The convenient fluorescence turn-on detection of heparin with a silole derivative featuring an ammonium group†

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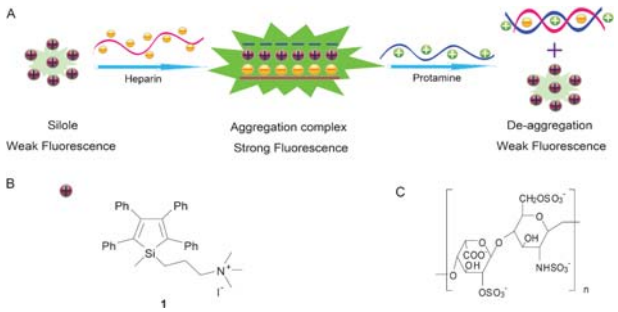
A new silole derivative with an ammonium group was designed and studied with a view to developing a convenient fluorescence turn-on assay for heparin by taking advantage of the aggregation-induced enhanced emission (AIE) feature of silole (silacyclopentadiene) molecules.

Heparin, known as the most negatively charged biological macromolecule,[1] is a highly sulfated linear glycoaminoglycan (GAG) consisting of repeating units of 1→4-linked pyranosyluronic acid and 2-amino-2-deoxyglucopyranose residues. It is crucial to monitor and control the level and activity of heparin during and after surgery, and manipulate the amount of heparin for anticoagulant therapy in order to avoid complications such as hemorrhage or thrombocytopenia[2] induced by heparin-overdose. Traditional methods for heparin assay include activated clotting time (ACT), activated partial thromboplastin time (aPTT), potentiometric assays and protamine complexation; however, these methods are usually inaccurate and expensive.[1,2] Optical sensors that give colorimetric or fluorescence responses with heparin binding have been developed recently by using a tripodal boronic acid,[3] heparin-specific peptide,[4a] fluorescent copolymers with binding sites tailored for sulfated sugars[4b] or polycationic calix[8]arenes,[4c] benzimidazolium dyes,[4d] and polymethinium salts[4e] based on electrostatic interactions. But, sensitive fluorescence turn-on sensors for heparin, which can be easily accessible and can be used for direct monitoring of heparin in serum, still remain rare. Herein we report a convenient fluorescence turn-on sensor for heparin detection by making use of the unusual aggregation-induced enhanced emission (AIE) feature of silole (silacyclopentadiene) molecules.[5]

The design rationale is illustrated in Scheme 1. Silole derivatives are weakly fluorescent in solution but become highly fluorescent after aggregation. This intriguing phenomenon was referred to as aggregation-induced enhanced emission (AIE) first reported by Tang, Zhu *et al.* in 2001.[5,6] With a view to developing fluorescence turn-on detection of heparin, a new silole derivative featuring an ammonium group (compound **1**, Scheme 1B) was designed to bind heparin through an ion pairing mechanism.[7] Compound **1** is expected to show rather weak fluorescence in aqueous solution, however, in the presence of heparin, the ammonium group in **1** would interact with the negative sulfate and carboxylate groups in heparin through electrostatic interactions; as a result, aggregation of **1** would occur[8] and fluorescence of the ensemble would increase. Therefore, compound **1** can be employed for the fluorescence turn-on detection of heparin. It is known that heparin can specifically bind with proteins such as protamines and chemokines;[1b] thus, when protamine was added to the ensemble of **1** and heparin, competing interactions of heparin with protamine would reduce the aggregation of compound **1**, and as result the fluorescence of **1** decreased. Thus, compound **1** may also be used for studies of the interactions of heparin and heparin-binding proteins.



Scheme 1 (A) Illustration of the fluorescence turn-on sensor for heparin based on the AIE feature of silole and its application to probe interactions between heparin and protamine; (B) chemical structure of the silole derivative with an ammonium group (compound **1**); (C) chemical structure of the major unit of heparin.

Compound **1** was prepared by the reaction of 1-methyl-1-(3-amino)propyl-2,3,4,5-tetraphenyl-silole with excess amount of MeI.[9] As anticipated, the aqueous buffer solution of **1** [5.0×10^{-5} M in HEPES buffer solution (5.0 mM, pH = 7.4)] showed rather weak fluorescence, However, after mixing with heparin stock solution, significant fluorescence enhancement was observed for **1** as shown in Fig. 1. The fluorescence intensity of **1** at 480 nm increased 92 fold when the concentration of heparin in the solution reached 13 μM.[10] Moreover, the emission maximum was red-shifted from 480 nm to 488 nm. The fluorescence enhancement of **1** upon mixing with heparin can be easily distinguished with the naked eye under UV illumination as shown in the inset of Fig. 1. The fluorescence intensity of **1** increased with the concentration of heparin as shown in Fig. 2, where a linear plot ($I_{480} = 129.5 + 224.6 \times$ [heparin], $n = 12$, $r = 0.994$) of the fluorescence intensity of **1** at 480 nm *vs.* the concentration of heparin was displayed. The

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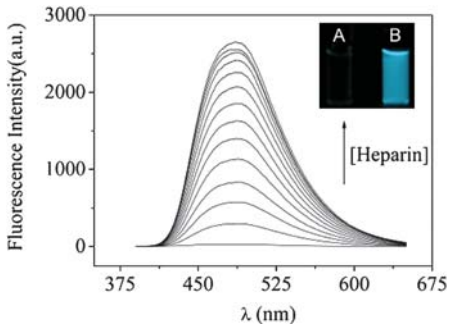

Fig. 1 Fluorescence spectra ($\lambda_{ex.}$ = 370 nm) of compound **1** [5 × 10^{-5} M in HEPES buffer solution (5.0 mM), pH = 7.4] in the presence of different amounts of heparin (from 0 to 13 μM); inset shows the photos of the solution of **1** (5.0 × 10^{-5} M in HEPES buffer solution, pH = 7.4) in the absence (A) and presence (B, containing 7 μM of heparin) under UV (365 nm) illumination.

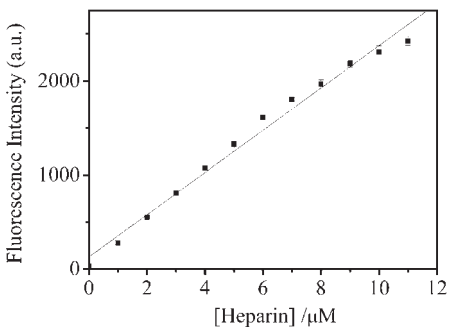

Fig. 2 Variation of the fluorescence intensity of **1** [5.0 × 10^{-5} M in HEPES buffer solution (5.0 mM), pH = 7.4] at 480 nm *vs.* the concentration of heparin; $\lambda_{ex.}$ = 370 nm.

detection limit for heparin with this fluorescence turn-on assay can reach 23 nM under this condition.

The fluorescence enhancement of **1** in the presence of heparin should be due to the aggregation of **1** induced by the electrostatic interactions between the ammonium group in **1** and sulfate and carboxylate groups in heparin. The absorption spectral variation of **1** after interaction with heparin also indicates the aggregation of **1**. As shown in Fig. S1 (in the ESI†), the absorption maximum of **1** was red-shifted upon mixing with heparin. For instance, the absorption maximum of **1** ascribed to the π–π transition of the silole moiety was shifted from 356 nm in the absence of heparin to 365 nm in the presence of 13 μM of heparin. According to a previous report,[6b] such an absorption spectral change is caused by the aggregation of silole molecules.

The fluorescence spectra of **1** in the presence of other two common less sulfated glycosaminoglycans: chondroitin sulfate (ChS) and hyaluronic acid (HA), as well as a simple neutral polyhydroxy sugar—dextran (Dex)—were measured to probe the effect of negative charge on aggregation of **1** and the selectivity of our fluorescence turn-on assay method. Fig. 3 shows the plot of the fluorescence intensity of **1** at 480 nm *vs.* concentrations of ChS, HA, Dex and heparin. Obviously, the fluorescence enhancement observed for **1** decreased in the following order under the same conditions: heparin > ChS > HA > Dex. The most intense fluorescence enhancement

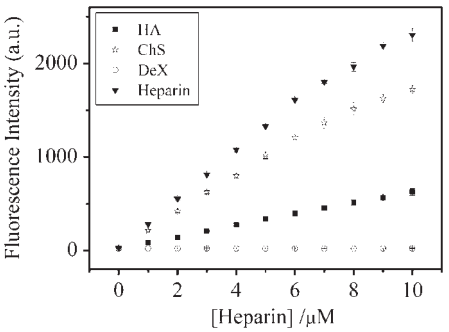

Fig. 3 Variation of the fluorescence intensity of **1** [5.0 × 10^{-5} M in HEPES buffer solution (5.0 mM), pH = 7.4] at 480 nm *vs.* concentrations of ChS, HA, DeX and heparin.

was observed for **1** in the presence of heparin, while the fluorescence enhancement of **1** with Dex could be neglected. These results are accountable by comparing the number of negative charges per molecule: heparin has more negative charge sites compared to ChS, HA for binding with **1**, and Dex has no anions. These results demonstrate that compound **1** can be used for the fluorescence turn-on detection of heparin selectively.

In order to demonstrate the practical usefulness of this fluorescence turn-on assay for heparin, we tried to detect the amount of heparin in serum using compound **1**. For convenience horse serum was used for the study. Horse serum contains proteins such as albumin, globulin and fibronectin and various anions including carboxylate ions. The fluorescence intensity of **1** was enhanced in matrix serum even without the addition of heparin. This is probably due to noncovalent interactions such as electrostatic attractions (especially for negatively-charged proteins) and the hydrophobic effect (particularly for the proteins with hydrophobic pockets), leading to the restriction of intramolecular rotations and thus fluorescence enhancement.[6d,g] Addition of heparin to the buffer solution of **1** containing serum led to further fluorescence enhancement. After subtracting the background intensity due to the presence of serum, the fluorescence intensity of **1** at 480 nm increased linearly with the concentration of heparin present in the serum as shown in Fig. 4 (I_{480} = −57.7 + 195.9 × [heparin], n = 12, r = 0.998). It is known that the concentration of heparin is in the range 0.8–3.2 μM

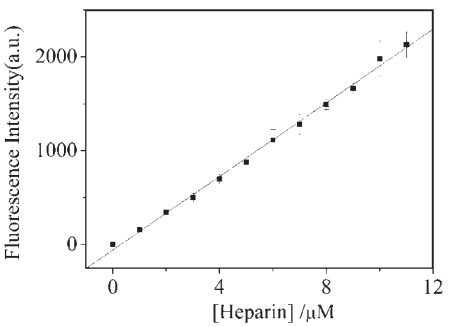

Fig. 4 Variation of the fluorescence intensity of **1** [5.0 × 10^{-5} M in HEPES buffer solution (5.0 mM), pH = 7.4 containing 200 μL of diluted serum] at 480 nm after subtracting the background intensity *vs.* the concentration of heparin.

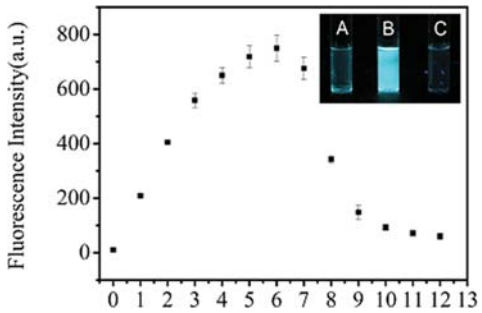

Fig. 5 Variation of the fluorescence intensity of **1** [1.25×10^{-5} M in HEPES buffer solution (5.0 mM), pH = 7.4] after addition of heparin (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 μM from points 1 to 7), followed by addition of protamine (0.9×10^{-3}, 1.8×10^{-3}, 2.7×10^{-3}, 3.6×10^{-3}, 4.5×10^{-3}, 5.2×10^{-3} mg mL^{-1} from points 8 to 13). Inset: photos of the solution of **1** [1.25×10^{-5} M in HEPES buffer solution (5.0 mM), pH = 7.4] under UV (365 nm) illumination: (A) in the absence of heparin and protamine; (B) in the presence of heparin (4.0 μM); (C) in the presence of heparin (4.0 μM) and protamine (2.8×10^{-2} mg mL^{-1}).

during surgery or clinical anticoagulant drug therapy;[2,3] therefore, compound **1** is a rather promising fluorescence probe for the detection of heparin in biological systems. In this way, a sample of serum free of heparin is needed for the quantitative detection of heparin in serum, since the background fluorescence intensity has to be measured. But, the background signal intensity can be largely reduced by performing the detection in a citric acid buffer solution with pH = 3.6, and as a result the detection of heparin in serum can be carried out even without the heparin free sample of the serum (for details see ESI†).

We further asked whether heparin-binding proteins will affect the fluorescence of compound **1**. Protamine is a positively charged peptide showing high binding affinity for heparin and it can be used to sequester heparin and release antithrombin III.[1b] It is expected that the fluorescence of the ensemble of **1** and heparin would be reduced after the addition of protamine, since protamine can strongly bind with heparin to weaken the interaction between compound **1** and heparin leading to de-aggregation of the ensemble of **1** and heparin and then fluorescence reduction (see Scheme 1). This is indeed in agreement with experimental findings as shown in Fig. 5. The fluorescence intensity of **1** [1.25×10^{-5} M in HEPES buffer solution (5.0 mM), pH = 7.4] at 480 nm increased firstly with the addition of heparin (3.0 μM); but, the fluorescence intensity started to decrease after addition of protamine to the ensemble of **1** and heparin. Similarly, the absorption maximum was also shifted from 362 nm in the presence of heparin (3.0 μM) to 353 nm in the presence of both heparin and protamine (Fig. S2 in ESI†). These spectral results indicate that molecules of **1** were almost released from the aggregation complex of **1** and heparin because of the stronger binding of protamine with heparin. The fluorescence intensity variation of the solution of **1**, after addition of heparin and further addition of protamine, can also be distinguished with the naked eye under UV illumination as shown in the inset of Fig. 5. Therefore, compound **1** could be a potentially useful fluorescent probe for investigation of the interactions of heparin and certain proteins.[1b]

In summary, we have developed a convenient fluorescence turn-on sensor for heparin with an easily accessible silole derivative (compound **1**) by taking advantage of the aggregation-induced enhanced emission (AIE) phenomenon. The detection limit for heparin with compound **1** can reach 23 nM in HEPES buffer solution. Moreover, this fluorescence turn-on sensor can be used to distinguish heparin from its analogues (ChS, HA and Dex). In addition, compound **1** has been successfully applied to heparin quantification in serum. Compound **1** could also be employed as a fluorescent probe for studies of the binding of heparin with protamine.

Notes and references

1 (*a*) D. L. Rabenstein, *Nat. Prod. Rep.*, 2002, **19**, 312–331; (*b*) I. Caplia and R. J. Linhardt, *Angew. Chem., Int. Ed.*, 2002, **41**, 390–412; (*c*) R. J. Linhardt and T. Toida, *Acc. Chem. Res.*, 2004, **37**, 431–438; (*d*) J. M. Whitelock and R. V. Iozzo, *Chem. Rev.*, 2005, **105**, 2745–2764.

2 (*a*) J. Hirsh, J. E. Dalen, D. Deykin and L. Poll, *Chest*, 1992, **102**, 337S–352S; (*b*) B. Girolami and A. Girolami, *Semin. Thromb. Hemostasis*, 2006, **32**, 803–809.

3 (*a*) A. T. Wright, Z. L. Zhong and E. V. Anslyn, *Angew. Chem., Int. Ed.*, 2005, **44**, 5679–5682; (*b*) Z. L. Zhong and E. V. Anslyn, *J. Am. Chem. Soc.*, 2002, **124**, 9014–9015.

4 (*a*) J. C. Sauceda, R. M. Duke and M. Nitz, *ChemBioChem*, 2007, **8**, 391–394; (*b*) W. Sun, H. Bandmann and T. Schrader, *Chem.–Eur. J.*, 2007, **13**, 7701–7707; (*c*) T. Mecca, G. M. L. Consoli, C. Geraci, R. L. Spina and F. Cunsolo, *Org. Biomol. Chem.*, 2006, **4**, 3763–3768; (*d*) S. L. Wang and Y. T. Chang, *Chem. Commun.*, 2008, 1173–1175; (*e*) T. Bříza, Z. Kejík, I. Císařová, J. Králová, P. Martásek and V. Král, *Chem. Commun.*, 2008, 1901–1903.

5 (*a*) J. Luo, Z. Xie, J. W. Y. Lam, L. Cheng, H. Chen, C. Qiu, H. S. Kwok, X. Zhan, Y. Liu, D. Zhu and B. Z. Tang, *Chem. Commun.*, 2001, 1740–1741; (*b*) J. Chen, C. W. Law, J. W. Y. Lam, Y. Dong, S. M. F. Lo, I. D. Williams, D. Zhu and B. Z. Tang, *Chem. Mater.*, 2003, **15**, 1535–1546.

6 By making use of the unique feature of silole derivatives, new chemical and biological sensors have been described: (*a*) Z. Li, Y. Dong, Y. Tang, M. Haüßler, H. Tong, Y. Dong, J. W. Y. Lam, K. S. Wong, P. Gao, I. D. Williams, H. S. Kwok and B. Z. Tang, *J. Phys. Chem. B*, 2005, **109**, 1006–10066; (*b*) S. J. Toal, K. A. Jones, D. Magde and W. C. Trogler, *J. Am. Chem. Soc.*, 2005, **127**, 11661–11665; (*c*) S. J. Toal, D. Magde and W. C. Trogler, *Chem. Commun.*, 2005, 5465–5467; (*d*) H. Tong, Y. Hong, Y. Dong, M. Häußler, J. W. Y. Lam, Z. Li, Z. Guo, Z. Guo and B. Z. Tang, *Chem. Commun.*, 2006, 3705–3707; (*e*) Y. Dong, J. W. Y. Lam, A. Qin, Z. Li, J. Sun, Y. Dong and B. Z. Tang, *Chem. Phys. Lett.*, 2007, **446**, 124–127; (*f*) Z. Ning, Z. Chen, Q. Zhang, Y. Yan, S. Qian, Y. Cao and H. Tian, *Adv. Funct. Mater.*, 2007, **17**, 3799–3808; (*g*) H. Tong, Y. Hong, Y. Dong, M. Häußler, Z. Li, J. W. Y. Lam, Y. Dong, H. H. Y. Sun, I. D. Williams and B. Z. Tang, *J. Phys. Chem. B*, 2007, **111**, 11817–11823.

7 R. H. Lorentsen, J. H. Graversen, N. R. Catter, C. Thøgersen and M. Etzerodt, *Biochem. J.*, 2000, **347**, 83–87.

8 P. Kaur, M. Wu, L. Anzaldi, D. H. Waldeck, C. Xue and H. Liu, *Langmuir*, 2007, **23**, 13203–13208.

9 Characterization data for compound **1**: δ_H (400 MHz; d^6-DMSO; Me_4Si) 6.81–7.16 (m, 20H, Ph), 3.21 (m, 2H), 2.93 (s, 9H), 1.64 (m, 2H), 0.93 (m, 2H), 0.50 (s, 3H); δ_C (100 MHz; d^6-DMSO; Me_4Si) 154.7, 139.9, 139.1, 138.2, 129.4, 128.4, 128.2, 127.5, 126.4, 125.8, 67.3, 52.1, 16.7, 9.1, −6.0; HR-MS(ESI) calcd. for $C_{35}H_{38}SiN^+$, 500.2768; found, 500.2786.

10 Since the heparin structure is complicated, a molecular weight of two repeating disaccharides = 644.2 g mol^{-1} was used as that of heparin for concentration calculations in this report.