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Electronic Supporting information (ESI)

A Novel DNA binding protein-based platform for electrochemical detection of miRNA

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Table of Contents

1. Experimental

1.1 Reagents and Materials

Unless otherwise stated, all reagents and chemicals were of analytical grade and were used without further purification. Reagent grade hexaammineruthenium(III) chloride (RuHex), phosphate buffer saline (PBS) tablets (0.01M phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride, pH 7.4 at 25 °C), mercaptohexanol (MCH), Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), Tris–HCl, and EDTA were purchased from Sigma-Aldrich (Sydney, NSW, Australia). Screen-printed gold electrodes (SPGE) with a three-electrode system printed on a ceramic substrate (length 34 × width 10 × height 5 mm) (DRP-250BT) from Metrohm Dropsens (Spain). In the three-electrode system, working (4 mm diameter), counter and reference electrodes are made of gold, platinum and silver respectively. Ultrapure™ DNase/RNase-free distilled water (Invitrogen, Australia) was used throughout the experiments. Probes and synthetic NAs were acquired from Integrated DNA Technologies, Singapore and sequences are shown in table S1. All electrochemical measurements were performed using a CH1040C potentiostat (CH Instruments, USA).

1.2 Capture and magnetic purification of target

Working concentration (10 µM) of biotinylated capture probe (CP1) and serial dilutions (1nM, 100pM, 10pM, 1pM, and 100fM) of synthetic miR-891-5p (samples) were prepared in nuclease free water H_2O. The target miRNA was captured and magnetically purified following our previously described protocol with slight modifications.[1, 2] Unless otherwise stated, all incubations in this section were carried out at room temperature with shaking at 300 rpm. Briefly, 15 µL of 10 µM CP1 solution were mixed with 10 µL of 5X saline-sodium citrate (SSC) buffer and 10 µL of each sample dilution in separate microcentrifuge tunes. For no-target control (NoT), 10 µL nuclease free water was used instead of sample. Separate reaction

tubes were prepared for end fill-in negative (EFN), no-HRP, and Bare controls where 1nM miRNA dilution was mixed CP1 and SSC buffer. For wrong probe (WP) control, 15 µL of 10 µM CP1-W were mixed with 10 µL SSC buffer and 10 µL of 1nM sample. All reactions were carried out in triplicate. The reaction tubes were vortex mixed and heated at 55°C for 2 minutes, followed by 1-hour incubation.

Dynabeads™ MyOne™ Streptavidin C1 [Invitrogen™ Cat # 65001] were prepared as per manufacturer's recommendations. Briefly, Dynabeads™ were resuspended by gently inverting stock bottles several times. Appropriate volume (10 µL per reaction) of Dynabeads™ was transferred to a microcentrifuge tube and washed 2 times with 500 µL of solution A (0.1 M NaOH, 0.05 M NaCl) and 2 times with 500 µL solution B (0.1 M NaCl). Beads were magnetically separated by placing on a magnetic rack, and supernatant discarded after every wash step. After final wash, beads were resuspended in appropriate volume of solution B (35 µL per 10 µL initial volume). 35 µL of resuspended beads was mixed with the target-capture probe mixture (35 µL) from previous step and incubated for one hour. After incubation, beads were magnetically separated, and supernatant was discarded, followed by 3 times washing with ≈500 µL 1X binding and washing (B&W) buffer (10 mM Tris-HCL, pH 7.5; 1 mM EDTA; 2 M NaCl). Beads were magnetically separated after every wash step as described above. The beads were placed on ice until further processing and end fill-in reaction mixture (see 1.3) was added directly to the beads after final wash.

1.3 5`-overhang end fill-in and heat release of captured target

End fill-in reaction mixture (30 µL per reaction) was prepared by mixing 3 µL of 10X T4 DNA polymerase (T4DP) reaction buffer (Thermo Scientific™, Cat# EP0061), 0.3 µL of 10 mM dNTP mix (Thermo Scientific™, Cat# R0192) to make a final concentration of 0.1 mM, 0.5U

of T4DP and the volume was made up to 30 µL using UltraPure™ water. No T4DP was added for EFN control reaction. The end fill-in reaction mixture was added directly to the beads from section 1.2 after last wash and incubated at 12 °C for 15 minutes. At the end of incubation, reaction tubes were immediately transferred to a magnetic rack placed on ice and the supernatant was magnetically separated from beads and discarded. Beads were washed 3 times with 500 µL 1X SSC buffer and finally resuspended in 10 µL of 5X SSC buffer. Captured and end filled-in target was heat released from the bead bound capture probe by heating at 95 °C for 5 minutes. The reaction tubes were then quickly transferred to a magnetic rack placed on ice where the supernatant containing end filled-in target (5`-miRNA-DNA-3` chimera) was collected and stored at -20˚C until further processing.

1.4 PCR verification of end fill-in reaction

Success of end fill-in reaction was verified by conventional PCR. Amplification was carried out using Applied Biosystems™ AmpliTaq Gold™ 360 Master Mix (Cat# 4398881). PCR reaction mixture contained 12.5 µL of 2X master mix, 0.5 µL each of 10 µM forward and reverse primers (table S1), and 5 µL of template (heat released purified miRNA-DNA chimera, 1 nM starting concentration, or EFN and NoT as described in section 1.2). Reaction volume was made up to 25 µL using PCR-grade distilled water. PCR was carried out at C1000™ Thermal Cycler (Bio-Rad™) using following reaction conditions: initial denaturation at 95˚C for 10 minutes, 25 cycles at 95˚C for 30 seconds, 61 °C for 30 seconds, and 72 °C for 1 minute with a final extension step at 72 °C for 7 minutes. The amplified fragments were visualized using 2% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer stained with SYBR Safe. Electrophoresis was carried out in 1X TAE at 90-95 V for 45 minutes. Invitrogen™ 50 bp DNA Ladder [Cat# 10416014] was included as size marker.

1.5 Sensor fabrication and characterization

Unless otherwise stated, electrodes were washed between each step using 10 mM PBS and dried by gentle air flow. Screen-printed gold electrodes (SPGEs) were pre-treated electrochemically as described earlier.[3] Cyclic voltammetry reaction for the one-electron reduction of $[Fe(CN)_6]^{3-}$ [2.0 mM in PBS (0.5 M KCl)] was carried out and the peak current as a function of scan rate was recorded.[4] The active surface area of working electrode was measured by using Randles -Sevcik equation:

$$i_p = (2.69 \times 10^5)\, n^{3/2}\, AD^{1/2}\, Cv^{1/2} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

where, i_p is the peak current (Amp), n is the number of electrons transferred ($Fe^{3+} \rightarrow Fe^{2+}$, n = 1), A is the effective area of working electrode. (cm^2), D is the diffusion coefficient of $[Fe(CN)_6]^{3-}$ (taken to be 7.60×10^{-5} cm^2 s^{-1}), v is the scan rate (V s^{-1}), and C is the concentration (mol cm^{-3}).

Self-assembled monolayer (SAM) of thiolated capture probes (CP2) was prepared as described earlier with slight modifications.[3, 5] TCEP solution was prepared fresh before each experiment and all TCEP solutions and reactions were protected from light. Disulfide bonds of thiolated CP2 were reduced by mixing 1 µL of 1 mM TCEP with 99 µL of 0.2 µM CP2 and incubating for 30 minutes. 6 µL of immobilization buffer (I-buffer) [10 mM Tris-HCl + 1 mM EDTA + 0.1 M NaCl + 10 mM TCEP (pH 7.4)] was mixed with to 4 µL of reduced CP2 and disposed on to the SPGE followed by 1 hour and incubation. The sensor surface was passivated by disposing 5 µL of 1 mM MCH on its surface and incubating for 20 minutes. Density of CP2 on electrode surface was measured by conducting chronocoulometry (CC) first in 50 µL of E-Buffer (10 mM Tris-HCl; pH 7.0) and then 50 µL of 50 µM ruthenium hexamine $[Ru(NH_3)_6]^{3+}$ (RuHex) solution. Charge (Q) versus $t^{1/2}$ for both CC experiments was plotted. Capacitive charge (Q_{dL}) and total charge (Q_{total}) were obtained from the intercept at t = 0, from the E-Buffer and RuHex plots respectively. Following parameters were used for CC: initial potential

0.2V, final potential -0.5V, number of steps 1, pulse width 0.25 sec, and sample interval 0.002 sec. (Figure S2).[5, 6] The charge corresponding to the electrostatic binding of RuHex to surface-confined CP2 (Q_{ss}) was calculated as described earlier.[7]

$$Q_{ss} = Q_{Total} - Q_{\mathrm{d}l} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

The probe density (Γss) was calculated by using the following equation:

$$\Gamma_{ss} = \left(\frac{Q_{ss}N_A}{nFA}\right)\left(\frac{z}{m}\right) \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (3)$$

where n is the number of electrons (n = 1), A is the area of the working electrode, m is the number of nucleotides in the DNA, z is the charge of the redox molecules, F is Faraday constant, and N_A is Avogadro's number.

1.6 Conjugation of p53 protein with HRP

Recombinant human p53 protein (ab84768, Abcam) was diluted to a concentration of 10 ngµL^{-1} in PBS and was conjugated with HRP using a commercial conjugation kit (ab102890, Abcam) as per manufacturer's instructions. Briefly, 1 µL of modifier reagent was added per 10 µL of protein and mixed gently. The protein-modifier mix was then pipetted directly onto the lyophilized HRP mix and resuspended gently by pipetting the liquid up and down a few times. The mix was incubated for 3 hours at the room temperature protected from light. The reaction was stopped by adding 1 µL of Quencher reagent (per 10 µL of the protein) and incubating for another 30 minutes. The conjugated protein was stored at 4 °C protected from light.

1.7 Electrochemical detection

Purified end filled-in samples (10 µL) were added on the fabricated sensor and incubated for 1 hour. Detection probe (DP) was diluted in hybridization buffer (10 mM PBS, 0.25 M NaCl; pH

7.4) to a final concentration of 1µM. 10 µL of diluted DP was added to the sensor surface and incubated for 1 hour. In next step, 12 µL of protein mixture (10 µL p53 binding buffer [10 mM tris buffer; pH 7.0, 0.5 mM EDTA, 20 mM KCl, 1 mM DTT, 0.2% Tween-20], 1 µL of 10 ng horseradish peroxidase (HRP) conjugated p53 and 1 µL of 1 µgµL-^{1} oligo-dTs (equimolar mixture of 15 and 20 nt long oligo-dTs) was added on to the SPGE surface and incubated for 30 minutes. Subsequently, 35 µL of 1 mM freshly prepared (in PBS) hydroquinone solution was added on to the electrode surface. Chronoamperometric measurements were carried out with a potential of -200 mV. Once the background current was stabilized (around 50 sec), 35 µL of 0.1 M H_2O_2 was injected to initiate the catalytic reaction. The electrochemical reaction was run up to 200 seconds and steady state current was recorded. All measurements were performed at room temperature.

2. Supplementary Tables

Table S1: Oligonucleotide sequences

	Oligonucleotide sequences (5′–3′)
Biotinylated capture probe (CP1)	CCGGACATGCCCGGGCATGTTCAGTGGCTCAGGTTCGTTGCA/3Bio/
Thiolated capture probe (CP2)	TCAGTGGCTCAGGTTCGTTGCA/3ThioMC3-D
Detection probe/Reverse Primer	CCGGACATGCCCGGGCATGT
Synthetic miR-891a-5p	UGCAACGAACCUGAGCCACUGA
miR-891a-5p-DNA/Forward Primer	TGCAACGAACCTGAGCCACTG A
m-891b	UGCAAC**UU**A**C**CUGAG**U**CA**U**UG**T**
Wrong Probe (CP1-W)	CCGGACATGCCCGGGCATGTCTCGGGGCAGCTCAGTACAGGA/3Bio/

Table S2: Comparison with recent electrochemical miRNA detection platforms

Method	**Linear range**	**LOD**	**Ref**
HCR electroanalytical assay	100 aM – 100 nM	53 aM	[8]
Bipedal DNA walkers and LNA modified toehold mediated strand displacement reaction (TMSDR) based ratiometric electrochemical sensor	100 aM – 100 fM	67 aM	[9]
Nucleic acid hybridization on electrically reconfigurable network of gold-coated magnetic nanoparticles	10 aM – 1 nM	10 aM	[10]
Gold-loaded iron oxide nanocubes mediated catalytic signal amplification strategy	100 fM – 1 µM	100 fM	[7]
miRNA detection based on their increased affinity with gold electrodes after polyadenylation	5fM – 5 pM	10 fM	[2]
Rolling circle amplification and Chronocoulometry (RCA–CC) based miRNA detection	100 fM – 1 nM	100 fM	[11]

Tetrahedron-based electrochemical miRNAs sensor (EMRS)	10 fM – 10 nM (avidin-HRP) 10 aM – 1 nM (poly-HRP80)	10 fM (avidin-HRP) 10 aM (poly-HRP80)	[12]
Three-Mode Electrochemical miRNA Sensing	10 aM – 1 μM	5 aM	[13]
Electronic microRNA detection with nanostructured microelectrode (NME) chips	10 aM – 1 fM	10 aM	[14]
Multifunctional DNA tetrahedron assisted catalytic hairpin assembly (MDTs-CHA)	10 aM – 100 pM	7.2 aM	[15]

3. Supplementary Figures

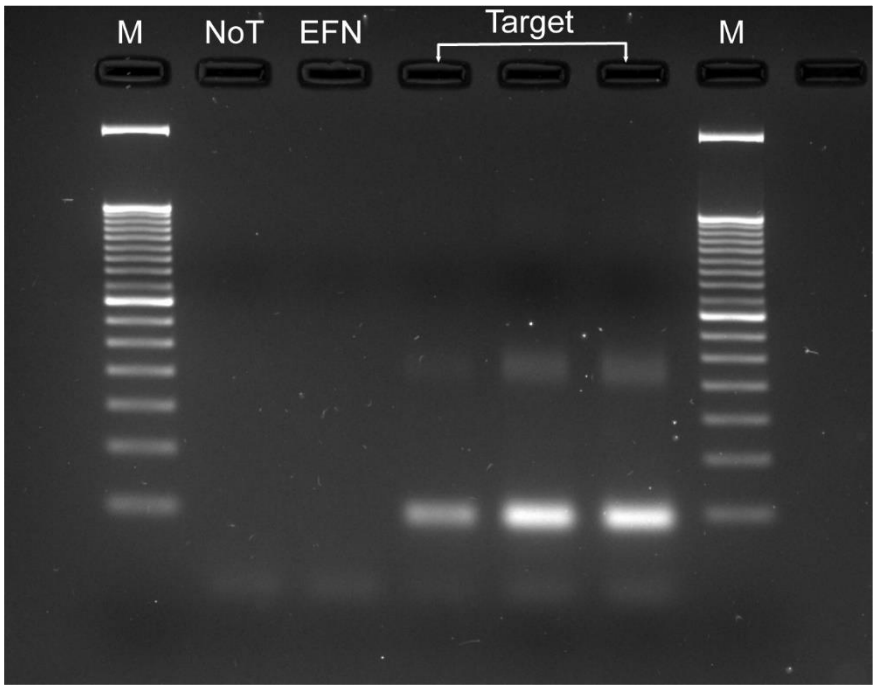

Figure S1: End-point PCR verification of end fill-in experiment. 2% Agarose gel stained with SYBR Safe. Wells 1 and 7 (M): 50 bp DNA ladder (Invitrogen Cat# 10416014). 42 bp sharp bands were observed for end-filled samples (target, lanes 4, 5, and 6) while no band was observed for end fill-in negative (EFN lane 3) and no-target control (NoT lane 2).

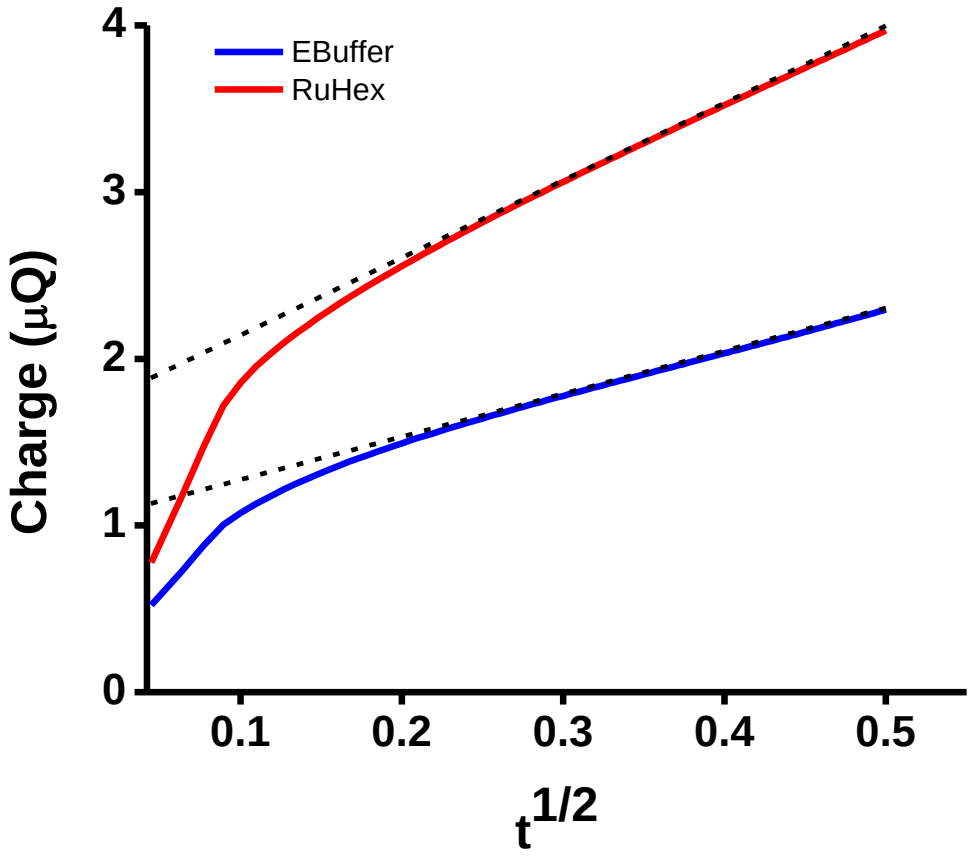

Figure S2: Chronocoulometric responses for screen-printed gold electrodes (SPGEs) modified with self-assembled monolayer (SAM) of thiolated capture probes in the presence of E-buffer (10 mM Tris-HCl; pH 7.0) and RuHex (50 µM ruthenium hexamine $[Ru(NH_3)_6]^{3+}$ solution in E-buffer).

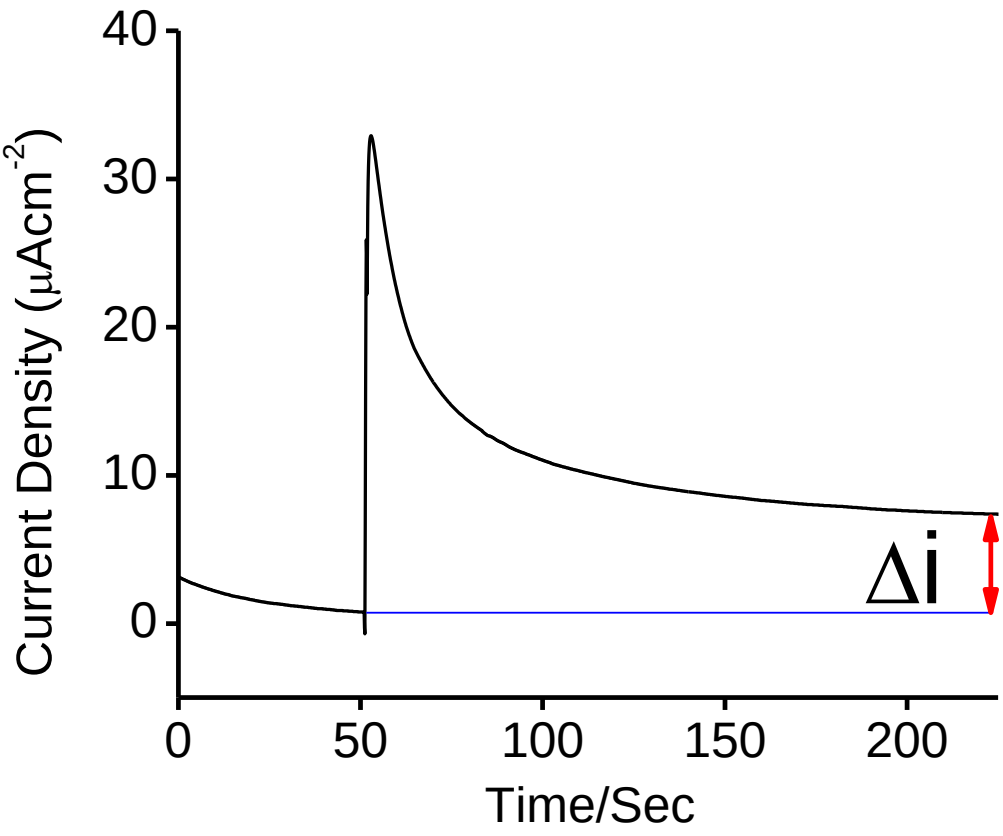

Figure S3: Calculation of the concentration dependent current response. Difference (Δi) between background current (B.C) and steady state current (S.S.C) was calculated as follows:

$$\Delta i = S.S.C - B.C$$

References

1 M. N. Islam, M. K. Masud, N.-T. Nguyen, V. Gopalan, H. R. Alamri, Z. A. Alothman, M. S. A. Hossain, Y. Yamauchi, A. K. Lamd and M. J. A. Shiddiky, *Biosens. Bioelectron.*, 2018, **101**, 275-281.

2 K. M. Koo, L. G. Carrascosa, M. J. A. Shiddiky and M. Trau, *Anal. Chem.*, 2016, **88**, 2000-2005.

3 J. Zhang, S. Song, L. Wang, D. Pan and C. Fan, *Nat. Protoc.*, 2007, **2**, 2888.

4 M. J. A. Shiddiky, A. A. J. Torriero, C. Zhao, I. Burgar, G. Kennedy and A. M. Bond, *J. Am. Chem. Soc.*, 2009, **131**, 7976-7989.

5 M. J. A. Shiddiky, A. A. J. Torriero, Z. Zeng, L. Spiccia and A. M. Bond, *J. Am. Chem. Soc.*, 2010, **132**, 10053-10063.

6 E. L. S. Wong and J. J. Gooding, *Anal. Chem.*, 2003, **75**, 3845-3852.

7 M. Kamal Masud, M. N. Islam, M. H. Haque, S. Tanaka, V. Gopalan, G. Alici, N.-T. Nguyen, A. K. Lam, M. S. A. Hossain, Y. Yamauchi and M. J. A. Shiddiky, *Chem. Commun.*, 2017, **53**, 8231-8234.

8 Q. Guo, Y. Yu, H. Zhang, C. Cai and Q. Shen, *Anal. Chem.*, 2020, **92**, 5302-5310.

9 J. Zhang, L.-L. Wang, M.-F. Hou, Y.-K. Xia, W.-H. He, A. Yan, Y.-P. Weng, L.-P. Zeng and J.-H. Chen, *Biosens. Bioelectron.*, 2018, **102**, 33-40.

10 R. Tavallaie, J. McCarroll, M. Le Grand, N. Ariotti, W. Schuhmann, E. Bakker, R. D. Tilley, D. B. Hibbert, M. Kavallaris and J. J. Gooding, *Nat. Nanotechnol.*, 2018, **13**, 1066-1071.

11 B. Yao, Y. Liu, M. Tabata, H. Zhu and Y. Miyahara, *Chem. Commun.*, 2014, **50**, 9704-9706.

12 Y. Wen, G. Liu, H. Pei, L. Li, Q. Xu, W. Liang, Y. Li, L. Xu, S. Ren and C. Fan, *Methods*, 2013, **64**, 276-282.

13 M. Labib, N. Khan, S. M. Ghobadloo, J. Cheng, J. P. Pezacki and M. V. Berezovski, *J. Am. Chem. Soc.*, 2013, **135**, 3027-3038.

14 H. Yang, A. Hui, G. Pampalakis, L. Soleymani, F.-F. Liu, E. H. Sargent and S. O. Kelley, *Angew. Chem. Int. Ed.*, 2009, **48**, 8461-8464.

15 Y. Zhang, X. Zhang, B. Situ, Y. Wu, S. Luo, L. Zheng and Y. Qiu, *Biosens. Bioelectron.*, 2021, **183**, 113205.