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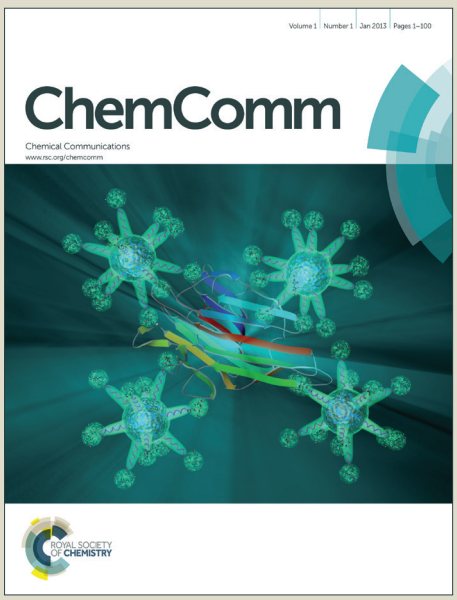

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Sensitive fluorescence detection of lysozyme using a tris(bipyridine)ruthenium(II) complex containing multiple cyclodextrins

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A new series of photoactive metallocyclodextrins with increased fluorescence intensity upon binding with ssDNA/aptamer has been demonstrated to sensitively and selectively detect lysozyme. The detection mechanism relies on the formation of aptamer/lysozyme complex, which leads to a reduction of fluorescence intensity.

Cyclodextrins (CDs) have attracted considerable attention because of their ability to encapsulate small molecules in their hydrophobic cavities.[1] The major driving force for this inclusion phenomenon is the hydrophobic interaction between the cavity of CDs and guest molecules.[2] CDs can form host-guest complexes with a wide range of guest species in aqueous solutions, which has many potential applications, in particular in supermolecular chemistry.[3] Introducing photoactive metal ions onto the CDs framework brings the new dimensions to metallocyclodextrins.[4] This methodology is increasingly attractive because the redox and photosensitization properties of the metal centers interact with the CD receptors, creating new opportunities for the fabrication of biosensors, molecular wires and energy transfer systems.[5] In a metallocyclodextrin system with metal cores, biomolecule sensing by the CD cavity can be realized.[6] There is increasing interest in the fluorescence changes of a sensing system that responds to the host–guest recognition between the metallocyclodextrin and a guest molecule.[7] Aptamers are one example of a guest molecule that can selectively bind to a variety of targets, including low molecular weight organic or inorganic substrates, macromolecules such as proteins,[8] small molecules.[9]

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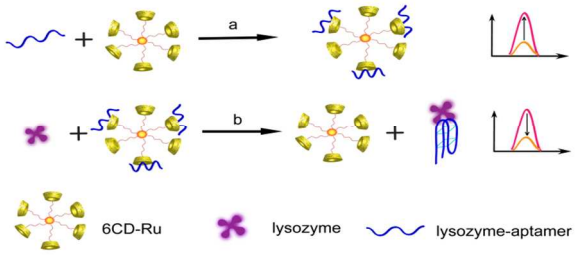
We have synthesized a series of metallocyclodextrins, having multiple ruthenium centers and multiple β-CD binding sites, respectively.[10] The former series were fabricated as biosensors for the detection of protein, small molecules and DNA based on its extraordinary electrochemiluminescence properties.[11] While the latter series (Fig. S1, ESI†), having a structure consisting of one ruthenium core and multiple β-CDs, including CD-Ru, 3CD-Ru and 6CD-Ru, were expected to exhibit distinctive electronic and photoactive characteristics, especially in fluorescence.[10a] Compared with the parent ruthenium center, the improvement in fluorescence intensity can be attributed to the presence of multiple β-CD rings, preventing fluorescence quenching of the ruthenium cores by oxygen in the solution.[12]

Here we present results demonstrating that single strand DNA (ssDNA) can significantly improve the fluorescence intensity of these metallocyclodextrin. A novel, sensitive strategy for lysozyme detection has been proposed. As illustrated in Scheme 1, the fluorescence intensities of the metallocyclodextrins were initially enhanced by binding ssDNA (aptamer in this case) via the host-guest recognition (Scheme 1a).



Scheme 1 Schematic representation of fluorescence detection of lysozyme based on the change of fluorescence intensity.

However, adding lysozyme into the solution resulted in preferential formation of aptamer/lysozyme complexes and a reduced fluorescence intensity (Scheme 1b).The reduction in the fluorescence intensity can be used for quantitative determination of lysozyme.

It has been reported that bases, the key components of ssDNA, can be encapsulated into CD cavities freely via host-guest recognition.[13] Thus, the fluorescence behavior of the metallocyclodextrins mixed with ssDNA was investigated. As shown in Fig.1, adding 1 μM ssDNA to the metallocyclodextrins solutions produced a large fluorescent enhancement (Fig. 1A, B and C, curves b) compared with the original fluorescence signals (Fig.1A, B and C, curves a). Details of the enhancement are shown in Table 1. Of the three metallocyclodextrins studied, 6CD-Ru exhibited the largest fluorescence enhancement (97.7%). The fluorescence intensities of 3CD-Ru and CD-Ru increased by 33.2% and 19.5%, respectively. While the fluorescence signal of the parent compound $Ru(bpy)_3^{2+}$ solution was enhanced by 13.8%, as ssDNA can effect $Ru(bpy)_3^{2+}$ through electrostatic binding.[14] Importantly, the enhancement of metallocyclodextrins, 3CD-Ru and 6CD-Ru especially, was much larger when compared with the parent compound $Ru(bpy)_3^{2+}$. The fluorescence signal enhancement resulting from these three metallocyclodextrins also

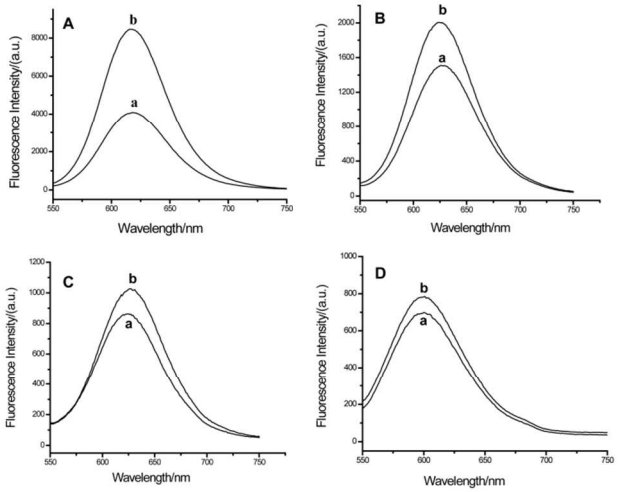
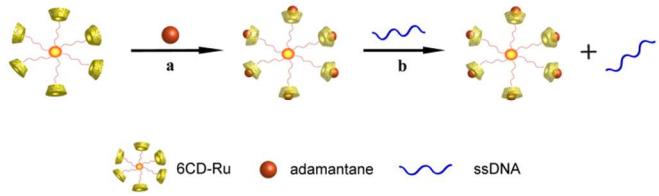


Fig. 1 Fluorescence spectra of (A): (a) 1 μM 6CD-Ru; (b) 1 μM 6CD-Ru with 1 μM ssDNA; (B): (a) 1 μM 3CD-Ru; (b) 1 μM 3CD-Ru with 1 μM ssDNA; (C): (a) 1 μM CD-Ru; (b) 1 μM CD-Ru with 1 μM ssDNA; (D): (a) 1 μM $Ru(bpy)_3^{2+}$; (b) 1 μM $Ru(bpy)_3^{2+}$ with 1 μM ssDNA.

Table 1 Comparison of the fluorescence intensities of the metallocyclodextrins after ssDNA addition in aqueous solution.

Enhanced I_f[a]	6CD-Ru (1 μM)	3CD-Ru (1 μM)	CD-Ru (1 μM)	$Ru(bpy)_3Cl_2$ (1 μM)
ssDNA	97.7%[b]	33.2%[b]	19.5%[b]	13.8%[b]

[a] $I_f = (I - I_0) / I_0$, where I and I_0 are the fluorescence intensities of the metallocyclodextrins in the presence and absence of ssDNA, respectively. [b] The concentration of ssDNA added into metallocyclodextrins solution (1 μM) was 1 μM.



Scheme 2 Schematic representations of (a) metallocyclodextrin recognition for adamantane and (b) the impact of ssDNA to the mixture of metallocyclodextrin and adamantane.

increases with the increasing number of β-CD units. We also investigated the stoichiometry of ssDNA binding to 6CD-Ru. Analysis of the complex stoichiometry by constructing a Job-plot suggested that a 1:2 complex was formed between 6CD-Ru and ssDNA(Fig. S2, ESI†).

As discussed above, ssDNA can bind with CD via host-guest interaction between the bases and CD cavities on the metallocyclodextrins.[15] To verify this host-guest recognition effect, adamantane, a typical guest molecule for CD in supramolecular chemistry,[16] has been added into the solution of metallocyclodextrins (6CD-Ru in this case) before the addition of ssDNA(Scheme 2). As illustrated in Fig. 2, the fluorescence intensity of 6CD-Ru (Fig. 2, curve a) was not significantly affected by the addition of adamantane (Fig. 2, curve b). Comparing curve b and curve c, we find that adding ssDNA to the solution of 6CD-Ru and adamantane did not result any fluorescence enhancement for the metallocyclodextrin compounds. In comparison, a large enhancement was obtained by adding ssDNA to metallocyclodextrins without adamantane (Fig. 2, curve d). Similar results were obtained when the same experiments were performed on the other two metallocyclodextrin compounds, CD-Ru and 3CD-Ru (Fig. S3A and B, ESI†).

Previously published data state that the association constant (K_a) for adamantane and bases on ssDNA with β-CD is approximately 10^4–10^5 M^{-1} [17] and 3×10^3 M^{-1} [18], respectively, This indicates a much stronger binding capacity for adamantane with CD. Adding ssDNA did not significantly affect the fluorescence intensities of metallocyclodextrins, which suggests that the encapsulation of CD cavities with adamantane restrained the host–guest recognition between bases and CDs. In the presence of adamantane the binding between metallocyclodextrins and ssDNA was reduced, leading to the disappearance of the fluorescence enhancement induced by the addition of ssDNA. In addition, there was no change in emission spectra when adamantane was added into the solution of $Ru(bpy)_3^{2+}$ and ssDNA (Fig. S3C, curve c, ESI†) compared with the mixed solution of $Ru(bpy)_3^{2+}$ and ssDNA without adamantane (Fig. S2C, curve d, ESI†). This suggests that adamantane does not affect the electrostatic binding between ssDNA and $Ru(bpy)_3^{2+}$ without the existence of CD, further demonstrating that the CD cavities play a vital role in the binding of ssDNA and metallocyclodextrins. These results verify the host–guest effect between the base of ssDNA and CD in the binding of ssDNA and metallocyclodextrins.

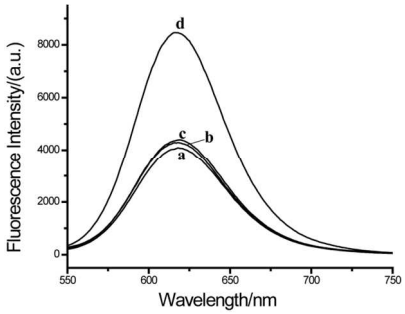


Fig. 2 Fluorescence spectra of (a) 1 μM 6CD-Ru, (b) 1 μM 6CD-Ru with 6 μM adamantane, (c) 1 μM 6CD-Ru with 6 μM adamantane and 1 μM ssDNA, (d) 1 μM 6CD-Ru with 1 μM ssDNA.

Because the fluorescence enhancement of metallocyclodextrin can be induced by ssDNA, we designed a sensitive, label-free sensing system for the detection of proteins as mentioned above. Lysozyme, an important protein widely studied in clinical research,[19] was taken as the model target to verify the concept. A fluorescence-based strategy, shown in Scheme 1, was developed to detect lysozyme. Fig. S4 (ESI†) demonstrates that 6CD-Ru was selected as the probe molecule based on its outstanding photophysical property and binding capacity, confirming the validity of this strategy. Compared with the original emission signal of 0.5 μM 6CD-Ru (curve a), an enhanced signal was obtained in the presence of 0.5 μM ssDNA (lysozyme-aptamer in this case) (curve c), which corresponds to the system described in Scheme 1a. After the addition of 50 nM lysozyme, a significant decrease in the fluorescent intensity (curve b) was observed. This decrease resulted from the formation of an aptamer/lysozyme complex with a lower dissociation constant (K_d=65 nM)[20] than that of aptamer/CD (K_d=3.3×10^5 nM),[18] as shown in Scheme 1b. As a comparison, lysozyme was added into free 6CD-Ru (curve d) and no increase or decrease was observed, which demonstrates that lysozyme does not impact the 6CD-Ru. This demonstrates that the simple and distinctive aptasening is able to accurately detect lysozyme.

When different amounts of lysozyme were added into the detection system comprising the lysozyme-aptamer and 6CD-Ru, the fluorescence intensity declined with the increase of lysozyme concentrations (Fig. 3A). The difference in the fluorescence signal exhibited a logarithmic correlation to the lysozyme concentrations in the range of 0.1 nM – 0.5 μM (Fig. 3B, curve a). The corresponding linear regression equation is ΔI = 305.684×lgC + 302.110 (correlation coefficient R=0.9829), where ΔI is the difference of fluorescence intensity in the absence and presence of lysozyme and C is the concentration of lysozyme. The detection limit is as low as 48 pM (S/N=3), demonstrating that this approach possesses high sensitivity without requiring subsequent signal labeling or amplification procedures and is also more sensitive than many other label-free lysozyme detection methods that have been proposed previously.[21]

The other metallocyclodextrins, CD-Ru and 3CD-Ru, were also tested as a signal probe molecule in the aptamer-based fluorescence detection of lysozyme. The calibration curves of the decreased fluorescence intensities with the logarithm of the lysozyme concentrations are presented in Fig. 3B. Detection limits of 27 nM and 239 nM were achieved for 3CD-Ru (curve b) and CD-Ru

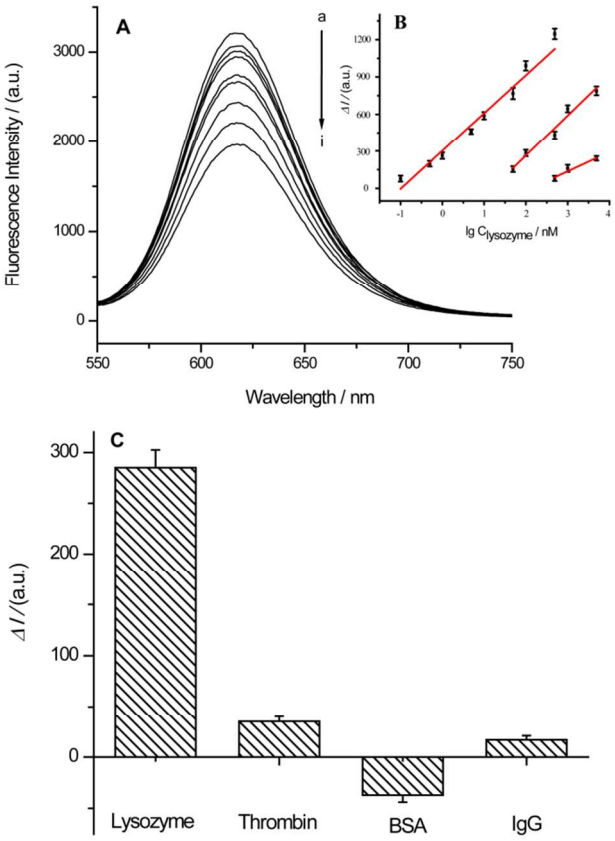


Fig. 3 (A) Changes in the emission spectra upon the addition of lysozyme to a mixture of 6CD-Ru (0.5 μM) and the lysozyme-aptamer (0.5 μM) at: a) 0 M, b) 0.1 nM, c) 0.5 nM, d) 1 nM, e) 5 nM, f) 10 nM, g) 50 nM, h) 0.1 μM and i) 0.5 μM. (B) The resulting calibration curves for the absolute difference of the fluorescence intensity as a function of the logarithm of the lysozyme concentrations (a) from 0.5 μM to 0.1 nM for 0.5 μM 6CD-Ru ; (b) from 5 μM to 50 nM for 5 μM 3CD-Ru; (c) from 5 μM to 500 nM for 5 μM CD-Ru. ($\Delta I = I_2 - I_1$, where I_2 and I_1 were the fluorescence intensity in the absence and presence of lysozyme, respectively.) The error bars shown were derived from the standard deviation of three repeated experiments. (C) Responses of the fluorescence detecting system containing 6CD-Ru (0.5 μM) and the anti-lysozyme aptamer (0.5 μM) with the addition of lysozyme (1 nM), thrombin (100 nM), BSA (100 nM) and IgG (100 nM), respectively. The error bars shown were derived from the standard deviation of three repeated experiments.

(curve c), respectively. The fluorescence detection using 6CD-Ru, with a detection limit of 48 pM, was the most sensitive to lysozyme.

To investigate the selectivity of the fluorescence strategy, some typical proteins, such as thrombin, bovine serum albumin (BSA) and human immunoglobulin G (IgG), were selected to serve as interference targets. The results clearly showed that this method is highly selective for lysozyme (Fig. 3C), even when the concentration of the interference proteins was 100 times that of lysozyme. Thus, high selectivity was proven to be another advantage of this detection system.

In conclusion, the newly synthesized series of metallocyclodextrins comprising CD-Ru, 3CD-Ru and

6CD-Ru were utilized for the detection of lysozyme. Combining a ruthenium core with multiple β-CD units, these compounds exhibited excellent photoactive characteristics. Because the host-guest recognition enables the fluorescence changes to the ruthenium core, a novel strategy for lysozyme detection has been proposed. The fluorescence was effectively enhanced following addition of lysozyme-aptamer to the CD cavities. When compared with the original fluorescence intensity, intensity reductions in the presence of lysozyme were proportional to the logarithm of the lysozyme concentrations. This label-free method does not require any complicated or subsequent signal labeling or amplification procedures, and has been demonstrated to be sensitive and selective for lysozyme detection. This method could be applied to various targets by using the appropriate aptamer sequences for a broad range of analytes.

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Notes and references

1 L. Szente and L. Szeman, *Anal. Chem.* 2013, **85**, 8024-8030.

2 [a] M. R. Eftink, M. L. Andy, K. Bystrom, H. D. Perlmutter and D. S. Kristol, *J. Am. Chem. Soc.,*1989, **111**, 6765-6772; [b] P. Falvey, C. W. Lim, R. Darcy, T. Revermann, U. Karst, M. Giesbers, A. T. M. Marcelis, A. Lazar, A. W. Coleman and D. N. Reinhoudt, *Chem. Eur. J.* 2005, **11**, 1171-1180; [c] M. V. Rekharsky and Y. Inoue, *Chem. Rev.,* 1998, **98**, 1875-1917.

3 [a] S. Li and W. C. Purdy, *Chem. Rev.* 1992, **92**, 1457-1470; [b] S. A. Nepogodiev and J. F. Stoddart, *Chem. Rev.* 1998, **98,** 1959-1976.

4 [a] J. M. Haider, M. Chavarot, S. Weidner, I. Sadler, R. M. Williams, L. D. Cola and Z. Pikramenou, *Inorg. Chem.* 2001, **40**, 3912-3921; [b] T. Ogoshi and A. Harada, *Sensors*, 2008, **8**, 4961-4982; [c] S. Menuel, Y. Corvis, E. Rogalska and A. Marsura, *New J. Chem.* 2009, **33**, 554-560.

5 [a] J. M. Haider and Z. Pikramenou, *Chem. Soc. Rev.* 2005, **34**, 120-132; [b] H. Takakusa, K. Kikuchi, Y. Urano, T. Higuchi and T. Nagano, *Anal. Chem.* 2001, **73**, 939-942.

6 [a] A. Ueno, A. Ikeda, H. Ikeda, T. Ikeda and F. Toda, *J. Org. Chem.,* 1999, **64**, 382-387; [b] J. M. Haider, M. Chavarot, S. Weidner, I. Sadler, R. M. Williams, L. D. Cola and Z. Pikramenou, *Inorg. Chem.,* 2001, **40**, 3912-3921; [c]M. J. J. Pereira Silva, J. M. Haider, R. Heck, M. Chavarot, A. Marsura and Z. Pikramenou, *Supramol. Chem.,* 2003. **15**, 563-571.

7 T. Ogoshi and A. Haider, *Sensors*, 2008, **8**, 4961-4982.

8 [a] A. D. Ellington and J. W. Szostak, *Nature,* 1990, **346,** 818-822; [b] C. Tuerk, and L. Gold*, Science*, 1990, **249,** 505-510; [c] J. Wang, Y. Cao, G. Chen and G, Li, *Chem. Bio. Chem,* 2009, **10**, 2171-2176; [d] Y. Cao, D. Chen, W. Chen, J. Yu, Z. Chen and G. Li, *Anal. Chim. Acta,* 2014, **812**, 45-49.

9 X. Zhu, B. Zhang, Z. Ye, H. Shi, Y, Shen and G. Li, *Chem. Comm.* 2015, **51**, 640-643.

10 [a] Y. T. Qi, X. H. Wang, H. Chen, J. Tang, F. Yang and P. G. He, Supramolecular Chemistry, 2015, **27**, 44-51; [b] Y. T. Qi, X. H. Wang, H. Chen, J. Tang, F. Yang and P. G. He, *Inorg. Chem. Commun.,* 2014, **40,** 11-14.

11 [a] Q. Chen, H. Chen, Y. Y. Zhao, F. Zhang, F. Yang, J. Tang and P. G. He, *Biosens. Bioelectron.* 2014, **54**, 547-552; [b] H. Chen, Q. Chen, Y. Y. Zhao, F. Zhang, F. Yang, J. Tang and P. G. He, *Talanta.* 2014. **121,** 229-233; [c] Y. Y. Zhao, H. Chen, Q. Chen, Y. T. Qi, F. Yang, J. Tang, P. G. He and F. Zhang, *Chin. J. Chem.* 2014. **32,** 1161-1168; [d] H. Chen, X. H. Wang, Y. T. Qi, S. S. Zheng, Q. Chen, P. G. He, F. Zhang, F. Yang, J. Tang and Y. Z. Fang, *Chem. Plus. Chem.* 2013, **78**, 780-784.

12 H. F. M. Nelissen, A. F. J. Schut, F. Venema, M. C. Feiters and R. J. M. Nolte, *Chem. Commun*., 2000, 577-578.

13 [a] A. Abbaspour and A. Noori, *Analyst,* 2012, **137**, 1680-1685; [b] X. Hao, C. Liang and C. Jian-Bin, *Analyst,* 2002, **127**, 834-837;

14 [a] V. A. Szalai and H. H. Thorp, *J. Phys. Chem. B,* 2000, **104**, 6851-6859; [b] T. W. Welch and H. H. Thorp, *J. Phys. Chem*. 1996, **100,** 13829-13836.

15 A. K. Vrkic, R. A. J. O'Hair and B. C. Lebrilla, *Eur. J. Mass Spectrom.* 2003, **9**, 563-577.

16 E. Blomberg, A. Kumpulainen, C. David and C. Amiel, *Langmuir,* 2004, **20**, 10449-10454.

17 D. Granadero, J. Bordello, M. J. Perez-Alvite, M, Novo and W. Al-Sourfi, *Int. J. Mol. Sci.* 2010, **11**, 173-188.

18 A. Abbaspour and A. Noori, *Analyst,* 2008, **133**, 1664-1672;

19 [a] S. S. Levinson, R. J. Elin and L. Yam, *Clin. Chem.,* 2002, **48**, 1131-1132; [b] J. F. Harrison, G. S. Lunt, P. Scott and J. D. Blainey, *Lancet,* 1968, **1**, 371-375; [c] M. Klockars, S. Reitamo, T. Weber and Y. Kerttula, *Acta Med. Scand*. 1978, **203**, 71-74.

20 J. C. Cox and A. D. Ellington, *Bioorg Med Chem.* 2001, **9**, 2525-2531.

21 [a] X. X. Han, G. G. Huang, B. Zhao and Y. Ozaki, *Anal. Chem.,* 2009, **81**, 3329-3333; [b] C. Y. Deng, J. H. Chen, L. H. Nie, Z. Nie and S. Z. Yao, *Anal. Chem*. 2009, **81**, 9972-9978.

