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Protein assisted fluorescence enhancement of a dansyl containing fluorescent reagent: Detection of Hg²⁺ ion in aqueous medium†

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Intramolecular charge transfer (ICT) based fluorescent reagents containing a dansyl fluorophore have been synthesized and characterized. The reagent **1** and its complex, **1**+Hg²⁺ in sodium acetate buffer (pH 6.7) revealed considerable fluorescence enhancement (switched-on) in the presence of bovine serum albumin (BSA) with 10 ppb detection sensitivity. ¹H NMR spectral analysis suggests complexation between **1** and Hg²⁺ ion involving the *N,N*-dimethylamino and carboxylic functions.

The development of a selective and sensitive fluorescent tool for detection of heavy and transition metal (HTM) ions is currently receiving great interest among the scientific community because of their related environmental and health problems.¹ The Hg²⁺ ion is commonly spread in the environment by anthropogenic and industrial releases and is converted to toxic methylmercury by bacterial and chemical actions. The bioaccumulation of such toxic stuff in living tissues of human and animal bodies, through the food chain, causes mercury poisoning and lethal diseases.² The maximum allowable concentration of mercury in food and the environment, as recommended by the FDA and EPA, is in the range of 1–2 ppb and beyond that it becomes toxic.^{1b}

Although several methods have been developed for the detection of HTMs at low concentrations, fluorescence based detection methods are more reliable, inexpensive, sensitive, easy to perform, and are suitable for real time monitoring of anions and metal ions.³ The poor solubility⁴ of sensing systems in aqueous medium and nonspecific fluorescence quenching induced by HTMs due to enhanced spin–orbit coupling^{5a} and electron transfer mechanism^{5b,c} make them impractical as good analytical tools. The conjugation of a fluorophore with a protein will be a promising way to overcome this limitation.^{5d} Therefore, a sensitive fluoroionophore having good optical properties in aqueous media and upon interaction with metal ions like Hg²⁺ showing fluorescence enhancement (FE) is essential for naked-eye visible recognition.⁶

In view of this new reagents have been synthesized by reacting dansyl chloride (DNS) with an analogous amino acid, *trans*-

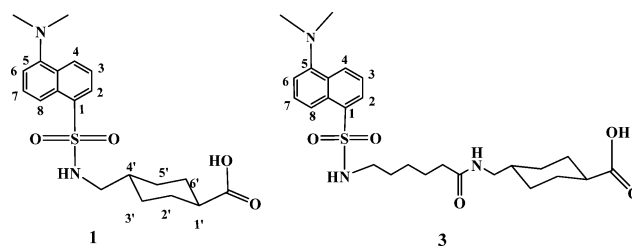


Fig. 1 Synthesized fluorescent reagents.

4-aminomethylcyclohexanecarboxylic acid (TAC).⁷ As a model, reagent **3** (Fig. 1) with an extended flexible linker arm was prepared by reacting DNS first with caproic acid to give **2**. The activated NHS ester of **2** subsequently coupled with TAC to obtain **3** by a carboxamide bond formation (Scheme 1, ESI†). The use of an amino acid not only enhances the aqueous medium compatibility of the probe but also the typical *trans* arrangement of the dansyl and carboxyl functions at the 1,4-termini will segregate the amino and carboxyl functions from self-interaction, and would also enhance the electrostatic interaction with protein molecules. Moreover, a *cis*-1,3 isomer based probe could also be employed, however, it has not been used because it is expected that in the *cis*-1,3 conformation the probability of self interaction between two functional groups will be high, and also complexation would be more favored in the close vicinity of sulfonamide and carboxylic functions rather than between the amino and carboxyl functions, and under these conditions the desired optical behavior might be hampered. Secondly, the synthesis of the 1,3 derivative is tedious and favors lactamization, and opening of the lactam most of the time leads to polymerization.^{7d,e} Dansyl as a fluorophore is implicated because it is sensitive to the variation in external environment and a slight variation in the medium would result in a dramatic change in its typical and strong fluorescence with high emission quantum yield, attributed to intramolecular charge transfer (ICT), involving the *N,N*'-dimethylamino and sulfonamide groups.^{8–9} Such typical optical behavior has led dansyl fluorophore to be a core structure in most fluorescent sensors for detection of cations,¹⁰ anions^{11a} and in supramolecular structures such as calixarenes^{11b–c} and dendrimers.^{11d}

The absorption spectra of reagents (1×10^{-4} M) in sodium acetate buffer (50 mM) illustrated the high and low energy bands in the range of ~247–244 ($\epsilon = 103\,200$, $83\,100$, $56\,500$ M⁻¹cm⁻¹) and 332–336 nm ($\epsilon = 29\,200$, $23\,500$, $13\,900$) due to π – π^* and

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$n-\pi^*$ electronic transitions, respectively. At 335 nm excitation wavelength reagents exhibited an emission band at ~ 555 nm (Fig. 2) in which the fluorescence intensity of **1** was relatively higher compared to **3** ($\Phi_1 = 0.65$ and $\Phi_3 = 0.54$ with respect to quinine sulfate in ACN). The low fluorescence intensity of **3** may obviously be attributed to restricted ICT, because of the extended flexible linker arm of TCA that favors more self interaction between the *N,N*-dimethylamino and carboxylic functions, and hence leads to diminished fluorescence due to hindrance in charge transfer from the amino to the sulfonamide unit.

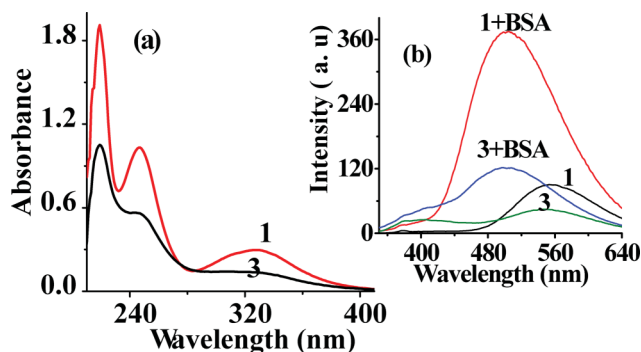


Fig. 2 UV-Vis absorption spectra. (b) Emission spectra of **1** and **3** with and without BSA (10 μ M) in AcONa buffer (50 mM; pH = 6.7).

Upon addition of different metal ions (Na^+ , K^+ , Ca^{2+} , Ag^+ , Co^{2+} , Pb^{2+} , Zn^{2+} , Cu^{2+} , Hg^{2+} , Ni^{2+} , Cd^{2+} ; 15 equiv, 12 μ L) to individual solutions of reagents (40 μ M) the emission spectra of **1** only revealed significant fluorescence quenching with Hg^{2+} ion (Fig. 3) while no significant change was observed with reagents **2** and **3** (Fig. S1†). A competitive metal ion study exhibited insignificant change in the fluorescence spectrum of complex **1**+ Hg^{2+} , either by the addition of Hg^{2+} (30 equiv) to the solution of **1** and then an excess of other metal ions, or by the addition of Hg^{2+} to a solution of **1** containing an excess of other tested metal ions (Fig. S2). Job's plot analysis from the fluorescence titration data have revealed a 1:1 stoichiometry between **1** and Hg^{2+} ion with a binding constant, $K_{\text{ass}} = 2.38 \times 10^4 \text{ M}^{-1}$ calculated by Benesi-Hildebrand (B-H) method. A good linear (R^2 0.992) Stern-Volmer plot with quenching constant, $K_{\text{S-V}} = 9000 \text{ M}^{-1}$ suggested a dynamic mode of fluorescence quenching with Hg^{2+} (Fig. S3b, c).

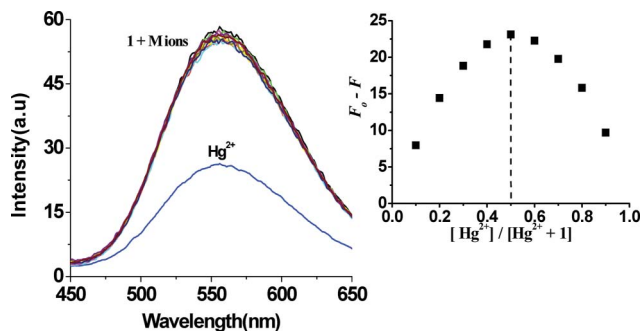


Fig. 3 Emission spectra of **1** upon interaction with various metal ions ($\text{M} = \text{Na}^+$, K^+ , Ca^{2+} , Ag^+ , Co^{2+} , Pb^{2+} , Zn^{2+} , Cu^{2+} , Hg^{2+} , Ni^{2+} , Cd^{2+} ; 15 equiv). Job's plot shows 1:1 equilibrium between **1** and Hg^{2+} ion in AcONa buffer.

In order to understand the possible reversibility and binding of **1** with Hg^{2+} ion, a strong chelating reagent, EDTA, was added to the solution of the probable complex, **1**+ Hg^{2+} . The revival of fluorescence of almost similar emission intensity suggested a reversible mode of complexation. Conversely, when excess of Hg^{2+} was added to the solution of **1** containing EDTA (50 equiv) the relative fluorescence intensity remained unaffected (data not shown). Thus, the preliminary observations suggested a high affinity of **1** for Hg^{2+} ion.

Further, bovine serum albumin (BSA) is known to generate well defined hydrophobic-hydrophilic cavities in aqueous media¹² and the microenvironment sensitive dansyl fluorophore exhibits enhanced fluorescence in hydrophobic media while the fluorescence quenching often occurs in hydrophilic media, and also in acidic conditions possibly due to the protonation of dimethylamino group, and consequently, hindrance in charge transfer to the naphthyl ring from the dimethylamino unit.⁸⁻⁹ Thus, keeping in mind the challenges of turn-on response to Hg^{2+} ions in aqueous medium, chemical sensors based on amino acids would be a possible route for development, in conjunction with proteins.¹³ Therefore, we observed the sensing behavior of reagents in the presence of proteins like BSA and fetal bovine serum (FBS).

The emission spectrum of BSA at excitation wavelength $\lambda_{\text{ex}} = 278$ nm shows a strong emission at ~ 345 nm due to tryptophan domains (Fig. S4†).¹⁴ Upon gradual addition of Hg^{2+} ions (0–44 equiv) to BSA solution significant fluorescence quenching was observed, in which the emission band centered at 345 nm was blue-shifted (~ 8 nm) to appear at 337 nm (Fig. S4). On the other hand, on a gradual increase in BSA concentration (0–30 μ M) in the solution of **1** (40 μ M), fluorescence enhancement (~ 5 fold) occurred with a blue shift of ~ 56 nm, and emission spectra were almost saturated after the addition of 20 μ M of BSA (Fig. 4a).

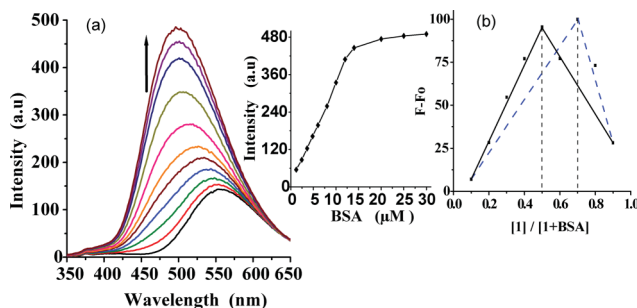


Fig. 4 Fluorescence titration spectra of **1** with BSA in AcONa buffer (50 mM; pH = 6.7). Inset shows the change in fluorescence intensity with respect to concentration of BSA. (b) Job's plot indicating 1:1 and 1:2 stoichiometry between **1** and BSA.

Similarly, **3** also shows a blue shift but the increase in intensity was relatively less (Fig. 2b). This is attributable to a structurally more favored interaction of **1** compared to **3** in the hydrophobic region of BSA. The observed 1:1 and 1:2 stoichiometries between **1** and BSA from the Job's plot have suggested concentration dependence. On increasing the concentration of either **1** or BSA, keeping the other one constant, the stoichiometry varies from 1:1 to 2:1 and *vice versa* with relative increase and decrease in fluorescence intensities. Since the titration curves could not be well fitted mixed interaction between **1** and BSA is expected.

At low concentration of **1** a good linear fit of the fluorescence titration curves favored a 1 : 1 equilibrium with an association constant $K_{\text{ass}} = 6.9 \times 10^4 \text{ M}^{-1}$ (Fig. 4b, 5a) while a low binding constant, $K_{\text{ass}} = 4 \times 10^3 \text{ M}^{-1}$ was obtained for **3**. Similarly, the interaction of **1** with FBS shows an increase in fluorescence intensity with a blue-shift (Fig. S5†). Further, the unfolding process of serum albumin on increasing the concentration of urea is very well established.¹² To see the denaturing effect of the protein on its binding affinity with **1** and related optical properties, a urea solution (0–3 M) was added gradually to the solution of protein-bound probe, **1**+BSA. The emission profile (Fig. 6) shows conversely a decrease in fluorescence intensity along with a red-shift. This clearly suggested about the weakening of probe–protein binding upon addition of urea, and hence, the release of the probe in a hydrophilic environment.

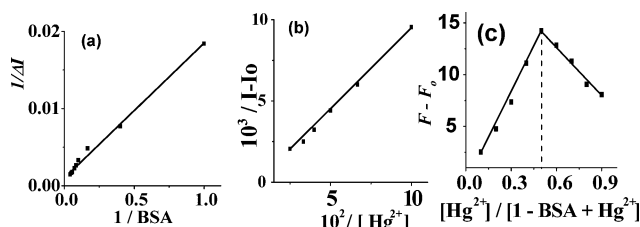


Fig. 5 Benesi–Hildebrand plots (a) **1**+BSA and (b) **1**+BSA+ Hg^{2+} (c) Job's plot indicates 1 : 1 equilibrium between **1**+BSA and Hg^{2+} ion.

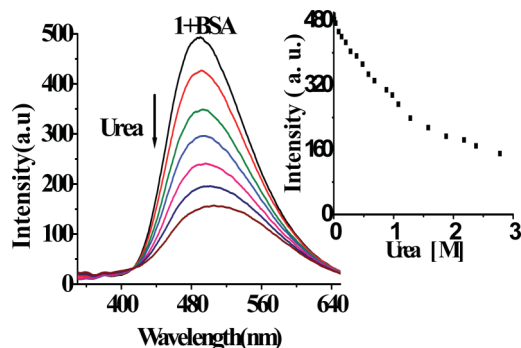


Fig. 6 Change in emission spectra of **1**+BSA upon increasing concentration of urea (0–3 M) in AcONa buffer (50 mM; pH = 6.7).

Thus, the turn-on fluorescence to detect Hg^{2+} ion using BSA as a support protein is expected. The optimum concentration of BSA for Hg^{2+} ion has been estimated by the addition of different concentrations (0–25 μM) of BSA to a solution of **1** (40 μM). After addition of Hg^{2+} (in excess) in each vial, emission spectra were recorded and found a maximum fluorescence enhancement (~4.5 fold) with a blue shift of ~56 nm for the solution containing 10–12.5 μM (0.25–0.3 equiv) of BSA, and a further increase of BSA (15–30 μM) exhibited only ~1.2 fold rise in emission intensity (Fig. S6†). Therefore, keeping the favored 1 : 1 complexation, a metal ion (10 equiv) interaction study was performed by reducing the BSA concentration to 1 : 1/4 (v/v) in NaOAc buffer. Upon addition of Hg^{2+} (0–1 equiv) to **1**+BSA solution, ~10 fold fluorescent enhancement is observed in comparison to the probe without BSA, while the other tested metal ions including Cu^{2+} and Cd^{2+} have not induced any significant change (~1–1.2 fold) Also, the model reagent **3** does not show any significant change in the emission signal under the same conditions (Fig. 7).

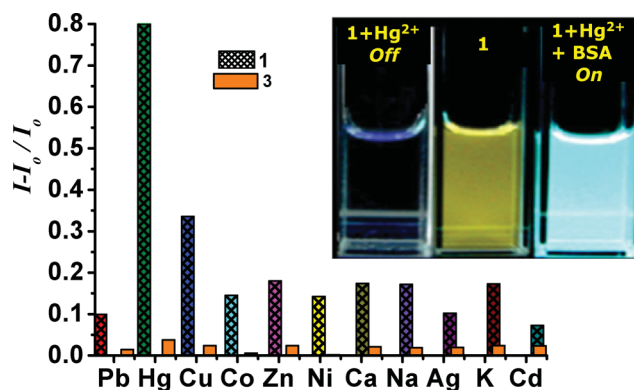


Fig. 7 Bar diagram showing interaction of **1** and **3** with tested metal ions in the presence of BSA in AcONa buffer. Inset shows change in color of **1**.

The competitive metal ion interaction studies of **1** in BSA revealed a high affinity for Hg^{2+} ion, and the emission generated in **1**+BSA+ Hg^{2+} solution remained unaffected when other metal ions tested were added, except for Cu^{2+} ion which shows a marginal decrease in the relative fluorescence intensity (Fig. 8b, Fig. S7 bar diagram). To observe the binding affinity of the protein-bound probe, **1**+BSA for Hg^{2+} ion, a fluorescence titration experiment was performed. The spectral data revealed fluorescence enhancement upon the addition of 1 equiv of Hg^{2+} ion. The Job's plot analysis revealed a 1 : 1 stoichiometry and the association constant estimated from fluorescence titration data was found as $K_{\text{ass}} = 2.2 \times 10^5 \text{ M}^{-1}$ (Fig. 5c, 5b) with 10 ppb level sensitivity ($\text{LOD}^{5d} = 0.05 \mu\text{M}$).

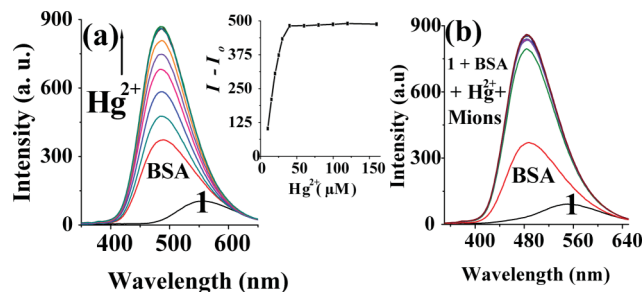


Fig. 8 Fluorescence titration spectra upon addition of Hg^{2+} ion (0–1.0 equiv) to **1** + BSA (1 : 1/4; v/v) in AcONa buffer (50 mM; pH = 6.7). (b) Interference study of **1** + BSA + Hg^{2+} with tested metal ions.

To arrive at the actual mechanism involved in Hg^{2+} ion interaction, ^1H NMR spectra were recorded in $\text{DMSO}-d_6$ (Fig. 9). The ^1H NMR spectrum of **1** shows a doublet at δ 8.48–7.29 ppm due to the H2, H8, H4, and H6 protons. The carboxylic group appears as a broad resonance at δ 12.0 ppm (Fig. S9). A triplet attributable to the -NH proton of sulfonamide appeared at δ 7.91 ppm and H3, H7 as a multiplet at δ 7.64 ppm. The aliphatic protons of the cyclohexane ring resonated in the range of δ 2.61–0.75 ppm. The -N(CH₃)₂ and methylene (-CH₂NH) protons appeared at δ 2.84 and 2.61 ppm as a singlet and triplet respectively. Upon addition of 2 equiv of Hg^{2+} ion, the H2 and H8 protons merged together to appear at δ 8.56 ppm ($\Delta\delta = 0.086$), whereas the resonance of the carboxylic function disappeared (Fig. S11). The resonances of the cyclohexane ring and methylene protons more or less shifted upfield, and the H2' and H3' protons exhibited maximum upfield shifts of 0.028 and 0.031 ppm respectively. The

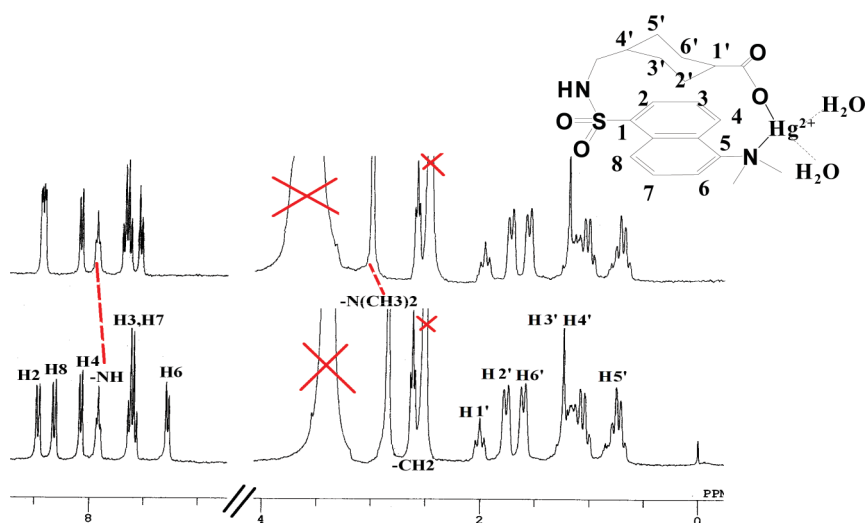


Fig. 9 ^1H NMR spectra of **1** in $\text{DMSO}-d_6$ and after addition of Hg^{2+} ion (1.5 equiv). Plausible mode of complexation between **1** and Hg^{2+} ion.

H3 , H7 ($\Delta\delta=0.12$), H6 ($\Delta\delta=0.35$), $-\text{NH}$ ($\Delta\delta=0.08$), and $-\text{N}(\text{CH}_3)_2$ ($\Delta\delta=0.24$) protons exhibited downfield shifts respectively. This clearly suggested the interaction of Hg^{2+} ion was most favorable through the N,N' -dimethylamino and carboxyl functions of the cyclohexane ring, without deprotonation of sulfonamide, hence the fluorescence quenching, due to hindrance in ICT. An almost similar behavior was observed when we measured the ^1H NMR spectra of reagent **2** in the presence of Hg^{2+} ion (Fig. S14).

Since, ^1H NMR titration spectral data revealed no dramatic variations in the resonances of the coupled cyclohexane ring, the retention of the typical conformation of probe and the possibility of the formation of any strained geometry after complexation with Hg^{2+} ion is therefore ruled out. Thus, taking into account the planar structure of the naphthalene ring we proposed a plausible mode of complexation with Hg^{2+} ion laterally, between the two functionalities as depicted in Fig. 9. The projected geometrical arrangement in that sense would generate no significant strained structure in which interaction between the two moieties is expected, as observed in the ^1H NMR spectra. Further, it is noteworthy to add that the typical conformation of the probe remained unchanged even before and after the interaction of Hg^{2+} ion in protein medium (BSA, FBS).

Additionally, the significant fluorescence enhancement in protein medium can be rationalized by assuming the interaction of **1** in the hydrophobic region of BSA and the further increase in fluorescence intensity upon interaction with Hg^{2+} ion is attributed to motional restriction exerted by the protein molecule on the favored geometrical conformation of the probe, which probably reduces vibrational-rotational energy interconversion of the excited and ground states of the complex, **1**+ Hg^{2+} of relatively high binding affinity. Therefore, a certain kind of rigidity experienced by the probe would more or less decrease the radiationless decay, and hence be responsible for fluorescence enhancement of the protein-bound molecule.¹²

Further, since the binding constant of **1** with BSA is very high compared to **3**, we can also infer a possible interaction of the carboxylate terminal of **1** with the BSA molecule and addition of Hg^{2+} ion leads to chelation/trapping through the sulfonamide function, well stabilised by sulfur atoms of the disulfide bonds, having one

free thiol of from cysteine amino acid residues present in BSA. Consequently, the increase in electron withdrawing effect on the sulfonamide group of naphthalene, and hence the disruption in internal charge transfer, causes fluorescence enhancement.^{14c} The least possibility of deprotonation was further evidenced when we carried out the pH titration study (Fig S8). In acidic medium **1** shows fluorescence quenching, possibly due to the protonation of the amino function of the probe,⁸ whereas in alkaline medium no significant fluorescence enhancement was observed.^{9c}

In conclusion, we have synthesised dansyl containing fluorescent reagents and demonstrated the turn-on fluorescence for detection of Hg^{2+} ion in aqueous media, using BSA as a support protein. Further, studies to deepen our understanding of the sensing mechanism are in progress.

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References

- (a) T. Gunnlaugsson, M. Glynn, G. M. Tocci, P. E. Kruger and F. M. Pfeffer, *Coord. Chem. Rev.*, 2006, **250**, 3094; (b) E. M. Nolan and S. J. Lippard, *Chem. Rev.*, 2008, **108**, 3443; (c) A. Renzoni, F. Zino and E. Franchi, *Environ. Res.*, 1998, **77**, 68.
- (a) P. Grandjean, P. Weihe, R. F. White and F. Debes, *Environ. Res.*, 1998, **77**, 165; (b) T. Takeuchi, N. Morikawa, H. Matsumoto and Y. Shiraishi, *Acta Neuropathol.*, 1962, **2**, 40.
- (a) R. Martinez-Manez and F. Sancenon, *Chem. Rev.*, 2003, **103**, 4419; (b) B. Ramachandram, G. Saroja, N. B. Sankaran and A. Samanta, *J. Phys. Chem. B*, 2000, **104**, 11824; (c) V. S. Jisha, A. J. Thomas and D. Ramaiah, *J. Org. Chem.*, 2009, **74**, 6667.
- (a) R. Metiver, I. Leray and B. Valeur, *Chem. Commun.*, 2003, 996; (b) J. Y. Kwon, Y. J. Jang, Y. J. Lee, K. M. Kim, M. S. Seo, W. Nam and J. Yoon, *J. Am. Chem. Soc.*, 2005, **127**, 10107.
- (a) D. S. McClure, *J. Chem. Phys.*, 1952, **20**, 682; (b) M. Yu, M. Shi, Z. Chen, F. Li, X. Li, Y. Gao, J. Xu, H. Yang, Z. Zhou, T. Yi and C. Huang, *Chem.-Eur. J.*, 2008, **14**, 6892; (c) G.-K. Li, Z.-X. Xu and C.-F. Chen, *Chem. Commun.*, 2008, 1774; (d) B. P. Joshi, C. r. Lohani and K. H. Lee, *Org. Biomol. Chem.*, 2010, **8**, 3220.
- J. V. Ros-Lis, M. D. Marcos, R. Martinez-Manez, K. Rurack and J. Soto, *Angew. Chem., Int. Ed.*, 2005, **44**, 4405.
- (a) A. Misra, S. Mishra and K. Misra, *Bioconjugate Chem.*, 2004, **15**, 638; (b) A. Misra, P. Dwivedi and M. Shahid, *Russ. J. Bioorg. Chem.*,

- 2009, **35**, 62; (c) A. Misra and M. Shahid, *Bioorg. Med. Chem.*, 2009, **17**, 5826; (d) H. K. Hall, Jr., *J. Am. Chem. Soc.*, 1958, **80**, 6412; (e) M. W. Cronyn, *J. Org. Chem.*, 1949, **14**, 1013.
- 8 (a) M. Majumdar, P. K. Parrack and B. Bhattacharyya, *Eur. J. Biochem.*, 1992, **204**, 127; (b) A. A. Bhattacharya, T. Grune and S. Curry, *J. Mol. Biol.*, 2000, **303**, 721; (c) X. M. He and D. C. Carter, *Nature*, 1992, **358**, 209; (d) T. Wu, Q. Wu Guan, H. Su and Z. Cai, *Biomacromolecules*, 2007, **8**, 1899; (e) U. P. Strauss and G. Vesnaver, *J. Phys. Chem.*, 1975, **79**, 1558.
- 9 (a) B. Valeur and I. Leray, *Inorg. Chim. Acta*, 2007, **306**, 765; (b) C. A. Munson, P. M. Page and F. V. Bright, *Macromolecules*, 2005, **38**, 1341; (c) C. A. Munson, M. A. Kane, G. A. Baker, S. Pandey, S. A. Perez and F. V. Bright, *Macromolecules*, 2001, **34**, 4624; (d) Y.-H. Li, L.-M. Chan, L. Tyer, R. T. Moody, C. M. Himel and D. M. Hercules, *J. Am. Chem. Soc.*, 1975, **97**, 3118; (e) T. Koike, T. Watanabe, S. Aoki, E. Kimura and M. Shiro, *J. Am. Chem. Soc.*, 1996, **118**, 12696.
- 10 (a) R. Metiver, I. Leray, B. Lebeau and B. Valeur, *J. Mater. Chem.*, 2005, **15**, 2965; (b) R. Metiver, I. Leray, B. Lebeau and B. Valeur, *Chem.-Eur. J.*, 2004, **10**, 4480; (c) G. K. Walkup and B. Imperiali, *J. Am. Chem. Soc.*, 1997, **119**, 3443.
- 11 (a) C.-F. Chen and Q.-Y. Chen, *Tetrahedron Lett.*, 2004, **45**, 3957; (b) A. Dhir, V. Bhalla and M. Kumar, *Org. Lett.*, 2008, **10**, 4891; (c) M. Kumar, R. Kumar and V. Bhalla, *Chem. Commun.*, 2009, 7384; (d) D. Astrue, E. Boissetier and C. Ornelas, *Chem. Rev.*, 2010, **110**, 1857.
- 12 (a) A. Mallick, B. Haldar and N. Chattopadhyay, *J. Phys. Chem. B*, 2005, **109**, 14683; (b) A. Chakrabarty, A. Mallick, B. Haldar, P. Das and N. Chattopadhyay, *Biomacromolecules*, 2007, **8**, 920; (c) K. A. Willets, O. Ostroverkhova, M. He and R. J. Twieg, *J. Am. Chem. Soc.*, 2003, **125**, 1174; (d) A. Wu, Y. Xu and X. Qian, *Bioorg. Med. Chem.*, 2009, **17**, 592.
- 13 (a) G. G. Talanova, N. S. A. Elkarim, V. S. Talanov and R. A. Bartsch, *Anal. Chem.*, 1999, **71**, 3106; (b) Y. Suzuki and K. Yokoyama, *J. Am. Chem. Soc.*, 2005, **127**, 17799; (c) L.-J. Ma, Y. Li, L. Li, J. Sun, C. Tian and Y. Wu, *Chem. Commun.*, 2008, 6345.
- 14 (a) K. Yamasaki, T. Maruyama, U. Kragh-Hansen and M. Otagiri, *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.*, 1996, **1295**, 147; (b) Y. Moriyama, D. Ohta, K. Hachiya, Y. Mitsui and K. J. Takeda, *J. Protein Chem.*, 1996, **15**, 265; (c) Y. Li, X. Yan, C. Chen, Y. Xia and Y. Jiang, *J. Proteome Res.*, 2007, **6**, 2277.