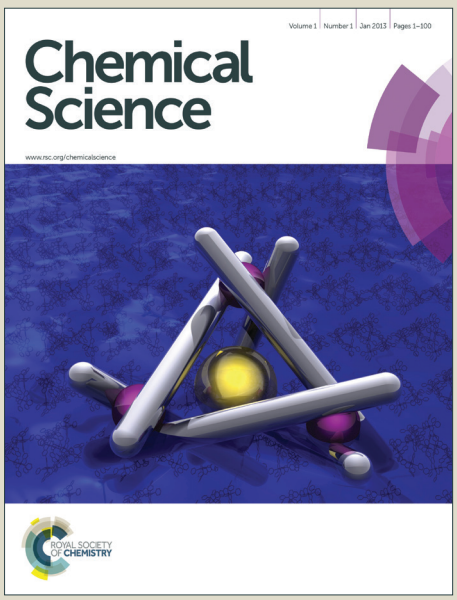
Chemical Science

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemicalscience

Journal Name

RSCPublishing

ARTICLE

Rational design of a turn-on fluorescent sensor for α-ketoglutaric acid in a microfluidic chip

Cite this: DOI: 10.1039/x0xx00000x

Pengwei Jin, Changhong Jiao, Zhiqian Guo,* Ye He, Shiqin Zhu, He Tian and Weihong Zhu*

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Detection of biomarkers *via* optical microfluidic chip is in great demand for high contrast biosensing and bioimaging. In distinct contrast with traditional chromatographic methods, which need tedious pretreatment and are not directly applicable in blood serum, the "turn-on" fluorescent sensor for cancer cell damaging agent α-ketoglutaric acid (α-KA) has been established. Hydrazino group is introduced into naphthalimide moiety as the reaction trigger for the specific fluorescence turn-on response. Under the rational design, probe **3** can successfully detect α-KA in complete aqueous system, along with about 7-fold fluorescence enhancement and rapid response with the help of micelle. The sensor exhibits good selectivity among 20 common amino acids, especially showing little interferences with various dicarbonyl derivatives and reactive oxygen species. Finally, the detection of α-KA in human serum is demonstrated in a microfluidic chip, indicative of a potential platform for high-throughput screening and kinetics monitoring especially in biological fields.

Introduction

Sensing of biomarkers has drawn considerable attention in recent years.[1-8] α-Ketoglutarate (α-KG, its acid form as α-KA) is an endogenous metabolite of the citric acid cycle (Krebs cycle) that occupies a central place in energy metabolism.[9] In particular, α-KG is suggested as a promising cancer cell damaging agent.[10,11] More recently, 2-hydroxyglutarate (2-HG) or the alteration of converting α-KG to 2-HG has been considered as biomarkers for diagnosis and classification of tumor-derived *IDH1* and *IDH2* mutations.[12-14] The present method for detecting 2-HG is based on the complicated and inconvenient 3T whole-body scanner (proton magnetic resonance spectroscopy, ^{1}H-MRS).[15,16] Also, the available methods for determining α-KA are confined to the traditional gas chromatography (GC) and high performance liquid chromatography (HPLC),[17-19] which need tedious pretreatment and are not directly applicable in blood serum. Accordingly, the development in fluorescent sensors for such biomarkers has become a focus of considerable research due to its extreme sensitivity, low background noise and wide dynamic ranges.[6,20,21] Herein, we present a novel alternative method for the detection of α-KA in human serum with "turn-on" fluorescent probe in a microfluidic chip. To the best of our knowledge, this is the first time that directly explores the fluorescence channel using molecular sensors to detect α-KA in blood serum with a microfluidic chip.

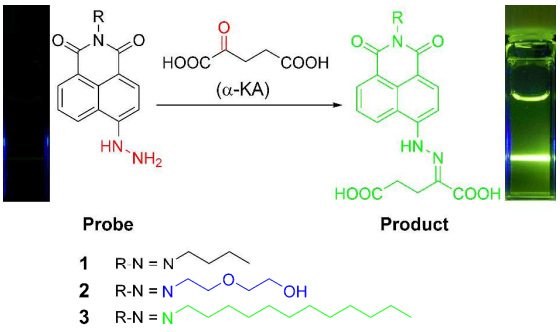


Fig. 1 Rational design of fluorescent chemosensors **1**-**3** for α-KA detection. Note: the hydrazino group is behaved as the reaction trigger for the specific fluorescence turn-on response.

The reactivity of carbonyl group in α-KA is preferably employed in the development of reaction-based sensors for measuring α-KA levels. Specifically, the generation of Schiff base between the reactive carbonyl and amino groups is a preferentially straightforward strategy.[22,23] Along these lines in hydrazino-substituted naphthalimide derivatives (Chemosensors **1**-**3**, Fig. 1), the hydrazino group as the specific functional unit is introduced for renewing turn-on fluorescence to α-KA *via* the

inhibition of photo-induced electron transfer (PET).[24-26] Inspired by the cassettes sold by Optimedical Inc.,[27] we seek microfluidics to realize determination of α-KA in serum. Microfluidic devices come into notice along with "lab-on-a-chip" concept emerging in bioassay.[28,29] Given the small amounts of samples required, we demonstrate well the detection of α-KA in human serum with a microfluidic chip, exhibiting a potential platform for high-throughput screening and kinetics monitoring especially in biological fields.[30]

Results and discussion

In the molecular design, naphthalimide is chosen as the fluorophore because of its chemical stability and high fluorescence efficiency.[31] Generally, modification of 4-position in naphthalimide is utilized as the PET trigger for realizing fluorescence "turn-off" or "turn-on". Meantime, the derivation of imido part is usually expected to facilitate functionality such as improving solubility or linking to polymers.[27] To realize measuring of α-KA in blood serum, three different alkyl amino groups were incorporated into the naphthalimide moiety for rationally designing chemosensors. Firstly, probe **1** was synthesized using commercially available intermediate of 4-bromonaphthalimide to check whether our proposed trigger unit of hydrazino group gets on work. As a result, upon addition of α-KA, the bright luminescence releases obviously in methanol solution. Probe **1** and corresponding Schiff base product **1** were well characterized by NMR and high-resolution mass spectra (HRMS, Figure S9-S11, S18-S19, ESI†). In addition, the quantum yields of probes and products were conducted and summarized in Table S1 (Figs. S5-S6†). Obviously, the high quantum yield ratios of product *vs* probe also confirmed our sensors with evident "turn-on" properties.

However, the butyl group in probe **1** brings forth its poor water solubility, which is critically limited to its further application. To enhance the solubility in aqueous environment,[32] diglycolamine (DGA) was introduced to develop probe **2** in two steps (Scheme S1†). However, as a typical Schiff base reaction, the reaction rate between carbonyl and amino groups is distinctly decreased in aqueous solution. To this end, probe **3** bearing a long carbon chain was developed for the detection of α-KA in blood serum with the help of micelle.

At the outset, the hypothesized competent probe **2** with excellent water solubility was studied. To get insight into the microenvironment influence on the generation of Schiff base, probe **2** was carried out under various conditions in the presence of α-KA (Fig. S1a-S1f†). Upon the addition of α-KA (100 μM), probe **2** (10 μM) in pure dimethyl sulfoxide (DMSO), ethanol (EtOH), acetonitrile (MeCN) and methanol (MeOH) exhibits different fluorescence response. Among these solvents, MeCN is an ideal solvent, especially with a 30-fold fluorescence enhancement (I/I_0, Fig. S1a†). Furthermore, water has distinct influence on the reaction between probe **2** and α-KA. As a matter of fact, upon the addition of α-KA (100 μM), the luminescence was largely discounted along with the increase of water portion. In order to keep the signal-to-noise ratio, the mixture of water-acetonitrile (volume ratio: 1:9) was taken as the optimization solvent (Fig. S1b†). Additionally, response time is another important parameter for chemosensors. In this case, 10 min is a favorable time for the detection of α-KA (Fig. S1f†). We also adopted the HPLC analysis to compare the performance of our sensor in HPLC (Figs. S7-S8). That demonstrated that our probe could be utilized for α-KA analysis with sufficient sensitivity by HPLC or fluorescence assay.

As a rule, reaction-based sensors are generally susceptible to microenvironment, especially in aqueous solution. However, the perfect luminescent properties with proper response time for probe **2** are carried out in 90% organic solvent, which is not environmental or suitable for its further application *in vivo*.[33] Herein, we sought cationic or anionic surfactant to minimize the negative effect with the aid of micelle (Fig. 2). In our system, probe **3** bearing a long carbon chain can easily form micelle in the presence of cetyltrimethyl ammonium bromide (CTAB). Upon formation of micelle, the positive charges on the micelle surface can help α-KA gather around (Fig. 2b). Here the cationic surfactant of CTAB can exactly enable or accelerate chemical reactions in aqueous solution by possibly providing a nonpolar solvent-like microenvironment,[34] resulting in concomitant prominently enhanced fluorescence with 3.2-fold enhancement in 10 min and a blue-shift in emission spectra with respect to its aqueous system (Fig. 2a and Fig. S4, ESI†). Moreover, an anionic surfactant (SDS, sodium dodecyl sulfate) with negative charges was also investigated in Fig. 2a. In contrast, the fluorescence was suppressed, and reaction time was delayed because the repulsion between surface-negative micelle and α-KA can make a decrease in the opportunity of α-KA to react with probe **3**. Thus, CATB is selected and utilized as an excellent auxiliary in the further experiments.

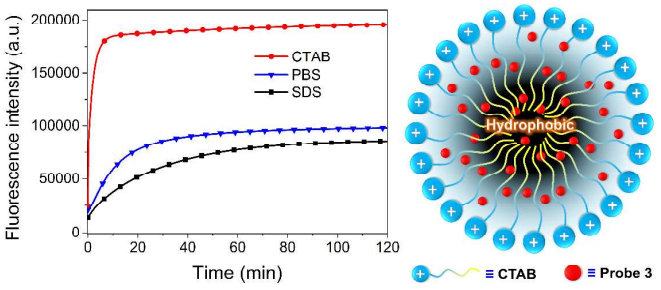


Fig. 2 (a) Fluorescence kinetics of probe **3** (10 μM) upon addition of α-KA (100 μM) with CTAB (1 mM), SDS (10 mM) and water buffered in PBS (pH 6.0), respectively. λ_{ex} = 430 nm, λ_{em} = 552 nm, 20 °C. (b) Schematic diagram of micelle-induced microenvironment (CTAB).

It should be noted that the optimum pH condition for probe **2** is 6.5 (Fig. S1c†). However, as shown in Fig. S2†, a little more acidic environment (pH 6.0) is conducive to the Schiff base generated reaction for probe **3**. The fact that the optimum pH for probe **3** is more acidic than probe **2** can be attributed to the surface positive charges of CTAB micelles. Such positive charges can appeal OH^- around, which cause the pH value

inside the micelle higher than the bulk solution.[35] Thus, the general environment for chemosensor **3** with micelle need more acidity than that for chemosensor **2** in aqueous system. As for temperature, it has an inconspicuous effect on the fluorescence and can be ruled out (Fig. S3†).

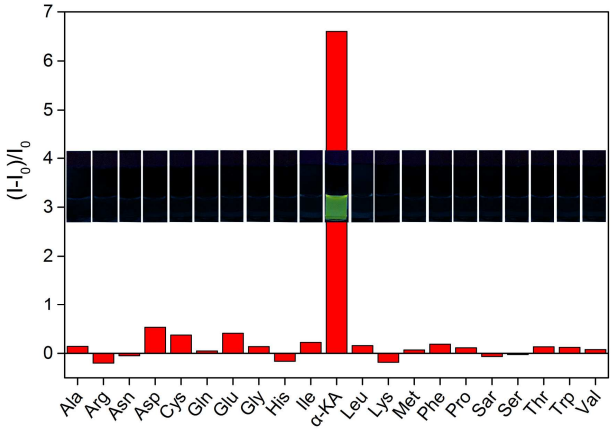


Fig. 3 Fluorescence response of probe **3** upon various amino acids. Probe **3** (10 μM) with CTAB (1 mM) was incubated with 100 μM substrate for 10 min at 20 ℃ in PBS (pH 6.0). Substrates are *DL*-Alanine (Ala), *L*-Arginine (Arg), *DL*-Asparagine monohydrate (Asn), *L*-Aspartic acid (Asp), *DL*-Cysteine (Cys), *L*-Glutamine (Gln), *L*-Glutamic acid (Glu), Glycine (Gly), *L*-Histidine (His), *L*-Isoleucine (Ile), *α*-Ketoglutaric acid (*α*-KA), *L*-Leucine (Leu), *L*-Lysine (Lys), *L*-Methionine (Met), *L*-Phenylalanine (Phe), *L*-Proline (Pro), Sarcosine (Sar), *L*-Serine (Ser), *L*-Threonine (Thr), *L*-Tryptophan (Trp), and *L*-Valine (Val), respectively. λ_{ex} = 430 nm, λ_{em} = 552 nm.

As *α*-KA exists in serum, its anti-interference ability against various amino acids remains very important. The selectivity of probe **3** (10 μM) was tested upon reaction with 10 equi. (100 μM) of various amino acids incubated for 10 min with CTAB (1 mM) at 20 ℃ in PBS (pH 6.0). Notably, only *α*-KA led to significant fluorescence increase with about 7-fold enhancement at 552 nm among 20 kinds of common amino acids (Fig. 3). This implies that probe **3** has good specificity and feasibility towards *α*-KA even in amino acids-surrounded environment. Actually in serum, there are other kinds of potential interferences with similar structures including various dicarbonyl compounds and reactive oxygen species (ROS).[2] We therefore compared its selectivity among some typical species (Fig. 4), including glyoxal (GO), hydrogen peroxide (H_2O_2), phenylglyoxal (PGO), methylglyoxal (MGO) and phenylpyruvic acid (PPA). After incubation of various dicarbonyls and ROS with probe **3** for 10 min, *α*-KA led to significant fluorescence increases with about 7-fold enhancement except two minor interferences of PPA and PAS (Fig. 4). The high selectivity of probe 3 towards dicarbonyl compounds with similar structure such as MGO could be attributed to the unique ketoacid group in *α*-ketoglutaric acid.

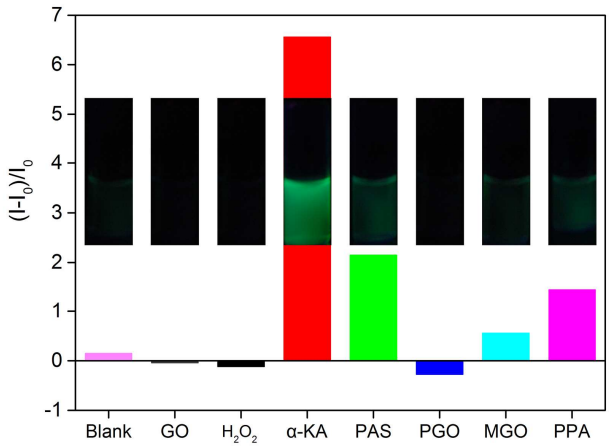


Fig. 4 Fluorescence response of probe **3** upon various dicarbonyls and ROS. Probe **3** (10 μM) with CTAB (1 mM) was incubated with 100 μM substrate for 10 min at 20 ℃ in PBS (pH 6.0). Substrates are glyoxal (GO), hydrogen peroxide (H_2O_2), *α*-ketoglutaric acid (*α*-KA), sodium pyruvate (PAS), phenylglyoxal (PGO), methylglyoxal (MGO) and phenylpyruvic acid (PPA).

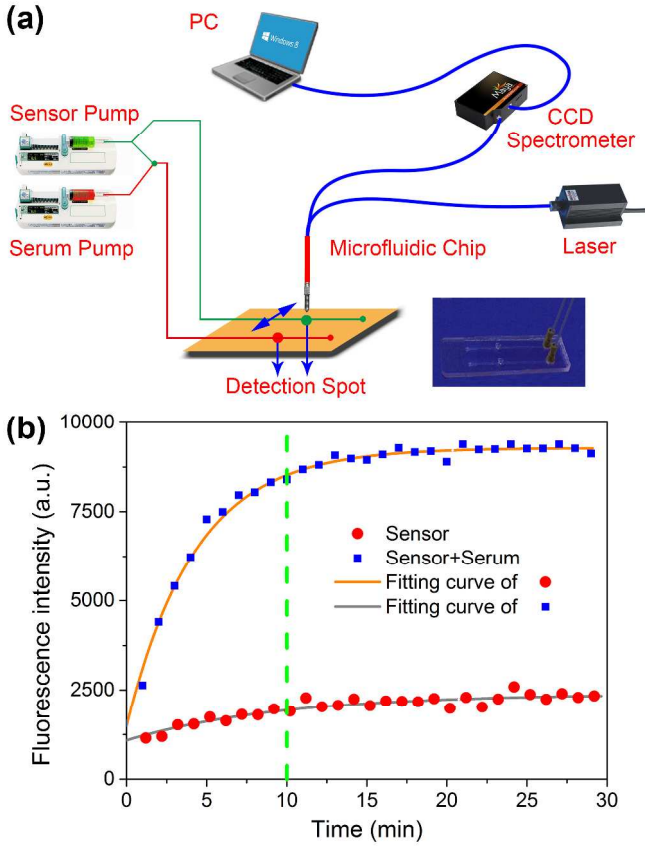


Fig. 5 (a) Schematic diagram of microfluidic system. Inset: picture of the microfluidic chip. (b) Fluorescence response of probe **3** (10 μM) with/without addition of human serum acquired in a microfluidic chip. λ_{ex} = 430 nm, λ_{em} = 552 nm.

Finally, in view of good performances in aqueous system, probe **3** for the detection of *α*-KA in human serum assay was then carried out in a microfluidic chip (Fig. 5a). Fresh blood was centrifuged first to separate the sediment, and protein was filtered quickly by microfiltration membrane. The

home-assembled microfluidic system uses two syringe pumps to push chemosensor (loaded with 1mM CTAB and PBS, pH 6.0) and pretreated serum liquid (loaded with α-KA as original content is very low). The sensor channel was divided equally to two branches. One branch channel is to react with sensor and the other branch channel is hereby designated as the reference. When two channels were filled up, pumps stopped and two channels were detected in turn by a mechanical switching device. The sensor tip of a QF600-8-VIS/NIR bifurcated optic fluorescence fiber bundle contains one flat fiber for detection, and seven angled fibers that direct excitation energy to the region in front of the detection fiber. Such sensor tip can distinctly avoid any of the direct incident light beam.[36] A CCD spectrometer MayaPro 2000 from Ocean Optics was used to realize online monitoring. The luminescence under the round spots was monitored at set intervals (1 min). The fitted curves indicated that the sensor response time was almost 10 min when 95% emission intensity changed (Fig. 5b). As demonstrated, probe **3** can function well in serum existed environment. Here the microfluidic chip and optical fiber tip were kept in dark during acquisition to avoid interference from the environment. Thus, with the help of CTAB, the additive α-KA in human serum could be detected with probe **3** in 10 min, exerting potential value in the real applications. Microfluidic chip with a high integration level will be developed in the future work.[37,38]

Conclusions

In summary, we presented a rational design of turn-on fluorescent chemosensors from probe **1** to probe **3** for monitoring α-KA, exhibiting good selectivity towards α-KA among amino acids, dicarbonyls and ROS. With the help of micelle, probe **3** can be applied in aqueous system with proper response time. It was successfully applied in the detection of α-KA in human serum with a microfluidic chip. We are exploring probe **3** as a low-cost indicator for α-KA in the practicable application.

Acknowledgements

This work was supported by National 973 Program (No. 2013CB733700), NSFC for Distinguished Young Scholars (Grant No. 21325625), NSFC/China, the Oriental Scholarship, Shanghai Pujiang Program (13PJD010), the Fok Ying Tong Education Foundation (142014), the Fundamental Research Funds for the Central Universities (WK1013002, 222201313010), and SRFDP 20120074110002.

Notes and references

Shanghai Key Laboratory of Functional Materials Chemistry, Key Laboratory for Advanced Materials and Institute of Fine Chemicals, East China University of Science and Technology, Shanghai 200237, P. R. China. E-mail: whzhu@ecust.edu.cn; guozq@ecust.edu.cn

Electronic Supplementary Information (ESI) available: chemicals and instruments, synthesis and characterization of probes, optimization of determination conditions and micelle-induced performances. See DOI: 10.1039/b000000x/

1 H. Shi, X. Chen, S. Liu, H. Xu, Z. An, O. Lang, Z. Tu, Q. Zhao, Q. Fan, L. Wang and W. Huang, *ACS Appl. Mater. Interfaces*, 2013, **5**, 4562.
2 T. Wang, E. F. Douglass, K. J. Fitzgerald and D. A. Spiegel, *J. Am. Chem. Soc.*, 2013, **135**, 12429.
3 H. Zhang, J. Fan, J. Wang, S. Zhang, B. Dou and X. Peng, *J. Am. Chem. Soc.*, 2013, **135**, 11663.
4 H. S. Jung, X. Chen, J. S. Kim and J. Yoon, *Chem. Soc. Rev.*, 2013, **42**, 6019.
5 D. D. Li, C. L. Miao, X. D. Wang, X. H. Yu, J. H. Yu and R. R. Xu, *Chem. Commun.*, 2013, **49**, 9549.
6 H. U. Khan, M. E. Roberts, O. Johnson, R. Forch, W. Knoll and Z. N. Bao, *Adv. Mater.*, 2010, **22**, 4452.
7 W. Xuan, Y. Cao, J. Zhou and W. Wang, *Chem. Commun.*, 2013, **49**, 10474.
8 Y. Huang, W. Ouyang, X. Wu, Z. Li, J. S. Fossey, T. D. James and Y. Jiang, *J. Am. Chem. Soc.*, 2013, **135**, 1700.
9 W. He, F. J. P. Miao, D. C. H. Lin, R. T. Schwandner, Z. Wang, J. Gao, J. Chen, H. Tian and L. Ling, *Nature*, 2004, **429**, 188.
10 B. Wagner, F. Donnarumma, R. Wintersteiger, W. Windischhofer and H. Leis, *Anal. Bioanal. Chem.*, 2010, **396**, 2629.
11 L. Kronberger, H. J. Semmelrock, R. J. Schaur, E. Schauenstein, W. Schreibmayer and E. Fink, *J. Cancer Res. Clin.*, 1980, **97**, 295.
12 P. S. Ward, J. Patel, D. R. Wise, O. Abdel-Wahab, B. D. Bennett, H. A. Coller, J. R. Cross, V. R. Fantin, C. V. Hedvat, A. E. Perl, J. D. Rabinowitz, M. Carroll, S. M. Su, K. A. Sharp, R. L. Levine and C. B. Thompson, *Cancer Cell*, 2010, **17**, 225.
13 W. Xu, H. Yang, Y. Liu, Y. Yang, P. Wang, S. Kim, S. Ito, C. Yang, P. Wang, M. Xiao, L. Liu, W. Jiang, J. Liu, J. Zhang, B. Wang, S. Frye, Y. Zhang, Y. Xu, Q. Lei, K. Guan, S. Zhao and Y. Xiong, *Cancer Cell*, 2011, **19**, 17.
14 A. Schulze and A. L. Harris, *Nature*, 2012, **491**, 364.
15 C. Choi, S. K. Ganji, R. J. DeBerardinis, K. J. Hatanpaa, D. Rakheja, Z. Kovacs, X. Yang, T. Mashimo, J. M. Raisanen, I. Marin-Valencia, J. M. Pascual, C. J. Madden, B. E. Mickey, C. R. Malloy, R. M. Bachoo and E. A. Maher, *Nat. Med.*, 2012, **18**, 624.
16 J. Kalinina, A. Carroll, L. Wang, Q. Yu, D. Mancheno, S. Wu, F. Liu, J. Ahn, M. He, H. Mao and E. Meir, *J. Mol. Med.*, 2012, **90**, 1161.
17 U. Langenbeck, H. U. Möhring and K. P. Dieckmann, *J. Chromatogr. A*, 1975, **115**, 65.
18 P. Montenegro, I. M. Valente, L. M. Goncalves, J. A. Rodrigues and A. A. Barros, *Anal. Methods*, 2011, **3**, 1207.
19 Z. Wang, K. Zaitsu and Y. Ohkura, *J. Chromatogr. B.*, 1988, **430**, 223.
20 J. Shao, H. Sun, H. Guo, S. Ji, J. Zhao, W. Wu, X. Yuan, C. Zhang and T. D. James, *Chem. Sci.*, 2012, **3**, 1049.
21 V. Kumar and E. V. Anslyn, *J. Am. Chem. Soc.*, 2013, **135**, 6338.
22 X. Shen, G. Zhang and D. Zhang, *Org. Lett.*, 2012, **14**, 1744.
23 J. A. Key, C. Li and C. W. Cairo, *Bioconjugate Chem.*, 2012, **23**, 363.
24 A. P. de Silva, H. Q. N. Gunaratne, J. Habib-Jiwan, C. P. McCoy, T. E. Rice and J. Soumillion, *Angew. Chem. Int. Ed.*, 1995, **34**, 1728.
25 X. Qian, Y. Xiao, Y. Xu, X. Guo, J. Qian and W. Zhu, *Chem.*

Commun., 2010, **46**, 6418.
26 S. Banerjee, E. B. Veale, C. M. Phelan, S. A. Murphy, G. M. Tocci, L. J. Gillespie, D. O. Frimannsson, J. M. Kelly and T. Gunnlaugsson, *Chem. Soc. Rev.*, 2013, **42**, 1601.
27 A. P. de Silva, T. P. Vance, M. E. S. West and G. D. Wright, *Org. Biomol. Chem.*, 2008, **6**, 2468.
28 A. Abou-Hassan, O. Sandre and V. Cabuil, *Angew. Chem. Int. Ed.*, 2010, **49**, 6268.
29 B. Jiang, W. Zheng, W. Zhang and X. Jiang, *Sci. China Chem.*, 2014, **57**, 356.
30 S. Kou, H. N. Lee, D. van Noort, K. M. K. Swamy, S. H. Kim, J. H. Soh, K. Lee, S. Nam, J. Yoon and S. Park, *Angew. Chem. Int. Ed.*, 2008, **47**, 872.
31 J. F. Zhang, C. S. Lim, S. Bhuniya, B. R. Cho and J. S. Kim. *Org. Lett.*, 2011, **13**, 1190.
32 J. Wang, Y. Xiao, Z. Zhang, X. Qian, Y. Yang and Q. Xu, *J. Mater. Chem.*, 2005, **15**, 2836.
33 A. D. Shao, Z. Q. Guo, S. J.Zhu, S. Q. Zhu, P. Shi, H. Tian and W. H. Zhu, *Chem. Sci.*, 2014, **5**, 1383.
34 H. Y. Tian, J. H. Qian, Q. Sun, H. Y. Bai and W. B. Zhang, *Anal. Chim. Acta*, 2013, **788**, 165.
35 H. Y. Tian, J. H. Qian, H. Y. Bai, Q. Sun, L. Y. Zhang and W. B. Zhang, *Anal. Chim. Acta*, 2013, **768**, 136.
36 L. J. Lucas, J. N. Chesler and J. Yoon, *Biosens. Bioelectron.*, 2007, **23**, 675.
37 Y. Yang, Q. Zhao, W. Feng and F. Li, *Chem. Rev.*, 2013, **113**, 192.
38 L. Yuan, W. Lin, K. Zheng, L. He and W. Huang, *Chem. Soc. Rev.*, 2013, **42**, 622.

Journal Name

RSCPublishing

ARTICLE

Graphics for Contents

Rational design of a turn-on fluorescent sensor for α-ketoglutaric acid in a microfluidic chip

Pengwei Jin, Changhong Jiao, Zhiqian Guo,* Ye He, Shiqin Zhu, He Tian and Weihong Zhu*

A rational design of turn-on fluorescent chemosensors for monitoring α-ketoglutaric acid has been developed with microfluidic chip, indicative of a potential platform for high-throughput screening and kinetics monitoring especially in biological fields.