SUPPORTING INFORMATION

Amperometric bioplatforms to detect regional DNA methylation with single-base sensitivity

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MATERIALS AND METHODS

Apparatus and electrodes

A potentiostat (model 812B, CH Instruments, Austin, TX) controlled by the CHI812B software was used for the amperometric measurements. Screen-printed carbon electrodes (SPCEs, DRP-110) and the specific cable (DRP-CAC) for connection with the potentiostat were from Metrohm-DropSens (Spain). A homemade Teflon casing with an embedded neodymium magnet (AIMAN GZ) was used for the reproducible magnetic capture of the modified MBs on the working electrode surface of the SPCEs. All the electrochemical measurements were performed at room temperature.

Other used apparatuses were: A Vortex (Bunsen AGT-9) for the homogenization of the solutions, a steam sterilizer (Raypa), a biological safety cabinet (Telstar Biostar), a thermocycler (SensoQuest LabCycler, Progen Scientific Ltd.), an incubator shaker (Optic Ivymen® System, Comecta S.A, Sharlab), and a magnetic particle concentrator (DynaMag™-2, 123.21D, Invitrogen Dynal AS).

Reagents and solutions

All reagents used were of the highest available analytical grade. Magnetic microbeads (MBs) modified with carboxylic groups (HOOC-MBs, 2.7 μm ϕ, 10 mg mL^{-1}, Cat. No: 14305D) and Streptavidin (Strep-MBs, 2.8 μm ϕ, 10 mg mL^{-1}, Cat. No: 11205D) were acquired from Invitrogen-ThermoFisher™, while Neutravidin-MBs (1.0 μm ϕ, 10 mg mL^{-1}, Cat. No: 78152104010350) were from SpeedBeads™, GE Healthcare. NaCl, KCl, NaH_2PO_4, Na_2HPO_4 and Tris–HCl were from Scharlab; *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC), *N*-hydroxysulfosuccinimide (Sulfo-NHS), ethanolamine, hydroquinone (HQ) and H_2O_2 (30 %, w/v) were purchased from Sigma-Aldrich and 2-(N-morpholino)ethanesulfonic acid (MES) was from Gerbu.

Anti-5-methylcytosine monoclonal antibody (anti-5-mC, Cat. No: A-3002), anti-5-hydroxymethylcytosine polyclonal antibody (anti-5-hmC, Cat. No: A-4001), 5-mC ELISA buffer (Cat. No: D5325) and the dsDNA 5-hmC standard (a linear 897 bp sequence containing 201 5-hmCs) from a DNA Standard Set (Cat. No: D5405) were purchased from Zymo Research.

A blocker casein solution (a 1 % w/v purified casein ready-to-use PBS solution) from Thermo Scientific was also used.

Anti-rabbit IgG-HRP (Cat. No: 170-6515, BioRad), anti-mouse IgG-HRP (Cat. No: D5325-3-30, Zymo Research), antifluorescein (FITC) Fab fragments conjugated with HRP (HRP-anti-FITC Fab fragments, Cat. No: 11426346910, Roche), Protein A, HRP conjugate (ProtA-HRP, Cat. No: P8651, Sigma-Aldrich), ProtA-poly-HRP_{40} and ProtA-poly-HRP_{80} (Cat. No: 201 01 003 CJG and ABIN929490, Senova and antibodies online, respectively) and Histostar (Cat. No: 8460, MBL Life Science) were used.

The following sterilized buffer solutions in Milli-Q water (18 MΩ cm at 25 °C) were used: 0.05 M phosphate buffer, pH 6.0; 0.1 M phosphate buffer, pH 8.0; Tris–EDTA buffer (TE) consisting of 0.01 M Tris–HCl solution containing 1.0 mM EDTA, pH 8.0; 0.1 M Tris-HCl buffer, pH 7.2, B&W buffer consisting of 0.01 M Tris-HCl solution containing 1.0 mM EDTA and 2.0 M NaCl, pH 7.5 (B&W); 0.025 M MES buffer solution pH 5.0.

All used synthetic oligonucleotides (sequences provided in **Table S1**) were purchased from Integrated DNA Technologies, reconstituted upon reception in TE buffer to 100 μM and stored at −80 °C into small aliquots.

Table S1. DNA sequences used in this work.

Name	**Sequence 5′ → 3′**
b-Cp-*MGMT**	Biotin-CACCAAGTCGCAAACGGTGCGCAC
FITC-Dp-*MGMT**	FITC-CACCAAGTCGCAAACGGTGCGCAC
Target *MGMT***	GTCCCGACGCCCGCAGGTCCTCGCGGTGCGCACCGTTTGCGACTTGGTG
Target 1×5-mC-*MGMT***	GTCCC(M)GACGCCCGCAGGTCCTCGCGGTGCGCACCGTTTGCGACTTGGTG
Target 4×5-mC-*MGMT***	GTCCC(M)GAC(M)GCCC(M)GCAGGTCCTC(M)GCGGTGCGCACCGTTTGCGACTTGGTG
Target 1×5-hmC-*MGMT***	GTCCC(hM)GACGCCCGCAGGTCCTCGCGGTGCGCACCGTTTGCGACTTGGTG
Target 4×5-hmC-*MGMT***	GTCCC(hM)GAC(hM)GCCC(hM)GCAGGTCCTC(hM)GCGGTGCGCACCGTTTGCGACTTGGTG

C(M): 5-methylcytosine (5-mC); C(hM): 5-hydroxymethylcytosine (5-hmC)

**Cp and Dp were designed to be fully complementary to a flanking region of the gene promoter regions where methylation is known to occur.*

***Unmethylated synthetic targets or methylated with 1 or 4 cytosines (5-mC or 5-hmC) in exon 1 of the human MGMT gene (genomic sequence on chromosome 10 from 131,265,519 to 131,265,537).*

RESULTS AND DISCUSSION

Optimal experimental variables used

The concentrations used for anti-5-hmC Ab,[1] ProtA-HRP and ProtA-polyHRP$_{40}$[2] were those optimized in previous works, while the experimental variables involved in the labeling with anti-rabbit IgG-HRP, ProtA-polyHRP$_{80}$ and Histostar were optimized in this work. To do that, the amperometric responses obtained in the absence (B) and in the presence (S) of 10,000 or 1,000 pM of synthetic target 1×5-hmC-*MGMT* and the corresponding S/B ratio were compared. The obtained results are discussed in Figures S1 and S2. The experimental variables used in the compared labeling strategies are summarized in Table S2. In addition, due to the reported advantages of using Neutravidin-MBs instead of Strep-MBs, in terms of low non-specific protein-protein interactions because of the nearly neutral charge of Neutravidin, and higher affinity toward biotin,[3,4] the performance of the platform constructed using ProtA-polyHRP$_{80}$ and Strep-MBs or Neutravidin-MBs was also compared. This comparison showed (results not shown) that the calculated S/B ratio for 1,000 pM of 1×5-hmC-*MGMT* was 6 times higher with the Strep-MBs and, therefore, these were used in further work.

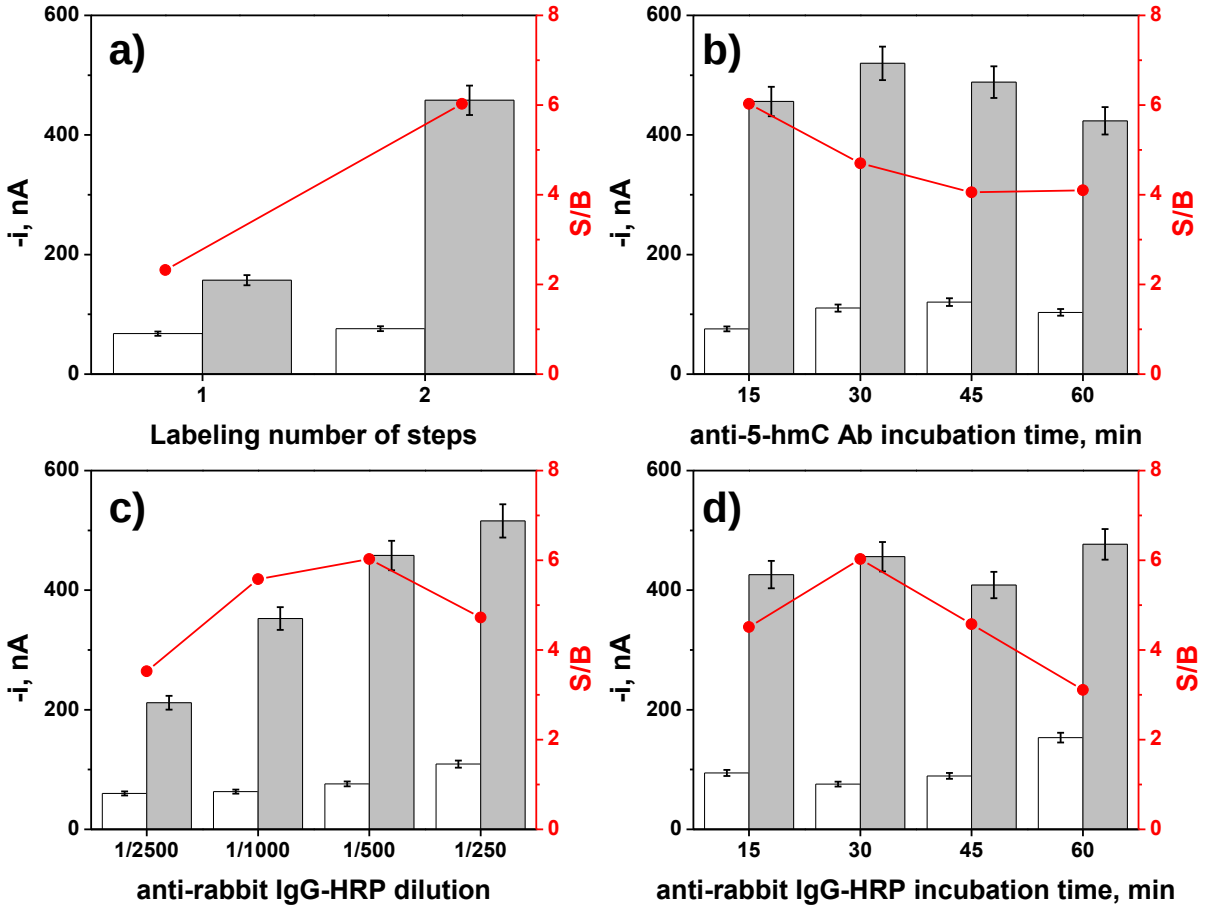

Figure S1. Optimization of different experimental variables involved in the enzymatic labeling with anti-rabbit IgG-HRP. Dependence of the amperometric responses measured in the absence (white bars) or in the presence (grey bars) of 10,000 pM 1×5-hmC-*MGMT* and the resulting signal-to-blank ratio (red lines) with: the number of steps involved in the labeling protocol (a); anti-5-hmC Ab incubation time (b); anti-rabbit IgG-HRP dilution (c); and incubation time (d). Error bars estimated as triple of the standard deviation of three replicates.

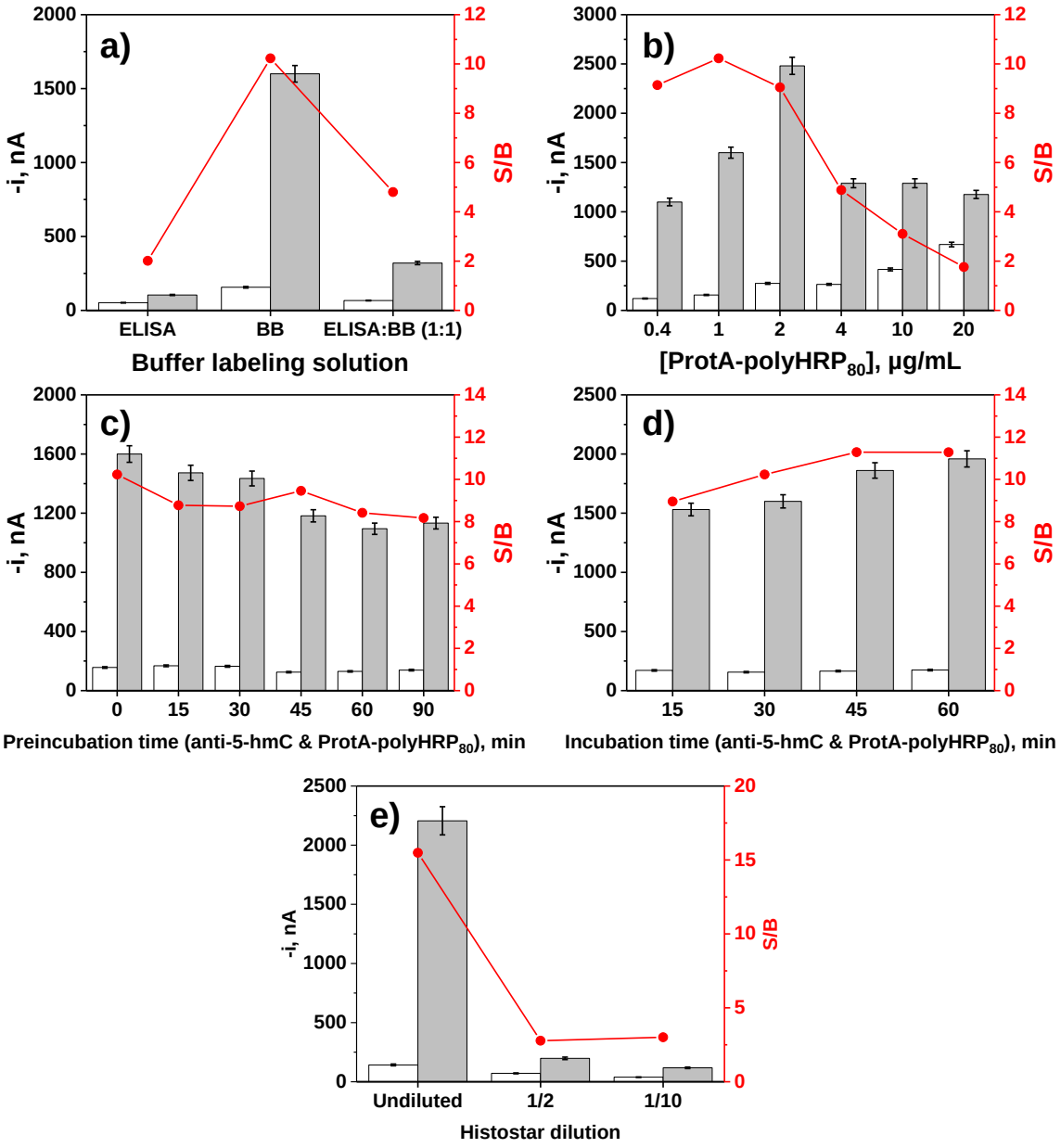

Figure S2. Optimization of the experimental variables involved in the enzymatic labeling with ProtA-polyHRP$_{80}$ (a-d) and Histostar (e). Dependence of the amperometric responses measured in the absence (white bars) or in the presence (grey bars) of 1,000 pM 1×5-hmC-*MGMT* and the resulting signal-to-blank ratio (red lines) with the: buffer labeling solution (a), ProtA-polyHRP$_{80}$ concentration (b), preincubation time of anti-5-hmC and ProtA-polyHRP$_{80}$ (c), incubation time of anti-5-hmC and ProtA-polyHRP$_{80}$ mixture solution with target/b-Cp-MBs (d), Histostar dilution (e). Error bars estimated as triple of the standard deviation of three replicates.

Table S2. Experimental variables used in the compared labeling strategies.

Variable	Evaluated range	Selected value
Strep-MBs, µL[2]	2.5-10.0	5.0
[b-Cp], µM[1]	0.01-0.5	0.1
b-Cp incubation time, min[1]	15-60	15
Target DNA incubation time, min[1]	15-60	30
anti-5-hmC Ab, µg/mL[1]	--	0.5
Conventional labelling: anti-rabbit IgG-HRP (i)		
Number of steps	1-2	2
anti-5-hmC Ab incubation time, min	15-60	15
anti-rabbit IgG-HRP, dilution	1/2,500-1/250	1/500
anti-rabbit IgG-HRP incubation time, min	15-60	30
Amplification strategies: ProtA-polyHRP$_{1,40,80}$ (ii-iv) or Histostar (v)		
Number of steps[2]	1-2	1
Histostar, dilution	Undiluted-1/10	Undiluted
ProtA-HRP, µg/mL[2]	0.2-2	1.0

ProtA-polyHRP$_{40}$, µg/mL[2]	0.2-2	1.0
ProtA-polyHRP$_{80}$, µg/mL	0.4-20	1.0
Labeling preincubation time, min*	0-90	0
Labeling incubation time, min*	15-60	30
Buffer labeling solution*	(BB, 5mC-ELISA Buffer, mixture of both (1:1)	BB

*Tested for ProtA-polyHRP$_{80}$

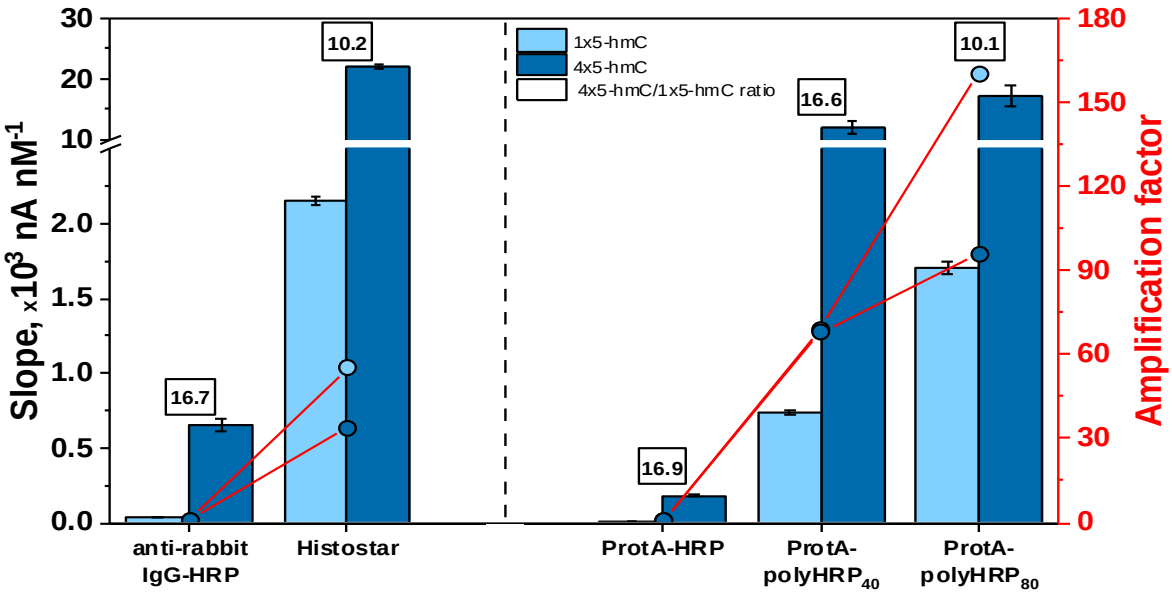

Figure S3. Dependence of the sensitivity provided by the bioplatform developed using different enzymatic labeling strategies for the amperometric determination of synthetic targets with (1 or 4)×5-hmCs. Error bars estimated as triple of the standard deviation of three replicates.

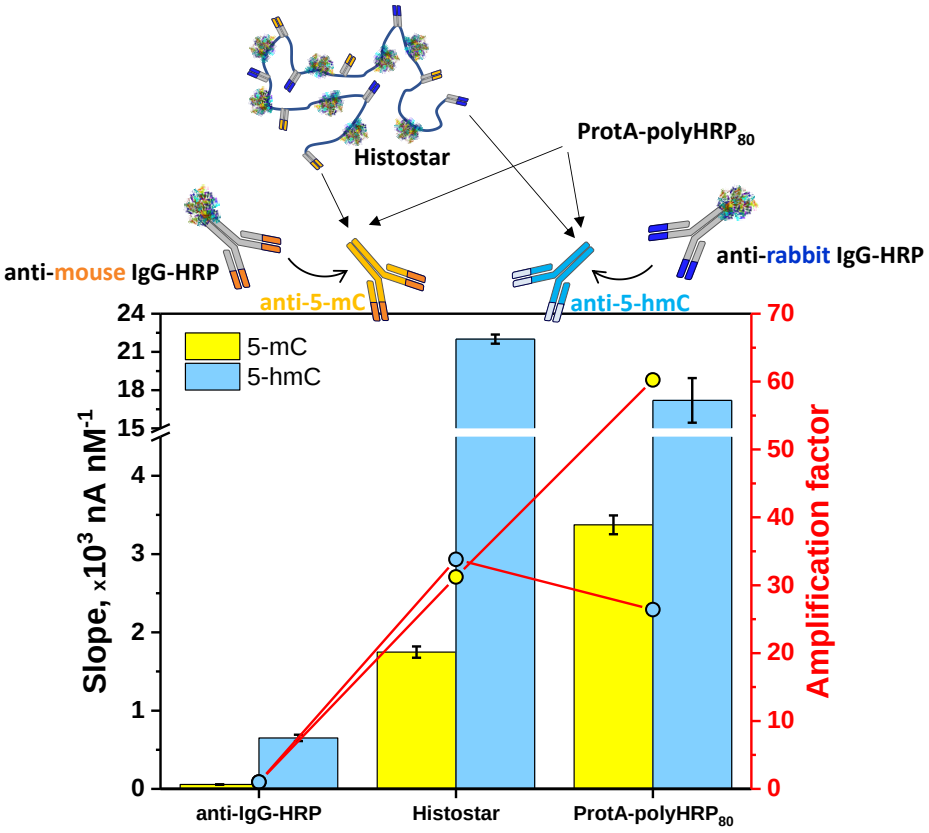

Figure S4. Comparison of the sensitivity provided by the bioplatforms developed involving labeling strategies using conventional secondary antibody, Histostar or ProtA-polyHRP$_{80}$ for the amperometric determination of synthetic targets with a single 5-mC or 5-hmC. Error bars estimated as triple of the standard deviation of three replicates. Amplification factors were calculated by comparison with the conventional enzymatic labeling with each secondary antibody.

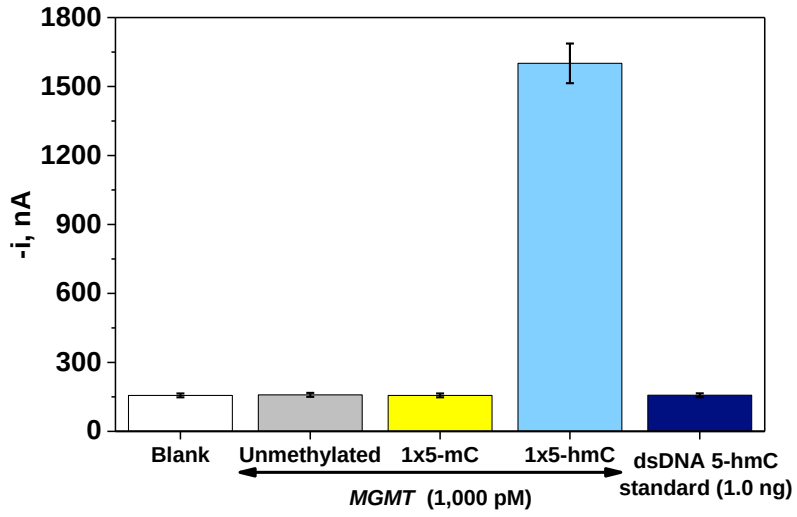

Figure S5. Comparison of the amperometric responses obtained with the bioplatform using labeling with ProtA-polyHRP$_{80}$ for 1,000 pM of the *MGMT,* 1×5-mC-*MGMT* and 1×5-hmC-*MGMT* sequences and 1.0 ng of the denatured dsDNA 5-hmC standard. Error bars estimated as triple of the standard deviation of three replicates.

References

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