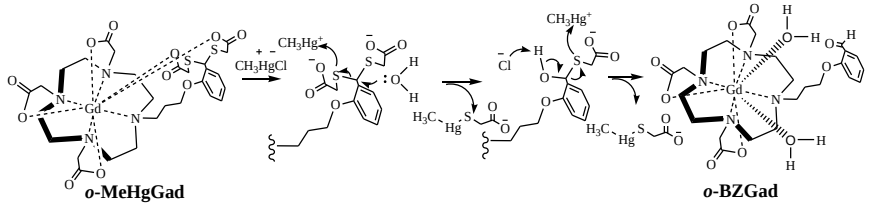
Next, we evaluated the parameters that influence the contrast enhancement of Gd(III) based MRI contrast agent. The efficiency of MR contrast agent is assessed in terms of relaxivity (r_1) and the observed relaxivity results from the contribution of the water molecules in the inner and outer coordination spheres.[16] Contribution of metal-bound water molecules to the relaxivity of Gd(III) complex is dominant and is referred as inner sphere relaxivity, given by equation 1[16]

$$r_1^{IS} = \frac{qC}{[H_2O]} \frac{1}{T_{1M} + \tau_M} \qquad (1)$$

where C represents the molar concentration of the Gd(III) complex i.e., CA, q is the number of water molecules bound to metal ions, T_{1M} is the longitudinal relaxation time of the inner-sphere water protons, and τ_M is the residence lifetime of the bound water. An obvious inference can be traced from the equation 1 that the image intensity can be modulated by altering the q value. In early reports a series of metal responsive MRI contrast agents have been developed which exploit alteration in the hydration state of Gd(III) complex.[17] While designing the molecular structure of *o*-MeHgGad we presumed that a pair appended acetate outside DO3A would saturate the coordination sphere around the Gd(III) and thereby cease the access of water molecules to the para-magnetic metal centre. However, it has been known that effective interaction between the Gd(III) and appended acetate is highly sensitive to the length and flexibility of the linker.[18] Therefore, to decisively determine the coordination status of *o*-MeHgGad, the number of water molecules coordinated directly to the Gd(III) ion was determined following previously reported method.[19] The hydration state of *o*-MeHgGad was found to be ca. 0.2 which upon addition of 2 equiv. of CH_3Hg^+ increase to 1.9 (Table S1, ESI†). Near zero inner sphere coordinated water molecule in *o*-MeHgGad clearly assures that the length and flexibility of linker is optimum to allow effective coordination of appended acetate to Gd(III) and further tuning in the structure was not required to achieve complete dormancy of *o*-MeHgGad in terms of water proton relaxivity. To further support the hydration state, relaxivity of *o*-MeHgGad was determined and it was found to be 2.3 $mM^{-1}s^{-1}$ which is comparable to the macrocyclic Gd(III) complexes with the saturated coordination profile[13,20] and lower than that of DOTAREM® (q = 1, Fig. S4, ESI†). The lower relaxivity and saturated coordinated sphere of *o*-MeHgGad strongly suggest that it is in dormant state and it will not reduce the longitudinal relaxation time (T_1) of water protons significantly. In addition, an attempt was made to identify the components of CH_3Hg^+ triggered hydrolysis of *o*-MeHgGad by FAB mass spectroscopy (Fig. S28, ESI†) and the peaks detected at m/z 663 and m/z 307 support our perceived assertion, which corresponds to *o*-BZGad (refer Scheme 2) and $C_3H_5HgO_2S^-$, respectively. Based on these results we envisage and propose the mechanism as shown in Scheme 2

ChemComm RSCPublishing

COMMUNICATION



Scheme 2 Systematic representation of proposed mechanism.

In order to evaluate practical applicability of *o*-MeHgGad as a CH_3Hg^+ sensor, changes in the relaxivity as a function of CH_3Hg^+ concentration were studied under physiologically simulated conditions. Fig. 2 represents a plot of relaxivity versus variable concentrations of CH_3Hg^+ in HEPES buffer (20 mM, pH 7.4). The results presented in Fig. 2 show that an equimolar amount of CH_3Hg^+ evokes 62% gain in the water-proton relaxivity of *o*-MeHgGad and relaxivity reaches a maximum value of 5.9 (145%) at 3 equiv. of CH_3Hg^+. The maximum observed relaxivity of *o*-BZGad (refer Scheme 2) is slightly lower than that of *p*-MeHgGad (r_1 = 6.4 $mM^{-1}s^{-1}$, Fig. S4, ESI†). The difference in the relaxivity of *o*-BZGad and *p*-MeHgGad possessing almost similar hydration state (q ~ 2) can be justified by taking into account the molecular weight of these two complexes, which is 663 and 813, respectively (Fig. S28, ESI†). Moreover, significant increase in relaxivity was also observed with inorganic mercury ions (Fig. S5, ESI†). Only one equimolar of inorganic mercury ion is sufficient to elicit almost ~ 145% change in relaxivity and this can be a concern regarding specificity of *o*-MeHgGad toward different mercury species. Nevertheless, it should be noted here that 90-100% of mercury content found in sea foods, especially in fishes is in the form of CH_3Hg^+. Thus, for the purposes of analysis any mercury content in fish should be considered CH_3Hg^+ regardless of species as prescribed in an advisory presented by US Environmental Protection Agency (EPA 2006).[21]

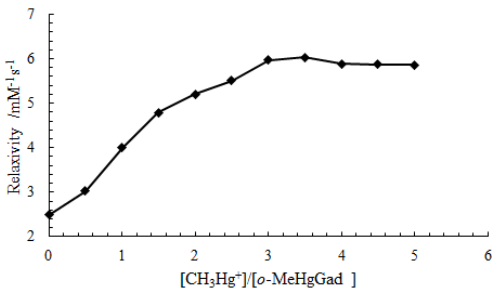


Fig. 2 Relaxivity response of *o*-MeHgGad (0.6 mM) to various concentration of CH_3Hg^+ at 37.0 ± 0.1 °C and 20 MHz in 20 mM HEPES buffer pH 7.4.

We further investigated the specificity of *o*-MeHgGad for CH_3Hg^+ by measuring relaxivity changes in the presence of biologically relevant metal ions. Unlike the response observed with CH_3Hg^+, no noticeable increase in water proton relaxivity of *o*-MeHgGad was observed in the presence of competitive metal ions except Cu(II), as depicted in Fig. 3 (white bar). Upon subsequent addition of 3 equiv. of CH_3Hg^+ to the metal ion containing solutions relaxivity values approximately similar to that observed for *o*-MeHgGad alone were obtained (black bar), confirming *o*-MeHgGad is highly selectivity toward CH_3Hg^+ and the presence of other metal ions does not influence the inherent detection capacity of the *o*-MeHgGad.

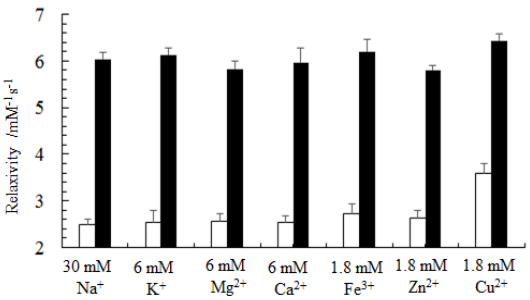


Fig. 3 Relaxivity responses of *o*-MeHgGad to various metal ions. Grey bars represent the addition of an excess of the appropriate metal ion to a 0.6 mM solution of *o*-MeHgGad. Black bars represent the subsequent addition of 1.8 mM (3 equiv.) CH_3Hg^+ to *o*-MeHgGad. Relaxivity measurements were acquired at 37.0 ± 0.1 °C in 20 mM HEPES buffer (pH 7.4) at 20 MHz.

Finally, MR imaging studies were carried out to investigate the merits of using *o*-MeHgGad as CH_3Hg^+ responsive contrast agent. Fig. 4 shows T_1-weighted MR images of six eppendorf tubes. Tubes A and B were control and contain HEPES buffer (20 mM) and *o*-MeHgGad (0.6 mM), respectively. Tubes C-F contained *o*-MeHgGad (0.6 mM) with CH_3Hg^+ added at 1, 2, 3, and 4 equiv. As can be seen in Fig. 4, solutions of *o*-MeHgGad is visibly darker compared to the complex solution with added CH_3Hg^+. In addition, gradual intensification in MR signal intensity with the increase in CH_3Hg^+ concentration suggests that *o*-MeHgGad can readily visualize differences in CH_3Hg^+ levels. These results are consistent with the relaxivity experiments shown in Fig. 2

Fig. 4 CH_3Hg^+-mediated enhancement in MR images. Images were acquired at 3.0 T (TR/TE = 200/16.3).

The *in vivo* MR imaging experiment was performed on the mice intravenously injected CH_3Hg^+ (0.1 mmol/kg) via tail vein. Previous reports on pharmacokinetics and organ distribution of intravenous CH_3Hg^+ in the mice suggest elevated retention of CH_3Hg^+ in liver, kidney, and intestine.[22] Therefore, T_1- weighted

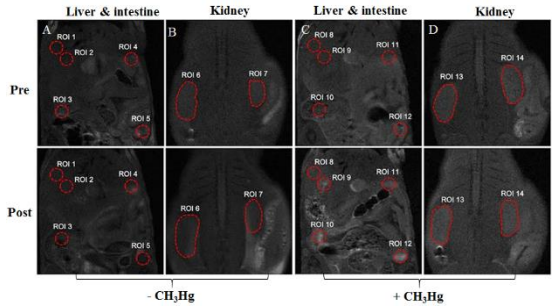


Fig. 5 Representative T_1-weighted MR images of C57BL/6JNarl mice after injection of *o*-MeHgGad at the dose of 0.1 mmol/kg. Upper and lower panels show precontrast and postcontrast, respectively.

contrast enhancement in liver, kidney, and intestine of control and CH_3Hg^+ treated mice was assessed after intravenous injection of *o*-MeHgGad (0.1 mmol/kg). As can be viewed in Fig. 5A and 5B, contrast enhancement in the organs under investigation was not observed at 30 min post injection of *o*-MeHgGad in the mice not treated with CH_3Hg^+. On the contrary, at the same detection time and dose of *o*-MeHgGad, MR contrast enhancement can be notice in liver (Fig. 5C), intestine (Fig. 5C), and kidney (Fig. 5D) of the mice earlier intravenously injected with CH_3Hg^+. For quantitative signal enhancement analysis, fourteen regions of interest (ROI) were drawn manually and contrast enhancement within the ROI was calculated (Table S2, ESI†). An average contrast enhancement of 12, 15, and 22% was recorded in liver, kidney, and intestine of mice exposed to CH_3Hg^+, which is higher than the contrast enhancement observed in control mice (Table S2, ESI†). Taken together, MR imaging results clearly demonstrate potential of using *o*-MeHgGad as a MR contrast agent for the detection of CH_3Hg^+. Finally, tissue samples from liver, kidney, and intestine were collected and Gd(III) and Hg(II) ion contents in these tissue were analysed by ICP-MS (Table S3, ESI†). Relatively higher concentration of Gd(III) was found in kidney suggesting *o*-MeHgGad is filtered and excreted through the kidney.

In conclusion, a newly designed MRI contrast was successfully synthesized and characterized for the selective detection of toxic CH_3Hg^+. The practical usability of *o*-MeHgGad was demonstrated by *in vivo* MR imaging study on BALB/c nude mice intravenously exposed to CH_3Hg^+. We believe that results presented in this report will push the limit of designed probe towards the practical utility in preclinical research endeavour focusing on various aspects of CH_3Hg^+ toxicity.

This work was supported by Ministry of Science and Technology and Ministry of Health and Welfare of the Republic of China for financial support (Health and welfare surcharge of tobacco products) under contract no. NSC 103-2627-M-009-001, NSC 101-2923-M-009-002, and MOHW 104-TDU-B-212-124-003.

Notes and references

1 M. T. Tsui, J. C. Finlay, S. J. Balogh and Y. H. Nollet, *Environ. Sci. Technol.*, 2010, **44**, 6998.
2 (a) M. D. Esteban-Vasallo, N. Aragonés, M. Pollan, G. López-Abente and B. Perez-Gomez, *Environ. Health Perspect*, 2012, **120**, 1369; (b) Z. Chen, R. Myers, T. Wei, E. Bind, P. Kassim, G. Wang, Y. Ji, X. Hong, D. Caruso, T. Bartell, Y. Gong, P. Strickland, A. Navas-Acien, E. Guallar and X. Wang, *J. Expo. Sci. Environ. Epidemiol*., 2014, **24**, 537.
3 P. Grandjean and K. T. Herz, *Mt. Sinai. J. Med*., 2011, **78**, 107-118.
4 C. T. Driscoll, R. P. Mason, H. M. Chan, D. J. Jacob and N. Pirrone, *Environ. Sci. Technol*., 2013, **47**, 4967.
5 J. H. Tidwell and G. L. Allan, *EMBO Rep*., 2001, **2**, 958.
6 (a) M. Santra, B. Roy and K. H. Ahn, *Org. Lett*., 2011, **13**, 3422; (b) Y. Yan, Y. Zhang and H. Xu, *ChemPlusChem*., 2013, **78**, 628; (c) M. H. Lee, S. W. Lee, S. H. Kim, C. Kang and J. S. Kim, *Org. Lett*., 2009, **11**, 2101; (d) M. H. Lee, B.-K. Cho, J. Yoon, J. S. Kim, *Org. Lett*., 2007, **9**, 4515; (e) K. C. Song, J. S. Kim, S. M. Park, K. C. Chung, S. Ahn and S. K. Chang, *Org. Lett*., 2006, 8, 3413; (f) M. G. Choi, Y. H. Kim, J. E. Namgoong and S. K. Chang, *Chem. Commun*., 2009, 3560.
7 (a) Y. K. Yang, S. K. Ko, I. Shin and J. Tae, *Org. Biomol. Chem*., 2009, **7**, 4590. (b) M. Santra, D. Ryu, A. Chatterjee, S. K. Ko, I. Shin and K. H. Ahn, *Chem. Commun*., 2009, 2115.
8 Z. Zhang, B. Zhang, X. Qian, Z. Li , Z. Xu , and Y. Yang, *Anal. Chem*., 2014, **86**, 11919.
9 S. Mizukami, R. Takikawa, F. Sugihara, Y. Hori, H. Tochio, M. Wälchli, M. Shirakawa and K. Kikuchi, *J. Am. Chem. Soc*., 2008, **130**, 794.
10 B. Weiss, T. W. Clarkson and W. Simon, *Environ. Health Perspect*., 2002, **110**, 851.
11 M. G. Shapiro, G. G. Westmeyer, P. A. Romero, J. O. Szablowski, B. Küster, A. Shah, C. R. Otey, R. Langer, F. H. Arnold and A. Jasanoff, *Nat. Biotechnol*., 2010, **28**, 264.
12 (a) M. Woods, G. E. Kiefer, S. Bott, A. Castillo-Muzquiz, C. Eshelbrenner, L. Michaudet, K. McMillan, S. D. Mudigunda, D. Ogrin, G. Tircsó, S. Zhang, P. Zhao and A. D. Sherry, *J. Am. Chem. Soc*., 2004, **126**, 9248; (b) S. M. Vibhute, J. Engelmann, T. Verbić, M. E. Maier, N. K. Logothetis and G. Angelovski, *Org. Biomol. Chem*., 2013, **11**, 1294.
13 (a) E. L. Que, E. Gianolio, S. L. Baker, A. P. Wong, S. Aime and C. J. Chang, *J. Am. Chem. Soc*., 2009, **131**, 8527; (b) E. L. Que and C. J. Chang, *J. Am. Chem. Soc*., 2006, **128**, 15942; (c) W.-H. Li, S. E. Fraser and T. J. Meade, *J. Am. Chem. Soc*., 1999, **121**, 1413.
14 (a) J. A. Duimstra, F. J. Femia and T. J. Meade, *J. Am. Chem. Soc*., 2005, **127**, 12847; (b) S. H. Chen, Y. T. Kuo, G. Singh, T. L. Cheng, Y. Z. Su, T. P. Wang, Y. Y. Chiu, J. J. Lai, C. C. Chang, T. S. Jaw, S. C. Tzou, G. C. Liu and Y. M. Wang, *Inorg. Chem*., 2012, **51**, 12426; (c) F. Arena, J. B. Singh, E. Gianolio, R. Stefania and S. Aime, *Bioconjug Chem.*, 2011, **22**, 2625.
15 (a) H. Dai, Y. Yan, Y. Guo, L. Fan, Z. Che and H. Xu, *Chem. Eur. J*., 2012, **18**, 11188; (b) X. Cheng, S. Li, H. Jia, A. Zhong, C. Zhong, J. Feng, J. Qin and Z. Li, *Chem. Eur. J*., 2012, **18**, 1691; (c) X. Cheng, Q. Li, C. Li, J. Qin and Z. Li, *Chem. Eur. J*., 2011, **17**, 7276; (d) J. Ros-Lis, M. D. Marcos, R. Martinez-Manez, K. Rurack and J. Soto, *Angew. Chem. Int. Ed.*, 2005, 44, 4405.
16 Y. H. Chang, C. Y. Chen, G. Singh, H. Y. Chen, G. C. Liu, Y. G. Goan, S. Aime and Y. M. Wang, *Inorg. Chem*., 2011, **50**, 1275.
17 E. L. Que and C. J. Chang, *Chem. Soc. Rev*., 2010, **39**, 51.
18 (a) K. D. Verma, A. Forgács, H. Uh, M. Beyerlein, M. E. Maier, S. Petoud, M. Botta and N. K. Logothetis, *Chem. Eur. J*, 2013, **19**, 18011; (b) L. M. Matosziuk, J. H. Leibowitz, M. C. Heffern, K. W. MacRenaris, M. A. Ratner and T. J. Meade, *Inorg. Chem.*, 2013, **52**, 12250.
19 (a) A. Beeby, I. M. Clarkson, R. S. Dickins, S. Faulkner, D. Parker, L. Royle, A. S. d. Sousa, J. A. G. Williams and M. Woods, *J. Chem. Soc., Perkin Trans. 2*, 1999, 493; (b) W. D. Horrocks and D. R. Sudnick, *J. Am. Chem. Soc.* 1979, **101**, 334.
20 M. Polasek and P. Caravan, *Inorg. Chem.*, 2013, **52**, 4084.
21 Chapter 12 of the Volunteer Estuary. Monitoring Manu-al, A. Methods Manual, Second Edition, EPA-842-B-06-003.
22 (a) G. Zareba, E. Cernichiari, L. A. Goldsmith and T. W. Clarkson, *J. Appl. Toxicol*., 2008, **28**, 535; (b) S. Omata, M. Sato, K. Sakimura, H. Sugano, *Arch. Toxicol*., 1980, **44**, 231.

