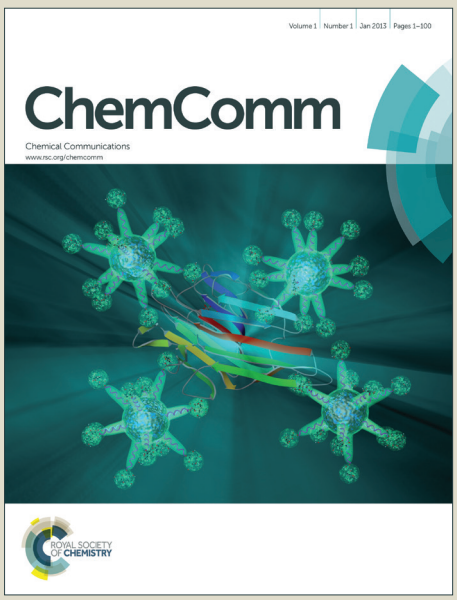
ChemComm

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Please do not adjust margins

Journal Name

COMMUNICATION

Target-induced three-way G-quadruplex junction for 17β-estradiol monitoring with naked-eye readout

Received 00th January 20xx,

Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Junhua Chen,* Junlin Wen, Guiqin Yang, and Shungui Zhou*

A label-free and enzyme-free three-way G-quadruplex junction sensing system for the amplified detection of 17β-estradiol has been constructed by the ingenious coupling of split G-quadruplex DNAzyme with toehold-mediated strand displacement. The biosensor is ultrasensitive, enabling the visual detection of 17β-estradiol concentrations as low as 1 fM without instrumentation.

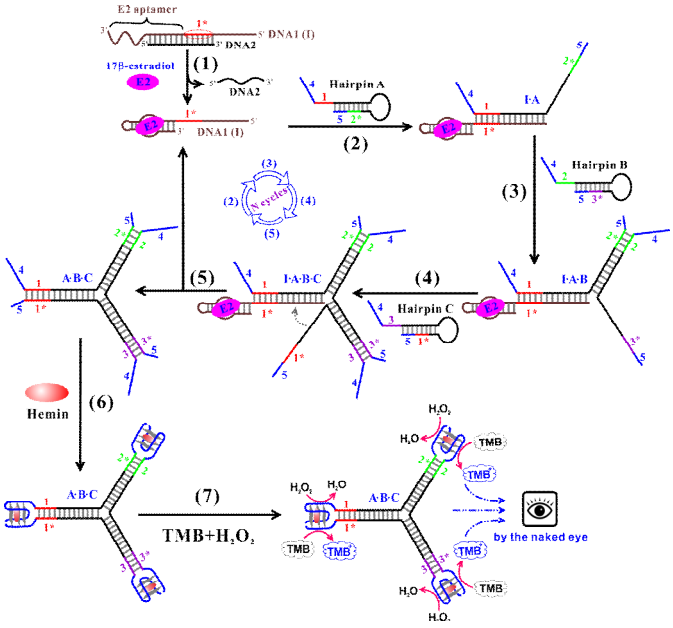
As an environmental endocrine disrupting chemical (EDC), 17β-estradiol (E2) is considered to be the most active estrogen that can cause adverse health effects even at low concentrations.[1-3] Therefore, routine detection of trace amounts of E2 with high sensitivity and selectivity is of critical importance for protecting environmental and public health. In recent years, various types of sensors with elegant design strategies have been proposed for E2 detection using fluorescent[1,4] and electrochemical[2,3a,5] methods. Despite significant contributions have been made to E2 detection, the readout signals of these methods rely on specialized analytical instruments that are not suitable for on-site monitoring in remote settings without infrastructure. As an alternative, colorimetric biosensors possess many attractive features that are required for point-of-use (POU) detection (*e.g.,* low cost, rapid response time, portability, and ease of use).[6,7] However, colorimetric assay is often confronted with unsatisfactory sensitivity. Thus, it is urgently needed to develop a colorimetric sensing system for E2 detection with high sensitivity and simplified operation.

One way to improve the sensitivity is through signal amplification. Most ultrasensitive detectors depend on amplification labels (*e.g.,* nanoparticles),[8] which require intricate synthetic processes and multiple operation steps that are inappropriate for highly demanding POU settings. Recent advance in the field of DNA nanotechnology[9] have generated a series of DNA nanostructures and nanodevices, including catalytic amplifiers,[10a,b] logic cascades,[10c,d] and autonomous molecular motors,[10e,f] most of which are driven by toehold-mediated DNA strand displacement reactions.[11,12] This method exhibits some intriguing characteristics such as continuous signal turnover capability, inherent modularity, environmental robustness, and easy to scale up.[13] These unique attributes make toehold-mediated strand displacement to be an ideal candidate for exquisitely sensitive molecular detection.[14] For example, Xiang's group has successfully developed a novel "DNAzyme ferris wheel" nanostructure for miR-141 detection with high sensitivity.[14a] Ellington and co-workers have designed a simplified DNA circuit for analyte detection in a "plug-and-play" fashion.[14b] To explore new dimensions in EDCs detection, it would be of great interest to combine toehold-mediated strand displacement with colorimetric assay in the fabrication of an ultrasensitive sensing platform without any label or enzyme for E2 monitoring.

As an excellent signal reporter, G-quadruplex DNAzyme is frequently utilized as a sensing element in colorimetric biosensors.[15,16] It was found that the G-quadruplex sequence may be split into two fragments that lack catalytic activity, but upon template-assisted formation of intact G-quadruplex DNAzyme from the split fragments, the peroxidation activity is reactivated,[17] which can effectively catalyze the H_2O_2-mediated oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) to generate a color change. In this study, we combined split G-quadruplex DNAzyme with toehold-mediated strand displacement to fabricate a label-free and enzyme-free sensing platform with cascaded signal amplification and naked-eye readout for E2 analysis on the basis of target-induced three-way G-quadruplex junction.

Guangdong Key Laboratory of Agricultural Environment Pollution Integrated Control, Guangdong Institute of Eco-Environmental and Soil Sciences, Guangzhou 510650, China.
E-mail: jhchen@soil.gd.cn; sgzhou@soil.gd.cn
† Electronic Supplementary Information (ESI) available: [Experimental details and supplementary figures and tables]. See DOI: 10.1039/x0xx00000x

ChemComm Accepted Manuscript



Scheme 1 Schematic illustration of the design principle for construction of the E2 sensing platform based on three-way G-quadruplex junction. Three hairpins (A, B, and C) are used as the building blocks to construct the signal amplification platform *via* a series of toehold-mediated strand displacement reactions (2, 3, 4, and 5). Toeholds and toehold binding regions are indicated in red (domains 1 and 1*), green (domains 2 and 2*), and purple (domains 3 and 3*). The split G-quadruplex fragments are marked in blue (domains 4 and 5).

The design principle for construction of the three-way G-quadruplex junction is schematically illustrated in Scheme 1. The aptamer sequence[5a] of E2 was extended at the 5'-end to form a chimeric strand (DNA1) where domain 1* is occluded by hybridization with a blocking strand (DNA2) (All DNA sequences were listed in Table S1, ESI†). Therefore, in the absence of target, domain 1* of DNA1 is inaccessible. The introduction of E2 to the sensing system induces dehybridization of the partially complementary duplex between DNA1 and DNA2. As a consequence, domain 1* of DNA1 is exposed and can serve as a toehold for initiating the subsequent amplification reaction (Scheme 1, reaction 1). Three hairpin structures were selected as the building blocks (hairpins A, B, and C) to construct the three-way G-quadruplex junction. The two G-rich fragments of the 3:1 split G-quadruplex sequences are distributed at the 5' and 3' ends of those hairpins (domains 4 and 5, indicated in blue), respectively. The exposed domain 1* of the initiator DNA1 strand (I) first binds to the domain 1 of hairpin A, potentially initiating a branch migration (toehold-mediated DNA strand displacement reaction) to open hairpin A, resulting in the formation of an I·A intermediate where the domain 2* of hairpin A is open (Scheme 1, reaction 2). Then, domain 2 of hairpin B interacts with the newly opened toehold 2* of hairpin A, again initiating a branch migration to open hairpin B and form an I·A·B hybrid where the domain 3* of hairpin B is no longer occluded (Scheme 1, reaction 3). Similarly, domain 3 of hairpin C can then hybridize to the newly accessible toehold 3* of hairpin B, activating a strand displacement that opens hairpin C and generates an I·A·B·C complex (Scheme 1, reaction 4). This complex is inherently unstable, resulting in a spontaneous disassembly step in which hairpin C displaces I from the complex, freeing I to catalyze the self-assembly of additional branched junctions (Scheme 1, reaction 5). The formed A·B·C product (three-way G-quadruplex junction) will bring the 3:1 split G-quadruplex fragments into close enough proximity and induce the formation of intact G-quadruplex probes at each end of the three arms. Upon incubation with hemin, the catalytic G-quadruplex/hemin peroxidase-mimicking DNAzymes are anticipated to be formed (Scheme 1, reaction 6). The active DNAzyme can effectively catalyze the H_2O_2-mediated oxidation of TMB to generate colored signal readout, which can be easily recognized by the naked eye (Scheme 1, reaction 7). In the absence of E2, the initiator I should be catalytically inactive. The three hairpins A, B, and C do not interact with each other to initiate the amplification reaction. In this state, no colored signal could be observed due to the failure in the formation of intact G-quadruplex DNAzyme in the system.

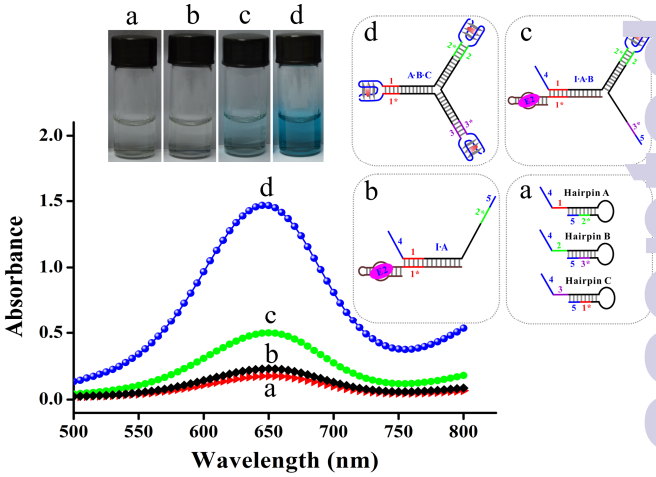


Fig. 1 UV-vis absorbance spectra of samples under different conditions: (a) no E2, DNA1-DNA2, hairpins A, B, and C, (b) 10 pM E2, DNA1-DNA2, hairpin A, (c) 10 pM E2, DNA1-DNA2, hairpins A and B, and (d) 10 pM E2, DNA1-DNA2, hairpins A, B, and C. Experiments are performed in TMB-H_2O_2 reaction solution. The inset shows a naked-eye color difference and the corresponding products.

The formation of the three-way junction structure based on toehold-mediated strand displacement reactions was confirmed by native polyacrylamide gel electrophoresis (PAGE) (Fig. S1, ESI†). To further verify the feasibility and signal amplification capability of our protocol, the UV-vis absorption spectra and color changes of the solution under different conditions were also investigated. As shown in Fig. 1, the mixture containing DNA1-DNA2 and hairpins A, B, and C exhibited a weak characteristic absorption peak at 650 nm without E2 introduction (the background signal of the TMB-H_2O_2 detection system), indicating that the hairpins could coexist in solution with negligible intermolecular hybridization in the absence of target analyte (curve a). Upon the addition of E2 to the system containing DNA1-DNA2 and only hairpin A, the absorbance (curve b)

ChemComm Accepted Manuscript

This journal is © The Royal Society of Chemistry 20xx

showed almost no change relative to curve a. This result confirmed that the generated I·A could not draw the split G-quadruplex fragments together to form intact DNAzyme. Significantly, when both hairpins A and B were present in the mixture, the absorbance intensity increased obviously (curve c). Such increase was basically due to the formation of active DNAzyme in the I·A·B compound. Most importantly, when the incubation solution simultaneously contained hairpins A, B, and C, the absorption signal heavily increased (curve d). The reason might be most likely a consequence of the fact that our rational designed hairpins could induce the autonomous cyclic formation of A·B·C product, thereby generated numerous G-quadruplex DNAzyme molecules to give an amplified colorimetric signal. The corresponding color changes of the solution (inset in Fig. 1) further demonstrated the feasibility of our protocol. Thus, the experimental results clearly indicated that the proposed three-way G-quadruplex junction sensing system can be adopted for sensitive E2 detection.

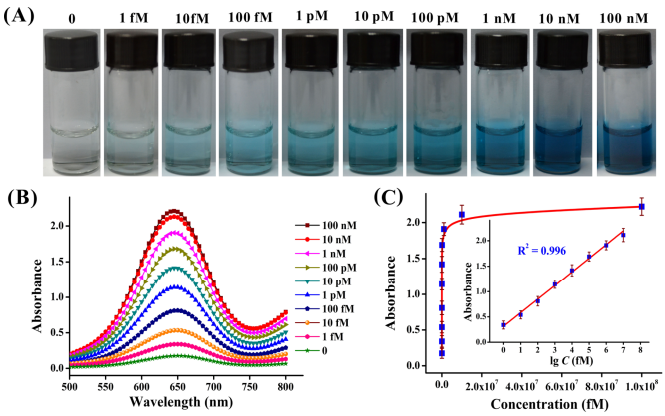


Fig. 2 (A) Photo images of the sensing system with different concentration of E2. The images were recorded with a digital camera. (B) Their corresponding UV-vis absorbance spectra. (C) Plots of the absorbance intensity at 650 nm as a function of the E2 concentration. Inset: linear relationship between the absorbance intensity and the logarithm of E2 concentration in the range from 1 fM to 10 nM. The error bars represent the standard deviation of three independent measurements.

Under the optimal conditions (Fig. S2-S6, ESI†), the sensitivity and dynamic range of the proposed three-way G-quadruplex junction sensing platform were investigated with different concentrations of E2. As shown in Fig. 2A, the color of the solution turned gradually to deep blue with increasing E2 concentration. In the absence of E2, there was no significant color change observed in the reaction system and the solution was still colorless. Their corresponding colorimetric responses were further confirmed by recording the UV-vis absorbance spectra of the solution (Fig. 2B). The absorbance values at 650 nm were also recorded in Fig. 2C. The resulting calibration curve shows that the absorbance values are proportional to the logarithm of E2 concentrations in the range from 1 fM to 10 nM with a correlation coefficient (R^2) of 0.996 (Fig. 2C, inset). Importantly, the presence of as low as 1 fM E2 can be easily identified by the naked eye according to the distinct color difference between the sample and the blank test. Such low visual detection limit (1 fM) is 6 orders of magnitude better than the aptamer-based optical biosensor for E2 without signal amplification.[1] Compared with previously reported E2 detection based on immunoassay, the new three-way G-quadruplex junction sensing system developed here also possesses much higher sensitivity.[4b] The significant improvement of the sensitivity can be primarily attributed to the continuous turnover capability of the toehold-mediated strand displacement reaction to generate numerous three-way G-quadruplex junctions even at low concentration of target E2. Besides the impressive visual detection limit, our method is essentially simple by avoiding the involvement of any DNA immobilization or conjugate procedure and any complicated signal transduction instrument, which holds great promise for on-site monitoring of E2 in environmental samples.

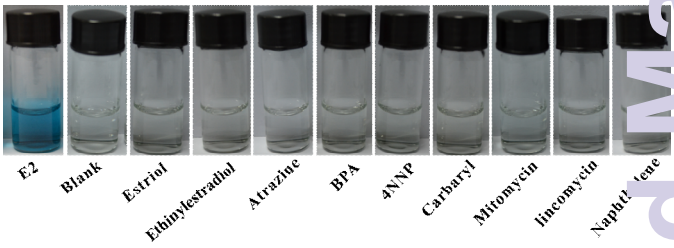


Fig. 3 Selectivity investigation of the constructed biosensor for E2 against other control molecules. The concentration is 1 nM for E2 and 100 nM for other molecules.

The selectivity of the constructed biosensor is examined by monitoring the color change of the solution with the presence of E2 against other control molecules, including estriol, ethinylestriol, atrazine, bisphenol A (BPA), 4-n-nonylphenol (4NNP), carbaryl, mitomycin, lincomycin, and napthalene, which may coexist in environment or have similar structure with E2. As shown in Fig. 3, 1 nM E2 could produce a bright blue color change of the reaction solution, whereas all other control molecules at a concentration of 100 nM could hardly exhibit substantial responses during the colorimetric assay and the solution remained colorless. The results indicated that our fabricated sensing system possessed excellent selectivity for E2 detection. The assay selectivity of our sensing system toward the random DNA sequence and the E2 aptamer sequence was also investigated. As shown in Fig. S7 (ESI†), the presence of the random DNA sequence causes no obvious color change of the probe solution against the blank test while the presence of the E2 aptamer sequence results in a clearly intensified color change. Such high selectivity is attributed to the highly specific binding capability of the aptamer to the target analyte. In other words, only the presence of E2 can interact with the aptamer sequence and expose the toehold domain to initiate the toehold-mediated strand displacement to generate numerous G-quadruplex DNAzymes for visual readout.

To validate the practical application of our proposed biosensor, several environmental water samples (tap water, lake water, and river water) were tested using the present sensing system. These samples spiked with different concentrations of E2 were analyzed according to the general procedure with five replicates. The results were summarized in Table S2 (ESI†). Satisfactory recovery values

ChemComm Accepted Manuscript

between 88 and 107% were obtained for the recovery experiments and the relative standard derivations (RSD) was in the range of 1.5-5.6%. These results confirmed that the possible interference from different background composition of water samples on the sensor response was negligible. Thus, our introduced sensing platform can be successfully applied to E2 analysis in real environmental water samples. In order to reduce the reaction time and move the three-way G-quadruplex junction sensing platform much closer to field monitoring, future work will focus on developing an even faster DNA circuit with accelerated strand displacement ability.

In summary, we have successfully designed an ultrasensitive biosensor for E2 monitoring on the basis of target-induced three-way G-quadruplex junction. By employing the split G-quadruplex DNAzyme as the signal reporter, the output of the biosensor can be unambiguously read out by the naked eye. Three DNA probes (hairpins A, B, and C) were utilized as the building blocks to construct the label-free and enzyme-free sensing platform with cascaded signal amplification *via* a series of toehold-mediated strand displacement reactions. Due to the significant signal amplification efficiency, the presence of the proof-of-concept target E2 leads to the generation of numerous intact G-quadruplex/hemin DNAzymes, which result in intensified color change of the reaction solution for visual detection of E2 down to 1 fM without instrumentation. Meanwhile, our colorimetric protocol exhibits excellent selectivity for E2 against other possible competing interferents. Moreover, this sensing system is robust and can be applied to the reliable monitoring of spiked E2 in environmental water samples with good recovery and accuracy. The construction strategy herein is simple in design and economic in operation without labeling and immobilization procedures or separation and washing steps, making this sensing platform particularly suitable for routine on-site monitoring of environmental pollutants in resource-constrained settings. Significantly, the proposed method is modular and versatile and can be easily extended to the analysis of other analytes by simply substituting the target-specific aptamer sequence.

Financial support was provided by the National Natural Science Foundation of China (Grant Nos. 21407029 and 41401261), the Pearl River S&T Nova Program of Guangzhou (Grant No. 201506010093), the Natural Science Foundation of Guangdong Province (Grant No. 2014A030313705), the Science and Technology Program of Guangzhou (Grant No.201508020010), and the Key Projects in the National Science & Technology Pillar Program of China (Grant No. 2015BAD06B03).

Notes and references

1 N. Yildirim, F. Long, C. Gao, M. He, H. Shi and A. Z. Gu, *Environ. Sci. Technol.,* 2012, **46**, 3288.
2 L. Fan, G. Zhao, H. Shi, M. Liu, Y. Wang and H. Ke, *Environ. Sci. Technol.,* 2014, **48**, 5754.
3 (a) S. Zhang, B. Du, H. Li, X. Xin, H. Ma, D. Wu, L. Yan and Q. Wei, *Biosens. Bioelectron.,* 2014, **52**, 225; (b) J. E. Moethorst, N. Brande-Lavridsen, B. Korsgaard and P. Bjerregaard, *Environ. Sci. Technol.,* 2014, **48**, 14668.
4 (a) F. J. Cespedes-Guirao, A. B. Ropero, E. Font-Sanchis, A. Nadal, F. Fernandez-Lazaro and A. Sastre-Santos, *Chem. Commun.,* 2011, **47**, 8307; (b) T. Kokko, L. Kokko, T. Lovgren and T. Soukka, *Anal. Chem.,* 2007, **79**, 5935; (c) F. Long, H. [illegible] and H. Wang, *RSC Adv.,* 2014, **4**, 2935.
5 (a) Y. S. Kim, H. S. Jung, T. Matsuura, H. Y. Lee, T. Kawai and M. B. Gu, *Biosens. Bioelectron.,* 2007, **22**, 2525; (b) H. Kanso, N. Inquimbert, L. Barthelmebs, G. Istamboulie, F. Thomas, [illegible] Calas-Blanchard and T. Noquer, *Chem. Commun.,* 2014, **5**[illegible] 1658; (c) K. Huang, Y. Liu, J. Zhang, J. Cao and Y. Liu, *Biosens. Bioelectron.,* 2015, **67**, 184; (d) B. Zhu, O. A. Alsager, [illegible] Kumar, J. M. Hodgkiss and J. Travas-Sejdic, *Biosens. Bioelectron.,* 2015, **70**, 398.
6 (a) Y. Song, W. Wei and X. Qu, *Adv. Mater.,* 2011, **23**, 421[illegible]; (b) X. Liu, H. T. Ngo, Z. Ge, S. J. Butler and K. A. Jolliffe, *Chem. Sci.,* 2013, **4**, 1680.
7 (a) Y. Xianyu, Z. Wang and X. Jiang, *ACS Nano* 2014, **8**, 1274[illegible] (b) A. D. Warren, G. A. Kwong, D. K. Wood, K. Y. Lin and S. N. Bhatia, *Proc. Natl. Acad. Sci. U.S.A.,* 2014, **111**, 3671; (c) [illegible] Chen, Z. Fang, P. Lie and L. Zeng, *Anal. Chem.,* 2012, **84**, 6321; (d) K. Zagorovsky and W. C. W. Chan, *Angew. Chem. Int. Ed.* 2013, **52**, 3168; (e) O. A. Alsager, S. Kumar, B. Zhu, J. Travas-Sejdic, K. P. McNatty and J. M. Hodgkiss, *Anal. Chem.,* 2015, **87**, 4201.
8 Y. Zhang, Y. Guo, Y. Xianyu, W. Chen, Y. Zhao and X. Jiang, *Adv. Mater.,* 2013, **25**, 3802.
9 (a) F. Wang, C. Lu and I. Willner, *Chem. Rev.,* 2014, **114**, 2881; (b) H. Zhang, F. Li, B. Dever, X. Li and X. Chris Le, *Chem. Rev.* 2013, **113**, 2812.
10 (a) P. Yin, H. M. T. Choi, C. R. Calvert and N. A. Pierce, *Nature* 2008, **451**, 318; (b) L. Qian and E. Winfree, *Science* 2011, **332**, 1196; (c) J. Elbaz, O. Lioubashevski, F. Wang, F. Remacle, R. D. Levine and I. Willner, *Nat. Nanotechnol.,* 2010, **5**, 417; (d) J. Chen, S. Zhou and J. Wen, *Angew. Chem. Int. Ed.,* 2015, **54**, 446; (e) Y. Krishnan and F. C. Simmel, *Angew. Chem. Int. Ed.* 2011, **50**, 3124; (f) F. Zhang, J. Nangreave, Y. Liu and H. Yan, *J. Am. Chem. Soc.,* 2014, **136**, 11198.
11 D. Y. Zhang and G. Seelig, *Nat. Chem.,* 2011, **3**, 103.
12 (a) X. Chen, N. Briggs, J. R. McLain and A. D. Ellington, *Proc. Natl. Acad. Sci. U.S.A.,* 2013, **110**, 5386; (b) D. Y. Zhang, S. X. Chen and P. Yin, *Nat. Chem.,* 2012, **4**, 208.
13 (a) F. Huang, H. Xu, W. Tan and H. Liang, *ACS Nano* 2014, **8**, 6849; (b) T. Song, S. Xiao, D. Yao, F. Huang, M. Hu and H. Liang, *Adv. Mater.,* 2014, **26**, 6181; (c) T. Song and H. Liang, *J. Am. Chem. Soc.,* 2012, **134**, 10803.
14 (a) W. Zhou, W. Liang, X. Li, Y. Chai, R. Yuan and Y. Xia, *Nanoscale* 2015, **7**, 9055; (b) B. Li, A. D. Ellington and X. Chen, *Nucleic Acids Res.,* 2011, **39**, e110; (c) D. Han, G. Zhu, C. Wu, Z. Zhu, T. Chen, X. Zhang and W. Tan, *ACS Nano* 2013, **7**, 2312; (d) M. You, L. Peng, N. Shao, L. Zhang, L. Qiu, C. Cui and W. Tan, *J. Am. Chem. Soc.,* 2014, **136**, 1256; (e) J. Chen, S. Zhou and J. Wen, *Anal. Chem.,* 2014, **86**, 3108; (f) J. Chen, Y. Zhou and L. Zeng, *Chem. Commun.,* 2013, **49**, 984.
15 (a) Y. Weizmann, M. K. Beissenhirtz, Z. Cheglakov, R. Nowarski, M. Kotler and I. Willner, *Angew. Chem. Int. Ed.* 2006, **45**, 7384; (b) F. Wang, C. Lu, X. Liu, L. Freage and I. Willner, *Anal. Chem.,* 2014, **86**, 1614.
16 (a) L. Zhang, J. Zhu, T. Li and E. Wang, *Anal. Chem.,* 2011, **83**, 8871; (b) S. Shimron, F. Wang, R. Orbach and I. Willner, *Anal. Chem.,* 2012, **84**, 1042.
17 (a) J. Zhu, L. Zhang, T. Li, S. Dong and E. Wang, *Adv. Mater.,* 2013, **25**, 2440; (b) J. Zhu, L. Zhang, T. Li, S. Dong and E. Wang, *ACS Nano* 2013, **7**, 10211; (c) L. Hu, X. Liu, A. Cecconello and I. Willner, *Nano Lett.,* 2014, **14**, 6030.

ChemComm Accepted Manuscript

This journal is © The Royal Society of Chemistry 20xx