RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Journal Name

RSCPublishing

COMMUNICATION

Host-guest assemble of adamantyl tethered squaraine in β-cyclodextrin for monitoring pH in living cells

Cite this: DOI: 10.1039/x0xx00000x

Wenjian Xu,[a,b] Xiaochan Zhu,[a,b] Guimei Wang,[a,b] Chuanguo Sun,[a,b] Qingfeng Zheng,[a,b] Huanghao Yang[b,*] and Nanyan Fu[a,b,*]

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

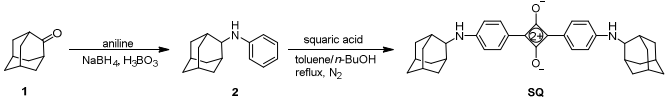
The formation of a 1:2 inclusion complex between adamantyl tethered squaraine and β-cyclodextrin inhibited the aggregation of SQ in aqueous solution, enhancing the fluorescent emission of the near-infrared dye. The resulting SQ⊂β-CD complex serves as a sensitive fluorescent probe for imaging intracellular pH in living cells.

Intracellular pH plays critical roles in enzymatic activity, ion transport, drug resistance, cell proliferation and apoptosis.[1] Abnormal pH values are associated with inappropriate physiological process and diseases such as cancer and Alzheimer's.[2] Monitoring pH changes inside living cells is important for studying cellular internalization pathways. Among the many methods used for measurement of pH, fluorescence based strategies have become the most attractive approach for pH detection in living cells because of their intrinsic selectivity and sensitivity, operational simplicity, and low cost, and are non-invasive, spatial and temporal observation techniques. Although a large number of fluorescent sensors for pH have been reported in the literature,[3] only a few of them worked in near-infrared band. [4] Because NIR radiation can penetrate deeply in tissues, minimizes damage to biological samples, and decreases the influence of background effects caused by autofluorescence from the biological samples in the tests, the probes that emit in the red and near-infrared region have attracted considerable attentions in recent decades, and there is however still a great need to develop high performance NIR fluorescent probes for intracellular pH detection.

Squaraines are a class of interesting organic functional dyes, which possess strong and intense absorption in the red to near-infrared regions which makes them suitable for fluorescent probes and biolabelling. Fluorescent squaraine has been widely used in metal ion sensing,[5] NIR fluorescent labelling[6] and detection of thiol-containing amino acids.[7] Some pH probes based on squaraine have been reported,[8] but none of them have been used in intracellular pH detection due to the stability and aggregation problems. The central electron deficient cyclobutenedione ring (C4O2) acts as an electron acceptor easily attacked by nucleophiles,[9] and the planar D-A-D configuration of squaraines have a strong tendency to aggregate. This causes fluorescence quenching,[10] which limits their practical application especially in aqueous solution. Therefore, the performance improvement of the squaraine dyes by structural modification,[11] surfactants,[12] nano materials[8] and molecules container[13] attracts significant attention.

β-Cyclodextrin (β-CD) is water-soluble, cyclic oligosaccharides containing seven α-1, 4-linked D-glucopyranose units and has a cavity with pore sizes ranging from 6.0 to 7.0 Å. One of most interesting properties of β-CD is that the internal cavity is hydrophobic and can accommodate suitably sized hydrophobic molecules.[14] Adamantane (AD) is a pseudo-spherical function-free hydrocarbon with a diameter approximately of 6.5 Å. It is well known that the solubility of AD in water is significantly enhanced when the AD molecules are included in the hydrophobic cavities of the β-CD molecules.[15] Supramolecular assembles formed by β-CD and AD complex are widely investigated to improve the water solubility of guest fluorophores for use for specific detection.[16] In this work, we report the synthesis of a novel adamantyl substituted squaraine **SQ** and the investigation of the interaction between **SQ** and β-CD. The interaction between **SQ** and β-CD forming a 1:2 supramolecular assembly can inhibit the aggregation of the squaraine in aqueous solution. The resulting **SQ⊂**β-CD inclusion complex can be used as pH probe in fluorescence imaging of living cells.

The adamantyl substituted squaraine dye **SQ** was synthesized through two steps from the commercially available 2-adamantanone (**1**) (Scheme 1). The synthetic sequence was initiated with a direct reductive amination of ketone **1** by solid-activated sodium borohydride under solvent-free conditions[17] to afford N-(2-adamantyl)aniline (**2**) with a yield of 62%. The steric hindrance of the adamantyl group decreased the nucleophilicity of aniline **2**, thus avoiding nucleophilic addition of **2** to the electron deficient central four membered ring of squaric acid, and the condensation between **2** and squaric acid in the mixture of toluene/*n*-butanol under reflux occurred smoothly, affording a novel squaraine dye **SQ** in 35% yield. To the best of our knowledge, the formation of squaraine dyes containing secondary amine moieties is relative rare.



Scheme 1. Synthesis of **SQ**.

The photophysical properties of **SQ** in different solvents are summarized in Table 1. **SQ** showed intense absorption at 614-627 nm and strong emission in 625-643 nm. The absorption maximum of **SQ** is red-shifted in polar solvents, indicating a positive solvatochromism.

Table 1. The photophysical properties of **SQ**.

Solvent	λ_{max} (nm)	λ_{em} (nm)	Stokes Shift (nm)	ε ($\times 10^5$ $M^{-1}cm^{-1}$)	φ[a]
THF	620	634	14	2.25	0.86
$CHCl_3$	615	630	15	2.30	0.78
CH_3CN	621	641	20	1.85	0.12
EtOH	627	645	18	1.95	0.26
CH_3OH	627	645	18	1.80	0.10

[a] With reference to ZnPc in DMF ($\varphi_F = 0.28$).

Squaraines have a high tendency to form aggregates in aqueous solution due to their planar structure and D-A-D interaction between the molecules. The aggregates formed not only lead the maximum absorption shift, but also lead to dramatic fluorescence emission quenching. In ethanol, **SQ** exhibits a sharp absorption peak at 627 nm, which can be attributed to the squaraine monomer. As the percentage of water in ethanol increases to 60% slight absorbance decreases and red-shifts are observed. The N-H bond improves the water solubility of **SQ** by forming the intermolecular hydrogen bonds with water. Further increasing the water ratio from 60% to 70% leads to a dramatic decline the absorption. The absorbance decreases further as the percentage of water increases to 80% and the absorption band broadens, indicating aggregation of **SQ** (Fig. S9, ESI†).

The rigid structure of adamantyl conforms to the cavity of β-CD, so that β-CD can be added to further improve the solubility of **SQ** and prohibit the aggregation in aqueous solution (for DLS evidence see Fig. S10, ESI†). In the absence of β-CD **SQ** shows a broad absorption band in water which is attributed to the aggregate of **SQ**. Upon addition of β-CD, a sharp peak of **SQ** appears at 628 nm, indicating that the central chromophore is exposed in a polar environment and that the binding site of β-CD is at the adamantyl side arm of **SQ**. The absorbance increases with increasing concentration of β-CD, and is saturated when the concentration of β-CD reaches 3.0 mM (Fig. S11, ESI†). Further investigation shows that the fluorescence intensity of **SQ** is dramatically enhanced upon addition of β-CD. The binding interaction between **SQ** and β-CD in water was analyzed by the Benesi-Hildebrand equation, showing a 1:2 complex formation between **SQ** and β-CD, with a calculated equilibrium constant of 4.7×10^6 $L^2 \bullet mol^{-2}$ (Fig. 1). The complex formation was further confirmed by ESI-MS (Fig. S12, ESI†).

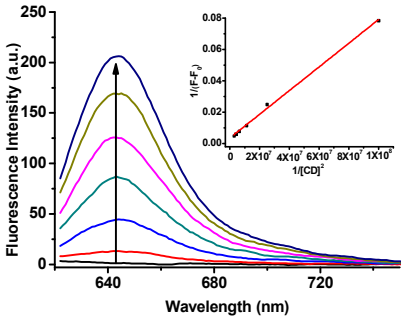


Figure 1. Fluorescent spectroscopy of **SQ** (2.0 μM) in pure water with difference concentrations of β-CD (0-0.6 mM). Insert: Benesi-Hildebrand plots for complexation between β-CD and **SQ** in pure water. (λ_{ex}=610 nm, λ_{em}=643 nm, slit: 5 nm/5 nm, PMT Volts: 650).

Trifloroacetic acid titrations of **SQ** in MeCN showed that protonation occurred at the oxygen atoms of the central ring first, leading to a bathochromic shift of absorption (Fig. S13, ESI†). At a high excess of acid, protonation of the nitrogen atom of the anilino side chain occurs, resulting in a new broad hypochromic band between 500 and 550 nm, formed with a loss of fluorescence (Fig. S14, ESI†). ^1H NMR experiment with D_2SO_4 confirmed the protonation of the anilino N (Fig. S15, ESI†). However, β-CD provided protection for **SQ** from the protonation of the anilino N and helped to keep high fluorescence of **SQ** in strong acidic condition.[18] Due to the electron deficient central ring, an adduct between OH and **SQ**⊂β-CD formed in basic condition and the conjugated chromophore was broken, leading to fluorescence quenching. This process can be confirmed by ESI-MS (Fig. S16, ESI†). pH-Dependent spectroscopic properties of **SQ**⊂β-CD further showed that only a slight red-shift occurred in both fluorescence and absorption as pH decreased from 5.0 to 2.0, and the fluorescence emission was kept "on" even in a strong acidic condition (pH 2.0). However, when the pH increased from 5.0 to 8.0, both emission and absorption intensity decreased dramatically and the maximum shifted to 643 nm and 628 nm, respectively. Upon increasing the pH from 8.0 to 10, no significant change in spectroscopic properties was observed. These results demonstrated that **SQ**⊂β-CD can serve as a fluorescent pH probe (Fig. 2 and Fig. S17, ESI†). After nonlinear data fitting of fluorescence spectra, the pKa value of **SQ**⊂β-CD is calculated as 6.33. The alternative addition of NaOH and HCl to **SQ**⊂β-CD showed highly reversible response to pH in both absorptive and fluorescent channels (Figs. S18 and S19, ESI†). The photobleaching experiments also confirmed a good photostability of **SQ** in soluton (Fig. S20, ESI†).

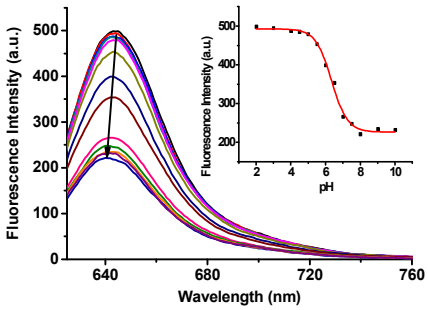


Figure 2. pH-Dependence of **SQ** fluorescence spectra (2.0 μM) in PB (20 mM) buffer solution with β-CD (2.0 mM). Insert: Fluorescence intensity changes in PB (20 mM) buffer solution. (λ_{ex}=610 nm, λ_{em}=643 nm ,slit: 5 nm/5 nm, PMT Volts: 650V.).

To test the practical application of **SQ**⊂β-CD further, competitive experiments were conducted to determine whether the existence of other metal ions or anions would interfere the probe's responses to pH. Either in the presence of K^+, Fe^{3+}, Ni^{2+}, Zn^{2+}, Na^+, Al^{3+}, Cu^{2+}, Mg^{3+}, Ca^{2+}, Ba^{2+}, Fe^{2+}, Li^+, Hg^{2+}, Pb^{2+} and Co^{2+} or of CO_3^{2-}, NO_2^-, HCO_3^-, S^{2-}, CH_3COO^-, ClO_4^-, I^-, SCN^-, Cl^-, SO_4^{2-}, and NO_3^- the fluorescence responses of **SQ**⊂β-CD in pH 5.0 PB buffer solution were examined. All cases showed very similar fluorescence spectra. These results implied that **SQ**⊂β-CD in aqueous media could respond to pH change exclusively in the presence of various metal ions or anions usually present in a complex system. The interference of these ions were also tested in pH 8.0, the fluorescence responses are the same between ions, which further confirmed the **SQ**⊂β-CD unique response toward pH (Fig. S21, ESI†).

The prominent spectral features of **SQ**⊂β-CD prompted us to test its potential applications for fluorescence imaging in living cells. The HeLa cells were incubated with 2.0 mM β-CD and 2.0 μM **SQ** for 20 min at 37 °C and then were washed three times with PBS buffer solution. Subsequently, two group cells were incubated in PBS medium at pH 4.5 and 8.0 with added nigericin (5 μg/mL^{-1}) that could rapidly equilibrate the intracellular and extracellular pH. The other group cells were incubated in culture medium. After 15 min, the cells were used for fluorescence imaging. As shown in Fig. 3, the cells incubated with **SQ**⊂β-CD in pH 4.5 PBS medium show bright red fluorescence emissions, while no fluorescence was observed in culture medium and pH 8.0 medium. This indicates **SQ**⊂β-CD is an excellent pH probe for living cells.

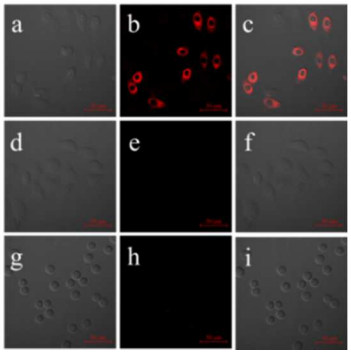


Figure 3. Fluorescence images of living HeLa cells. (a) Bright-field image of HeLa cells incubated with **SQ**⊂β-CD in PBS medium at pH 4.5; (b) Fluorescence image of (a); (c) the overlay image of (a) and (b); (d) Bright-field image of HeLa cells incubated with **SQ**⊂β-CD in culture medium; (e) Fluorescence image of (d); (f) the overlay image of (d) and (e); (g) Bright-field image of HeLa cells incubated with **SQ**⊂β-CD in PBS medium at pH 8.0; (h) Fluorescence image of (g); (i) the overlay image of (g) and (h).

In conclusion, a host-guest assemblage of adamantyl substituted squaraine in β-CD has been designed as an intracellular fluorescent pH probe. Encapsulation of β-CD improved the water solubility of squaraine, inhibited the aggregation of the dye, and protected it from protonation of the anilino side chain, leading to a high fluorescent emission in an acidic environment. It has been successfully applied for monitoring pH in living cells.

This work was financially supported by the National Science Foundation for Fostering Talents in Basic Research of National Natural Science Foundation of China (No. J1103303), the National Natural Science Foundation of China (No. 20702005), the Fujian Provincial Department of Science and Technology, P. R. China (No. 2013Y0062), Funding (Type A) from Fujian Education Department, P. R. China (No. JA12038, JA13043) and the Science and Technology Development Fund of Fuzhou University, P. R. China (No. 600902). We thank Fujian Institute of Research on the Structure of Matter, Chinese Academy of Sciences for the ESI-MS measurements.

Notes and references

[a] Research Institute of Photocatalysis, State Key Laboratory of Photocatalysis on Energy and Environment, Fuzhou University, Fuzhou 350002, P. R. China.

[b] Key Laboratory of Analysis and Detection for Food Safety, Ministry of Education & Fujian Provincial Key Laboratory of Analysis and Detection Technology for Food Safety, and Department of Chemistry, Fuzhou University, Fuzhou 350108, P. R. China. Tel: 86 13459108842; E-mail: nanyan_fu@fzu.edu.cn.

† Electronic Supplementary Information (ESI) available: Synthetic details along with spectroscopic data of **SQ** and **SQ**⊂β-CD. See DOI: 10.1039/c000000x/

1 M. Chesler, *Physiol. Rev.*, 2003, **83**, 1183; A. M. Paradiso, R. Y. Tsien, T. E. Machen, *Nature*, 1987, **325**, 447; I. Yuli, A. Oplatka, *Science*, 1987, **235**, 340.

2 H. Izumi, T. Torigoe, H. Ishiguchi, H. Uramoto, Y. Yoshida, M. Tanabe, T. Ise, T. Murakami, T. Yoshida, M. Nomoto, K. Kohno, *Cancer Treat. Rev.*, 2003, **29**, 541.

3 J. Han, K. Burgess, *Chem. Rev.*, 2010, **110**, 2709.

4 W. Liu, R. Sun, J.-F. Ge, Y.-J. Xu, Y. Xu, J.-M. Lu, I. Itoh, M. Ihara, *Anal. Chem.*, 2013, **85**, 7419; B. Tang, F. Yu, P. Li, L. Tong, X. Duan, T. Xie, X. Wang, *J. Am. Chem. Soc.*, 2009, **131**, 3016; L. Fan, Y.-J. Fu, Q.-L. Liu, D.-T. Lu, C. Dong, S.-M. Shuang, *Chem. Commun.*, 2012, **48**, 11202; Y. Ni, J. Wu, *Org. Biomol. Chem.*, 2014, **12**, 3774.

5 C. Chen, R. Wang, L. Guo, N. Fu, H. Dong, Y. Yuan, *Org. Lett.*, 2011, **13**, 1162; C. Chen, H. Dong, Y. Chen, L. Guo, Z. Wang, J.-J. Sun, N. Fu, *Org. Biomol. Chem.*, 2011, **9**, 8195; Y. Huang, Q. Lin, J. Wu, N. Fu, *Dyes Pigm.*, 2013, **99**, 699.

6 M. H. Sleimn, S. Ladame, *Chem. Commun.*, 2014, **50**, 5288; Y. Xu, Q. Liu, X. Li, C. Wesdemiotis, Y. Pang, *Chem. Commun.*, 2012, **48**, 11313.

7 Q. Lin, Y. Huang, J. Fan, R. Wang, N. Fu, *Talanta*, 2013, **14**, 66; J. Fan, Z. Wang, H. Zhu, N. Fu, *Sens. Actuators B: Chem.*, 2013, **188**, 886.

8 P. T. Snee, R. C. Somers, G. Nair, J. P. Zimmer, M. G. Bawendi, D. G. Nocera, *J. Am. Chem. Soc.*, 2006, **128**, 13320.

9 H. S. Hewage, E. V. Anslyn, *J. Am. Chem. Soc.*, 2009, **131**, 13099.

10 Y. Xu, Z. Li, A. Malkovskiy, S. Sun, Y. Pang, *J. Phys. Chem. B*, 2010, **114**, 8574.

11 Y. Zhang, B. Kim, S. Yao, M. V. Bondar, K. D. Belfield, *Langmuir* 2013, **29**, 11005.

12 Y. Xu, B. Li, L. Xiao, W. Li, C. Zhang, S. Sun, Y. Pang, *Chem. Commun.*, 2013, **49**, 7732; H. Zhu, Y. Lin, G. Wang, Y. Chen, X. Lin, N. Fu, *Sens. Actuators B: Chem.*, 2014, **198**, 201.

13 E. Arunkumar, C. C. Forbes, B. C. Noll, B. D. Smith, *J. Am. Chem. Soc.*, 2005, **127**, 3288.

14 Y.-C. Su, W.-C. Chen, F.-C. Chang, *Polymer*, 2005, **46**, 1617.

15 G. D. Enright, K. A. Udachin, J. A. Ripmeester, *CrystEngComm* 2010, **12**, 1450; J. Carrazana, A. Jover, F. Meijide, V. H. Soto, J. V. Tato, *J. Phys. Chem. B*, 2005, **109**, 9719.

16 Y. Chen, K.-Y. Han, Y. Liu, *Bioorg. Med. Chem.* 2007, **15**, 4537; G. Fang, M. Xu, F. Zeng, S. Wu, *Langmuir*, 2010, **26**, 17764; M. Xu, S. Wu, F. Zeng, C. Yu, *Langmuir*, 2010, **26**, 4529; K. T. Arun, D. T. Jayaram, R. R. Avirah, D. Ramaiah, *J. Phys. Chem. B*, 2011, **115**, 7122

17 B. T. Cho, S. K. Kang, *Tetrahedron*, 2005, **61**, 5725.

18 J. V. Ros-Lis, R. Martínez-Máñez, F. Sancenón, J. Soto, M. Spieles, K. Rurack, *Chem. Eur. J.*, 2008, **14**, 10101.