Electronic Supplementary Material (ESI) for Chemical Communications

Electronic Supporting Information

Simultaneous profiling of multiple gene-methylation loci by electrochemical methylation-specific ligase detection reaction

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Reagents and Materials. All of the synthetic single-stand DNAs (ssDNAs) were purchased from Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). The *E. coli* DNA ligase was purchased from TaKaRa Biotech. Co. Ltd. (Dalian, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and 3-mercaptopropanoic acid (MPA) were obtained from Alfa Aesar (Tianjin, China). *N*-hydroxysuccinimide (NHS) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Magnetic beads (MBs, carboxyl-coated, 1 μm) were obtained from Bangs Lab Co. Ltd. (IN, USA). EDAC/NHS solution (1:1 per 1 mg in 100 μL) was freshly prepared in 0.1 M phosphate buffer solution (PBS, pH = 8.6). All other chemicals were of analytical grade. All solutions were prepared with doubly distilled water.

Preparation of the CdS and PbS Quantum Dots (QDs). The preparation of the QDs was followed the reported procedure with a slightly-modification.[1] Sodium bis(2-ethylhexyl)sulfosuccinate (AOT) (14.0g) was first dissolved in a mixture of n-heptane:water (200 ml: 4 ml). The resulting solution was separated into two sub-volumes of 120 ml and 80 ml. A 0.48 ml aliquot of 1 M $Cd(NO_3)_2$ or $Pb(NO_3)_2$ solution was added to the 120 ml sub-volume, while a 0.32 ml of 1 M Na_2S solution was added to the 80 ml sub-volume. The sub-volumes were stirred for 1 h, and then mixed and stirred for another 1 h under nitrogen. The QDs were added with 0.34 ml of 0.32 M cysteamine solution and 0.66 ml of 0.32 M sodium 2-mercaptoethane sulfonate solution, and mixing under nitrogen for 24 h. The resulting QDs were

obtained by evaporating the heptane *in vacuo* and washing with pyridine, hexane and methanol.

Preparation of PbS-P1 and CdS-P2 Conjugates. The QDs of PbS and CdS were synthesized according to the literature (details in Supporting Information).[33] The PbS-P1 conjugates were prepared as follows: Ten-μL of 8 OD ml^{-1} P1 was added into 10 μL of 1 mg ml^{-1} PbS aqueous solution and the mixture was stirred for 24 h under the atmosphere of N_2. After that, the mixture was centrifuged and re-suspended in a 0.1 M PBS (pH = 7.4, with 0.24 M NaCl and 0.1% NaN_3), and dialyzed in a 0.1 M PBS (pH = 7.4, with 0.2 M NaCl) for 48 h. The resultant PbS-P1 conjugates were kept under 4 ^oC for further use. The CdS-P2 conjugates were prepared similar as described above.

Preparation of MB-P3P4 Conjugates. Ten-μL of a carboxyl-coated MBs suspension were washed twice by 100 μL of 0.1 M imidazole buffer (pH = 7.0). The resultant MBs were collected and re-suspended in 100 μL of 0.1 M PBS (pH = 8.6). After that, 100 μL of an EDAC/NHS solution was applied to the MBs and incubated for 30 min. Following the removal of the EDAC/NHS solution, 50 μL of 10 μM P3 and P4 were added to the MBs and stirred for 3 h at 50 ^oC. The MB-P3P4 conjugates were collected and washed by 0.1 M imidazole buffer (pH = 7.0, with 0.5% SDS) for three times and doubly distilled water for twice at 65 ^oC. The resultant MB-P3P4 conjugates were re-suspended in a 0.1 M PBS (pH = 7.4, with 0.2 M NaCl and 0.1%

NaN_3) and kept under 4 ^oC for further use.

Apparatus and Measurements. Electrochemical measurements were carried out on a CHI 660C electrochemical workstation (Chenhua, Shanghai, China) equipped with a three-electrode system, which consisted of a glassy carbon working electrode, a platinum wire auxiliary electrode, and a Ag/AgCl (saturated KCl) reference electrode. The electrochemical stripping detection involved a 600 s electrodeposition at −1.10 V, and stripping from −1.10 to −0.40 V using a square wave voltammetric waveform, with a 4 mV potential step, a 25 Hz frequency, and a 25 mV amplitude. The electrode was cleaned after each measurement by applying 0.3 V for 45 s in the electrolyte solution. Scanning electron microscopy (SEM) micrographs were recorded on a Hitachi S-3700N microscope (Hitachi, Japan). Scanning electron microscopy/energy-dispersive spectroscopy (SEM/EDS) analysis was performed on a Hitachi S-3700N (Hitachi, Japan) scanning electron microscope equipped with an EDS detector (HORIBA EMAX, Japan). The measurements were performed at 20 kV. Before the analysis, the samples were golden-coated to facilitate a conductive surface. All experiments were performed at room temperature.

Analysis Procedure. Samples were first treated with EpiTect Bisulfite kits (Qiagen, Shanghai, China) according to the recommended protocol. The treated samples were incubated at 38 ^oC with 10 μL of the MB-P3P4 solution for 2 h, and then with 10 μL of the PbS-P1 and CdS-P2 solution for 1 h. After washed twice by doubly distilled

water, the resultant MB-conjugates were incubated with 20 μL of an *E. coli* ligase solution in the ligation buffer (30 mM Tris–HCl, 4 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.005% BSA, and 0.2 mM NAD^+) for 1 h at 38 ^oC. The resultant mixture was heated at 90 ^oC for 10 min. Washed by a 0.1 M PBS (pH = 7.0, with 0.2 % Tween 20), and doubly distilled water at 90 ^oC, the obtained MB-conjugates were dissolved in 100 μL of 0.1 M HNO_3 for 1 h and detected in a 0.1 M acetate buffer (pH = 4.7, with 400 μg L^{-1} Bi^{3+}) by square wave voltammetry (SWV).

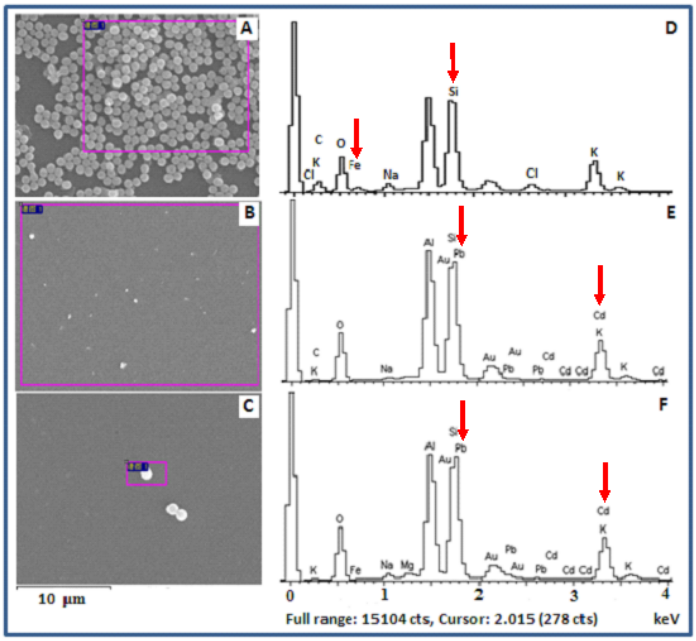

Fig. S1 SEM/EDS images of (A and D) MBs, (B and E) PbS-P1 and CdS-P2 conjugates, and (C and F) the MBs obtained from the analysis of 50 nM T1.

Table S1 EDS analysis of MBs, PbS- and CdS-conjugates, and MBs from the analysis T1

Elements	MBs		PbS-P1 and CdS-P2 conjugates		MBs from the analysis of T1	
	Weight %	Atom %	Weight %	Atom %	Weight %	Atom %

C K	22.36	33.90	0.77	1.53	37.14	55.55
O K	38.72	44.07	36.45	54.61	0.98	1.02
Mg K	—	—	—	—	0.65	0.64
Na K	2.41	1.91	0.43	0.45	—	—
Al K	—	—	18.21	16.17	17.16	15.22
Si K	20.59	13.35	22.94	19.57	22.60	19.25
K K	9.07	4.23	10.32	6.32	10.48	6.41
Cd L	—	—	0.20	0.04	0.48	0.10
Cl K	1.70	0.87	—	—	—	—
Fe K	5.15	1.68	—	—	1.74	0.75
Au M	—	—	10.29	1.25	8.43	1.02
Pb M	—	—	0.39	0.05	0.35	0.04
total	100.00		100.00		100.00	

Introduction of square-wave voltammetry. Square-wave voltammetry (SWV) is a technique beneficially used in analytical applications and in fundamental studies of electrode mechanisms. SWV is a further improvement of staircase voltammetry which is itself a derivative of linear sweep voltammetry. A square wave is superimposed on the potential staircase sweep as shown in Fig. S5. The current is measured at the end of each potential change, right before the next. Because the Faraday's current (i_f) is in proportion to $t^{(-1/2)}$, and the charging current (i_c) is proportion to $e^{(-t/RC)}$. The attenuation of i_f is slower than that of i_c. Therefore the contribution to the current signal from the i_c is minimized. The differential current is then plotted as a function of potential, and the reduction or oxidation of species is measured as a peak or trough. Due to the lesser contribution of charging current the detection limits for SWV are on the order of nanomolar concentrations.[2]

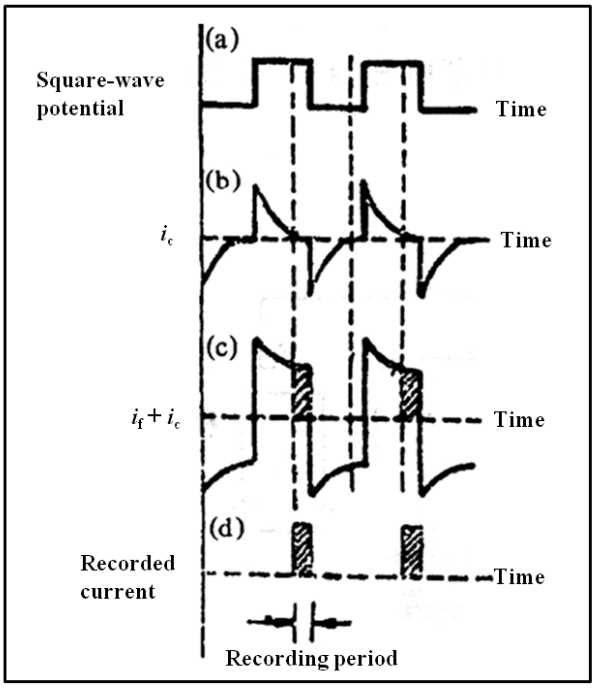

Fig. S2 The detection principle of square wave voltammetry. $i_{f,}$ Faraday's current. $i_{c,}$ charging current

Optimization of E-msLDR. It is worth noting that the precision of the E-msLDR method for gene-methylation analysis is affected by the following two factors: (a) the discrimination of the LDR to base-mismatched sequences during hybridization, (b) the release of the disjoined probes after the LDR. Wrong discrimination of LDR at mismatched sequences can cause false judgment of methylation status, and incomplete release of the disjoined probes can lead to positive error in the quantification of methylation level. Therefore, the hybridization and heating conditions of the E-msLDR were carefully optimized.

Hybridization Conditions. Temperature is one of the most important parameters in nucleic acid hybridization. The effect of hybridization temperature on the analytical performance of the E-msLDR is depicted in Fig. S3A. The SWV peak current from

the analysis of 50 nM T1 was recorded at various hybridization temperatures from 10 ^{o}C to 55 ^{o}C for 2 h. Small peak current was found at the hybridization temperature of 10 °C. With the increase in the hybridization temperature to 38 °C, the peak currents for both methylation loci increased and reached their maximal values. After that, both of the peak currents reduced rapidly. The average melting temperature (Tm) for the duplexes of probes and target p53 gene is around 36 ^{o}C. At low hybridization temperature, probes and target gene cannot easily form stable duplex. Raising the reaction temperature closer to the Tm of the duplexes allows for enhancement of the hybridization efficiency and specificity.[3] Besides, the operating temperature of the *E. Coli* ligase is 37 °C.[4] With a further increase in the hybridization temperature, the duplexes for cDNA will be partly transformed into ssDNA, resulting in lower detection signal.

The effect of hybridization time on the analysis performance of the E-msLDR was investigated by recording the SWV peak current from the analysis of T1 at 38 °C with different hybridization times (Fig. S3B). The peak currents for both methylation loci initially showed a linear rise with the increase in the hybridization time, indicating the accelerating temperature effect. After the hybridization time was longer than 2 h, the peak currents reached their maximum values and leveled off. Therefore, the hybridization procedure in the E-msLDR was performed at 38 ^{o}C for 2 h.

Heating Conditions. The effect of the heating temperature and the heating time on the analysis performance of the E-msLDR was investigated by recording the SWV peak current from the measurement of 50 nM T4. The SWV responses reduced with

the increase in the heating temperature from 70 to 90 ^{o}C. When the heating temperature was higher than 90 ^{o}C, negligible SWV responses were found and no overall trend of increase or decrease was observed (Fig. S3C), indicating the complete release of the disjoined probes together with tagged QDs.

The effect of heating time on the analysis of the E-msLDR was investigated at 90 ^{o}C with different heating time. As shown in Fig. S3D, a linear decrease of the peak intensity was found with the increase in the heating time up to 20 min. At longer heating time, the response leveled off and approached a minimum signal, indicating the complete release of the disjoined QDs. Therefore, the heating procedure in the E-msLDR was performed at 90 ^{o}C for 20 min.

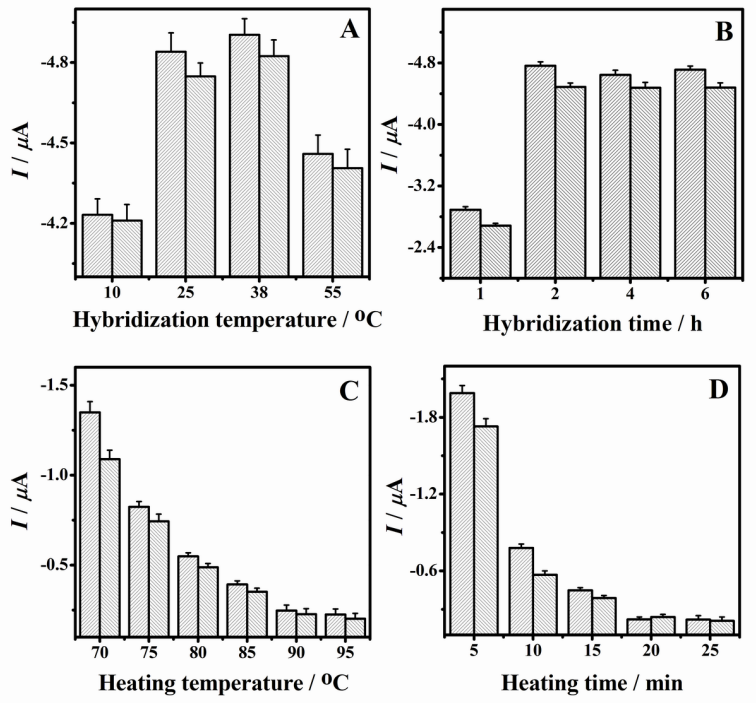

Fig. S3 Effect of (A) the hybridization temperature, (B) the hybridization time, (C) the heating temperature, and (D) the heating time on the analysis of the E-msLDR. (A) and (B) were recorded following the analysis of 50 nM T1, and (C) and (D) were recorded following the analysis of 50 nM T4.

Optimization of the Square Wave Voltammetry Detection. In order to achieve high sensitivity and reproducibility, the parameters for square wave voltammetry (SWV) analysis on bismuth film modified electrode (BiFE) were carefully optimized.

Deposition potential. The influence of the deposition potential on the stripping signals of Cd^{2+} and Pb^{2+} was investigated under the deposition time of 600 s with various deposition potentials from −0.85 to −1.15 V in Fig. S4A. The SWV peak current of 10 μg L^{-1} Pb^{2+} increased greatly and then reached its maximum value with the decrease of deposition potential from −0.85 to −0.90 V. After that, the SWV response slightly reduced with the decrease in the deposition potential to −1.15 V. Similarly, the SWV peak current of Cd^{2+} increased with the decrease of deposition potential from −0.85 to −1.10 V. After that, the SWV response leveled off. Therefore, all further measurements were carried out with a deposition potential of −1.10 V.

Deposition time. The influence of the deposition time upon the analytical signal of 10 μg L^{-1} Pb^{2+} and Cd^{2+} was examined under the deposition potential of −1.10 V with various deposition times from 50 to 800 s in Fig. S4B. The stripping currents of Pb^{2+} increased linearly along with the deposition time prolonged to 600 s. As the deposition time longer than 600 s, the peak current began to leave off and the peaks became distorted. Similarly, the SWV peak current of 10 μg L^{-1} Cd^{2+} increased and reached its maximum value with the increase in the deposition time to 450 s. After the deposition time was longer than 550 s, the SWV response decreased. Therefore, 600 s was adopted as optimal accumulation time for the following experiments.

Concentration of Bismuth. The thickness of the bismuth film was controlled by

varying the Bi^{3+} concentration in the plating solution. The effect of the concentration of bismuth on analytical signal was optimized by keeping deposition time (600 s) and deposition potential (−1.10 V) constant while changing the concentration of Bi^{3+} in the analysis solution. As shown in Fig. S4C, the response of Pb^{2+} initially increased quickly with the increase in the concentration of Bi^{3+} up to 200 μg L^{-1}, then increased slowly with the bismuth up to 600 μg L^{-1}. After that, the response of Pb^{2+} slightly decreased when the concentration of Bi^{3+} was more than 600 μg L^{-1}. Similarly, the response of Cd^{2+} increased rapidly with the concentration of Bi^{3+} up to 200 μg L^{-1}, and then nearly leveled off above 600 μg L^{-1} Bi^{3+}. After the concentration of Bi^{3+} more than 600 μg L^{-1}, the response of Cd^{2+} reduced. This may be attributed to the formation of bismuth multilayers on the electrode surface at high concentration of Bi^{3+}, which is not favorable for metal ions diffusing out of the bismuth film during the stripping step. Subsequently, all the following experiments were conducted using a bismuth-film electrode formed in a plating solution containing 600 μg L^{-1} bismuth, in connection to 600 s deposition at −1.10 V.

Precondition time. Between each determination, the BiFE was placed in 0.2 M acetate buffer (pH = 4.6) and applied at +0.3 V for certain time to renew the electrode surface. Fig. S4D shows the determination of 10 μg L^{-1} Pb^{2+} at a GCE treated with different precondition time. With the increase in the precondition time from 15 to 45 s, the mean of 5-time determinations of Pb^{2+} decreased with reductive error bars, indicating the improvement of the determination repeatability. Short precondition time may not be able to totally eliminate the residue of the metal in the bismuth film,

causing accumulation effect. When the precondition time was longer than 45 s, the mean of the response of Pb^{2+} treaded to be constant and the error bar for the determination reached its minimum. Therefore, a washing step of oxidation at +0.3 V for 45 s was performed between each determination.

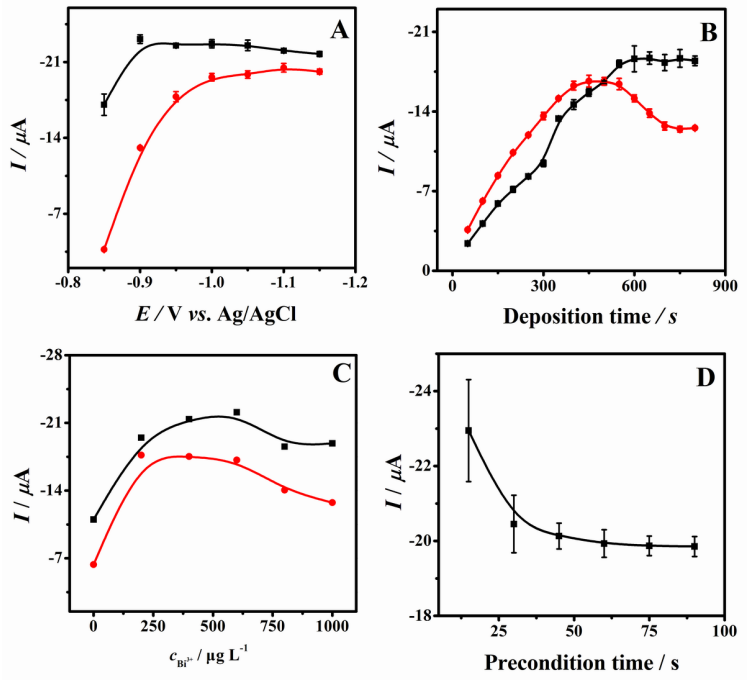

Fig. S4 Effect of (A) deposition potential, (B) deposition time, (C) concentration of bismuth, and (D) precondition time on the stripping signals of Cd^{2+} (red line) and Pb^{2+} (black line).

Methylation of Samples and Inhibition. Samples containing different methylation level of T2 were prepared as the following procedures. Ten-μL of 10 μM T4 were incubated with 10 μM H1 (5'-AGCAGCGCTCATGGT-3') at 38 ^{o}C for 2 h. The samples were then added with 8 μL of 10XNE Buffer [50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH = 7.9)], 10 μL of 1280 μM SAM, and 3.0 unit of M. *SssI*. The mixture was incubated at 37 ^{o}C for various times of 1 or 2 h. After

that, the samples were purified by DNA purification kit.

Samples containing different methylation levels of T3 were prepared similar as the above procedure except H2 (5'-CTTCCACTCGGATAA-3') was used to hybridize with T4. Samples containing different methylation levels of T1 were prepared by using H1 and H2 to hybridize with T4.

Procedure of clinic sample preparation. As for the analysis of clinical samples, routine sample preparation processes are required prior to electrochemical detection. In brief, venous blood and tissue samples can be collected from cancer patients and donors with the aid of cooperative hospital.[5,6] Then, genomic DNA extraction was able to be implemented by the standard phenol-chloroform procedure.[6-8] The PCR amplification can be performed according to literature method.[9,10] The detailed preparation procedures of these steps were carefully demonstrated in literatures.

Reference

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