Electronic Supplementary Information (ESI)

Approaching single DNA molecule detection with an ultrasensitive electrochemical genosensor based on gold nanoparticles and Cobalt-porphyrin DNA conjugates

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General

Commercially available reagents and solvents were used from the following suppliers without further purification unless stated otherwise: $NaAuCl_4$, trisodium citrate, bis(p-sulfonatophenyl)phenyl phosphine dipotassium salt (BSPP) were obtained from Sigma Aldrich UK; cyclohexane, 6-Mercaptohexan-1-ol (MCH), buffer components: sodium chloride (NaCl), sodium phosphate monobasic (NaH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4) were purchased from Sigma-Aldrich (Poznań, Poland). Potassium hydroxide (KOH), sulphuric acid (H_2SO_4), ethanol (EtOH) and methanol (MeOH) were obtained from Avantor Performance Materials (Gliwice, Poland). Oligonucleotides were purchased from Biomers (Germany) or synthesised in-house on Expedite DNA synthesiser. The oligonucleotide tethered with a thiol linker abbreviated as SH-ssDNA (5'-SH-$(CH_2)_6$-CTG TCT TTG ACA GTC CCA TT-3') was used as capturing probe for the immobilization of ssDNA functionalized AuNPs on the surface of gold electrodes. The oligonucleotide abbreviated as ssDNA (5'-$(SS\text{-}HEG)_2$ AAT(CoP) GGG ACT GTC AAA GAC AG-3') was used as the detection probe and modified with CoP, and then immobilized on the surface of AuNPs. Two unmodified oligonucleotides (analytes), c-ssDNA (5'-CTG TCT TTG ACA GTC CCA TT-3') and nc-ssDNA (5'-GAG AGA AAC TGT CAG GGT AA-3') were used as complementary and non-complementary hybridisation targets, respectively.

All aqueous solutions were prepared with deionized and charcoal-treated water (resistivity of 18.2 $M\Omega\cdot cm^{-1}$) purified with a Milli-Q reagent grade water system (Millipore, Bedford, MA). All

solutions were deoxygenated by purging with nitrogen (ultra-pure 6.0, Air Products, Poland) for 15 minutes. Each step of modification and hybridisation processes were performed in PB containing 2.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4 and 50 mM NaCl, pH 7.0.

UV-visible spectra were recorded on a Cary 300 bio UV-vis spectrophotometer over a range of 200-800 nm. Electrochemical measurements were performed using potentiostat-galvanostat AutoLab (Eco Chemie, Utrecht, The Netherlands). Atomic force microscope (AFM) used was from Universal SPM Quesant (Agoura Hills, CA, USA).

13 nm AuNPs[1a] and porphyrin-modified DNA[1b,c] prepared according to literature procedures.

Synthesis of the DNA modified gold nanoparticles.

A solution of AuNPs (100 µL, 0.2 µM) in phosphate buffered saline (PBS) (20 mM phosphate, 5 mM NaCl, pH 7.4) was added to three vials, followed by disulfide-DNA (100 µL) of 2 µM, 20 µM and 40 µM separately, to give three final concentrations of 1 µM, 10 µM, 20 µM for the DNA, and 0.1 µM for the AuNPs. NaCl solution (200 µL, 0.125 M) and sodium phosphate buffer (100 µL, 25 mM) were added to the NP-DNA solutions so that the total volume of each was increased to 500 µL. The solutions were then left for two hours then their volume was slowly reduced to ~100 µL by vacuum centrifugation, to gradually increase the ionic strength and concentration of DNA. The samples were purified by centrifugation (13000 rpm, 15 minutes) twice. The conjugates were resuspended and stored at 4 °C in PBS buffer (pH 7.4, 80 mM phosphate, 6 mM NaCl).

UV-vis (H_2O): AuNPs λ_{max} = 521 nm. In the AuNP porphyrin-DNA conjugates, the porphyrin absorbance is strongly dominating, thus the AuNP absorbances are masked in the spectra of the purified samples.

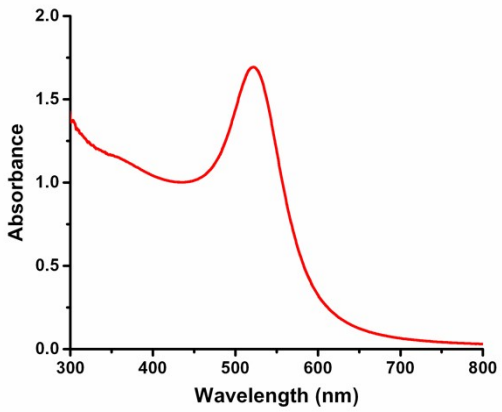

Figure S1. UV-vis spectrum of 13 nm gold nanoparticles.

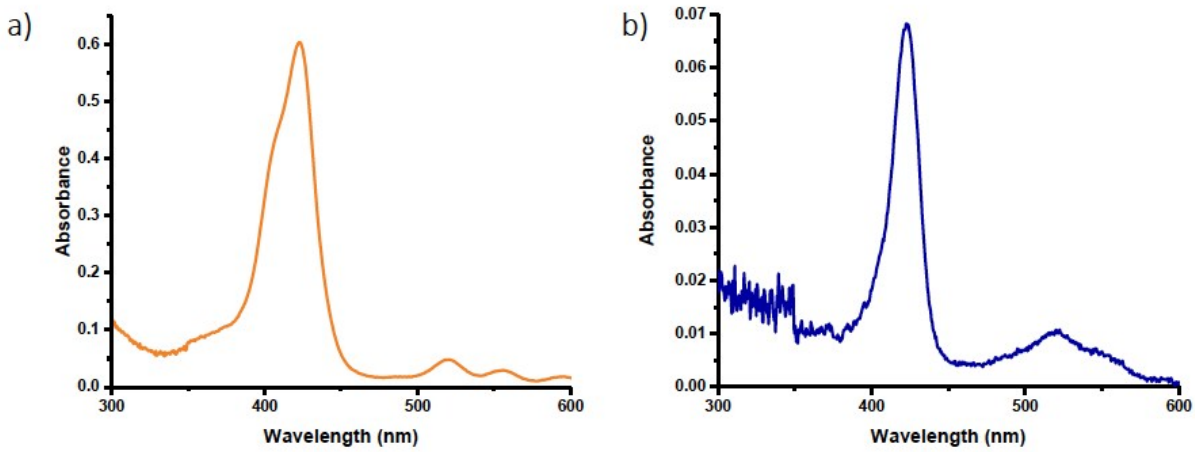

Figure S2. a) UV-vis absorbance spectrum of the single-stranded CoP-DNA; b) UV-vis absorbance spectrum of the conjugated CoP-DNA–AuNP after purification from unbound DNA through centrifugation.

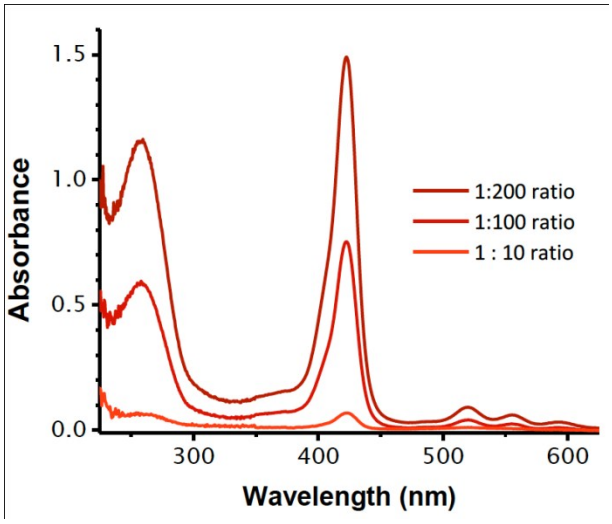

Figure S3. UV-vis spectra of CoP-DNA–AuNP conjugates with different DNA to NP ratios.

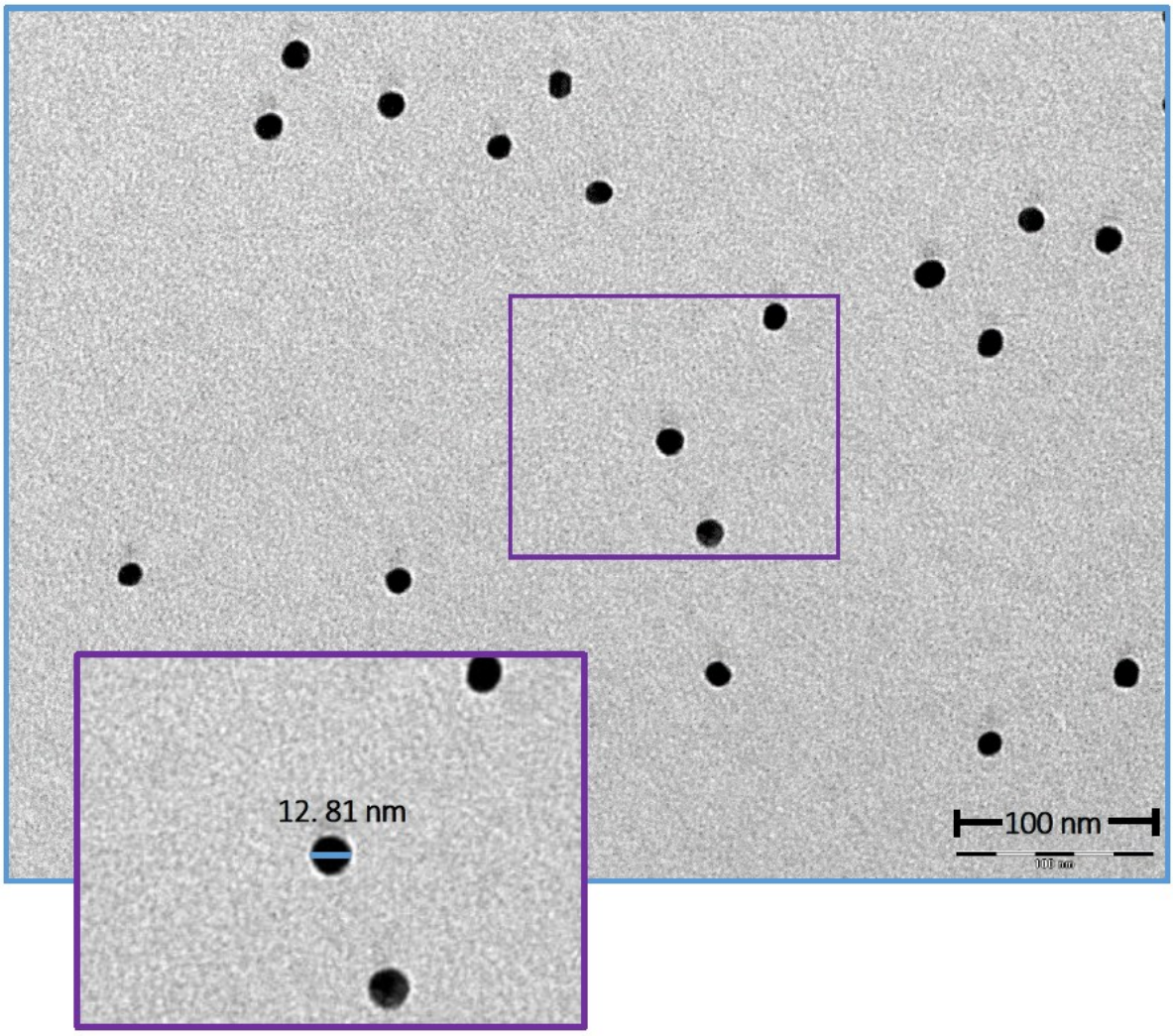

Figure S4. TEM image of the AuNPs.

Determining the maximum surface loading of DNA on the NPs

Attempts to determine the loading of the DNA on the NPs using either release of the DNA with DTT, or dissolving the AuNPs using KI, proved unsuccessful as the majority of the DNA was not recovered from the subsequent column purification, and the determined values were not reproducible. Therefore, the loading was determined by adsorbing the DNA (excess) onto the AuNP, and determining the remaining DNA in the supernatant after removal of the AuNP-DNA conjugates through ultra-centrifuging (supernatant 1), followed by a washing step of the AuNP-DNA conjugates (supernatant 2). A second washing step did not give any detectable DNA in the solution.

For the analysis, AuNPs (3.90 nM, 195.5 µL) and CoP-DNA (112 µM, 4.5 µL) were mixed, and buffer solution (total volume 300 µL) added as described above. The solution was left for two hours, then the volume was slowly reduced to ~100 µL by vacuum centrifugation. The final volume was determined to be 130 µL. The number of moles that were added to this sample were 0.76 pmoles AuNP and 0.56 nmoles CoP-DNA.

AuNP: 13 nm AuNP: $\varepsilon = 2.270 \times 10^8\ M^{-1}cm^{-1}$

$A_{520} = 1.33$; c = 5.87 nM; V = 130 µL

n = 0.764 pmoles

After centrifugation, the supernatant was measured:

Supernatant 1: DNA: $\varepsilon = 222\ 930\ M^{-1}cm^{-1}$

$A_{260} = 0.629$; c = 2.82 µM; V = 130 µL

n = 0.367 nmoles

The samples were re-dispersed in 130 µL buffer solution, and the supernatant measured after centrifugation:

Supernatant 2: DNA: $\varepsilon = 222\ 930\ M^{-1}cm^{-1}$

$A_{260} = 0.029$; c = 0.13 µM; V = 130 µL

n = 0.017 nmoles

Total nmoles of DNA remaining in solution: 0.384 nmoles

Total nmoles of DNA retained on NPs: 0.504 – (0.367 + 0.017) = 0.120 nmoles

Ratio of DNA : AuNP 0.120 nmoles : 0.764 pmoles = 157 : 1

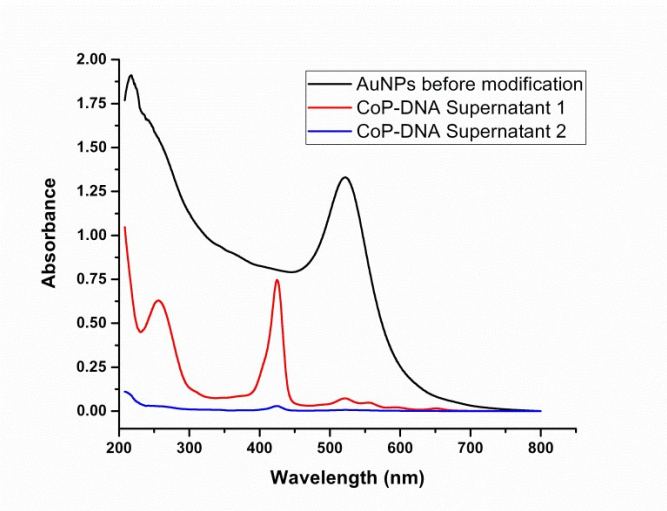

Figure S5. UV-vis spectra of the AuNPs before modification, and the supernatants after centrifugation and washing.

Preparation of genosensor: Successive steps of the modification of gold electrode

Gold disk electrodes with a radius of 1 mm (Bioanalytical Systems (BASi), West Lafayette, IN) were used for the experiments. The electrodes were polished with 0.3 µm and 0.05 µm alumina

slurries (α and γ micropolish; Buehler, Lake Bluff, IL) on a flat pad (BASi) for 5 min each and rinsed with Milli-Q water. They were further cleaned electrochemically by cyclic voltammetry (CV). At first, the electrodes were dipped in 0.5 M KOH solution and the potential was cycled between -0.4 V and -1.2 V (versus Ag/AgCl reference electrode) with a scan rate of 0.1 V/s with the number of cycles 6, 100 and 20. Subsequently, the electrodes were cleaned in 0.5 M H_2SO_4 solution in the potential window between -0.3 V and 1.5 V (versus Ag/AgCl reference electrode) with a scan rate of 0.1 V/s with number of cycles 6, 20 and 6. Before modification, the surfaces of the electrodes were refreshed in 0.5 M KOH solution for 20 cycles. After cleaning, they were rinsed with Milli-Q water followed by PB buffer solution (2.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4 and 50 mM NaCl, pH 7.0). Afterwards 10 µL of the following solution was dropped on each gold electrode surface: 0.1 µM SH-ssDNA and 10 µM MCH in PB buffer solution for 3h at room temperature (RT). Then the electrodes were rinsed with the same buffer solution.

In the second step of modification, 10 µL of 6 pM AuNPs-CoP-ssDNA (1:10 or 1:100 or 1:200) in PB solution were dropped on the gold electrodes surfaces for 2h at RT. Finally, they were washed with the PB solution and stored in the same buffer overnight at RT.

Electrochemical Measurements

All electrochemical measurements were performed with a potentiostat-galvanostat AutoLab (Eco Chemie, Utrecht, The Netherlands) with a three-electrode configuration. Potentials were measured *versus* the Ag/AgCl electrode, and a platinum wire was used as an auxiliary electrode. Cyclic voltammetry (CV) measurements were performed in the potential range from -100 mV to 750 mV for the modified gold electrodes. Osteryoung square wave voltammetry (OSWV) was performed with a potential from -100 mV to 750 mV for the modified gold electrodes with a step potential of 1 mV, a square-wave frequency of 50 Hz, and an amplitude of 50 mV. Differential pulse voltammetry (DPV) was performed with a potential scanned in the potential window: -100 mV to 750 mV (oxidation) or 750 mV to -100 mV (reduction), with a step potential of 1 mV, and an amplitude of 25 mV. All measurements were carried out in the presence of electrolyte purged with nitrogen for 15 min. A gentle nitrogen flow was applied over the sample solution during all measurements.

Hybridisation processes of the AuNPs-CoP-ssDNA probe and the target sequences

The target oligonucleotides (c-ssDNA and nc-ssDNA) were diluted with the PB buffer solution to the concentration of 0.05, 0.075, 0.1 and 0.2 fM. Hybridisation reactions were performed by dropping 10 µL of the solution containing targets: c-ssDNA and nc-ssDNA in buffer solution for 2h at room temperature on the modified gold electrode surface. After the hybridisation with the particular

concentration of targets, the electrodes were rinsed thoroughly with PB solution. Then, the electrodes were transferred to the electrochemical cell for electrochemical measurements. The hybridisation processes were monitored using OSWV. The electrode responses were expressed as: $\mathbf{(I_n – I_0)/I_0 \times 100\%}$, where I_n is the peak current measured in the presence of the analyte and I_0 the peak current before applying the analyte i.e. in pure buffer.

Atomic Force Microscopy

The gold substrate modified with Au/SH-ssDNA+MCH/AuNPs-CoP-ssDNA (1:200) was characterized by atomic force microscope (AFM) system from Universal SPM Quesant (Agoura Hills, CA, USA). As the gold substrates was used mica plates coated with 10 nm thick titanium under layer and 100 nm thick gold layer (IMEC, Belgium). All gold substrates were cleaned with UV/ozone chamber (Novascan Technologies, USA) for 20 min and annealed by hydrogen flame. Before use, the gold substrates were rinsed with cyclohexane, methanol and Milli-Q water and dried with streaming nitrogen. Modification layers were formed by immersion of the Au substrate in PB buffer containing, in the first step, mixture of 0.1 µM SH-ssDNA and 10 µM MCH (3h, RT). Then substrate was rinsed with the PB and dipped in the same buffer containing 6 pM AuNPs-CoP-ssDNA (1:200) for 2h at RT. Finally, it was washed with the PB and stored in this buffer overnight at room temperature.

The AFM images were obtained using the non-contact mode with NSC16 tip (W2C, Si3N4), which oscillates at *ca.* 170 kHz resonance frequency above the sample surface. The nominal spring constant of these cantilevers was equal 45 N/m. The radius of tip curvature was *ca.* 10 nm. The images were recorded in air with pixel resolution of 300 × 300.

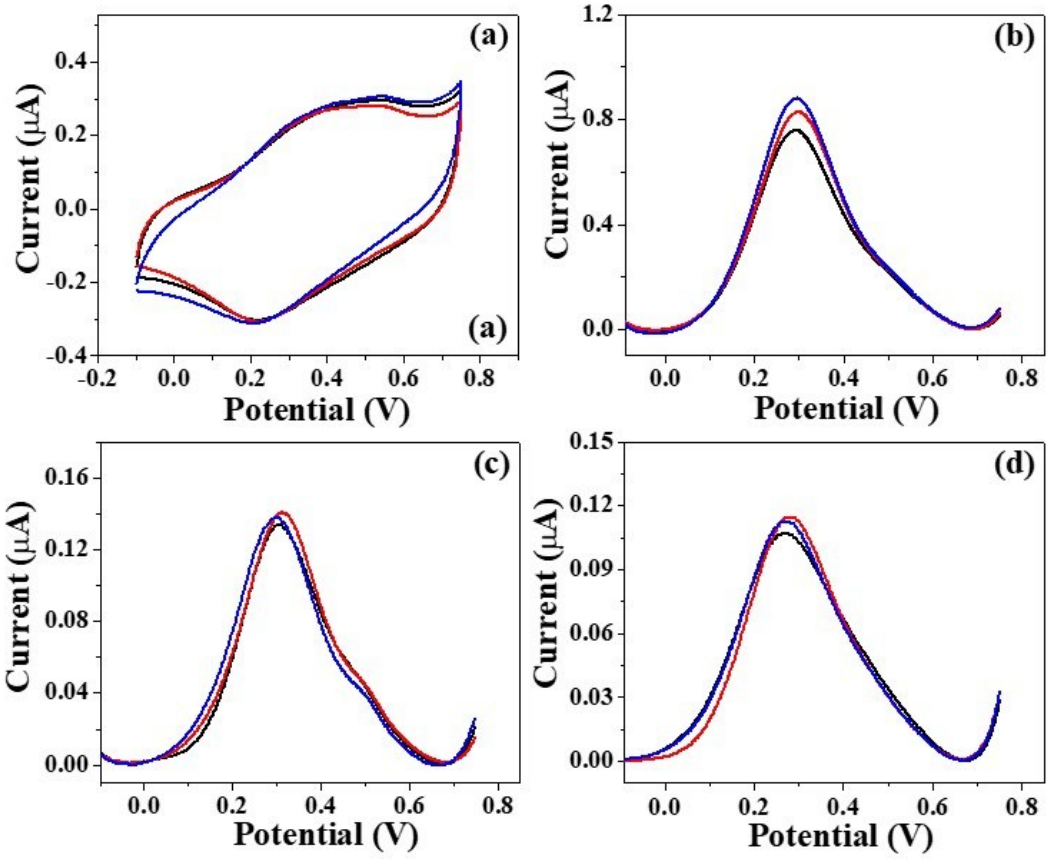

Figure S6. Representative (a) cyclic voltammograms, (b) Osteryoung square-wave voltammograms, (c) differential pulse voltammograms for oxidation and (d) differential pulse voltammograms for reduction recorded using gold electrode modified with layers: AuNPs-CoP-ssDNA (1:10) – black line, AuNPs-CoP-ssDNA (1:100) – red line or AuNPs-CoP-ssDNA (1:200) – blue line. Buffer composition: 2.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4 and 50 mM NaCl, pH 7.0

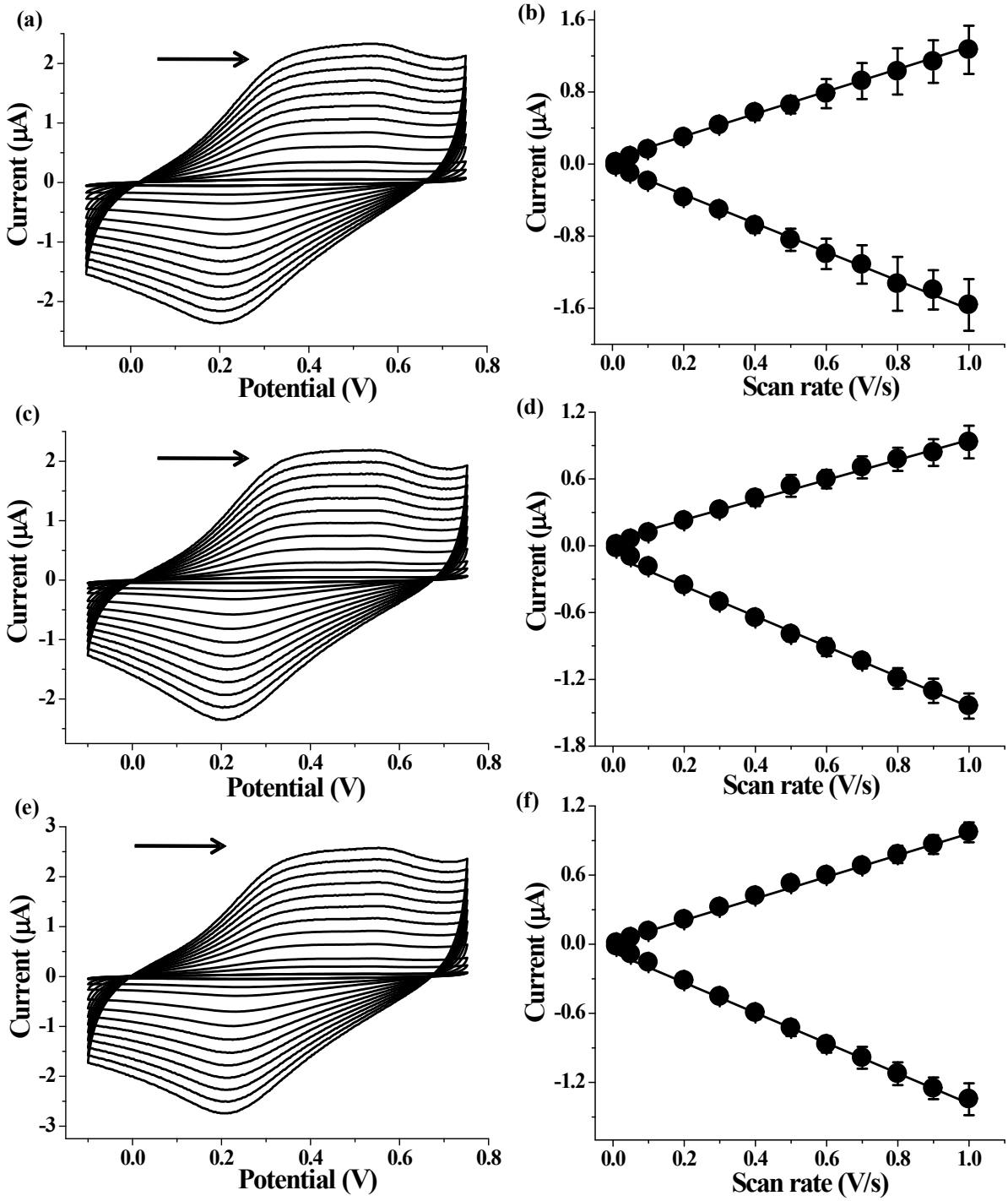

Figure S7. Representative cyclic voltammograms and corresponding plots of anodic and cathodic peak current vs scan rate recorded using gold electrode modified with (a,b) AuNPs-CoP-ssDNA (1:10), (b,c) AuNPs-CoP-ssDNA (1:100), or (d,e) AuNPs-CoP-ssDNA (1:200). Measuring conditions: scan rates from 0.01 to 1 V/s, buffer composition: 2.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4 and 50 mM NaCl, pH 7.0.

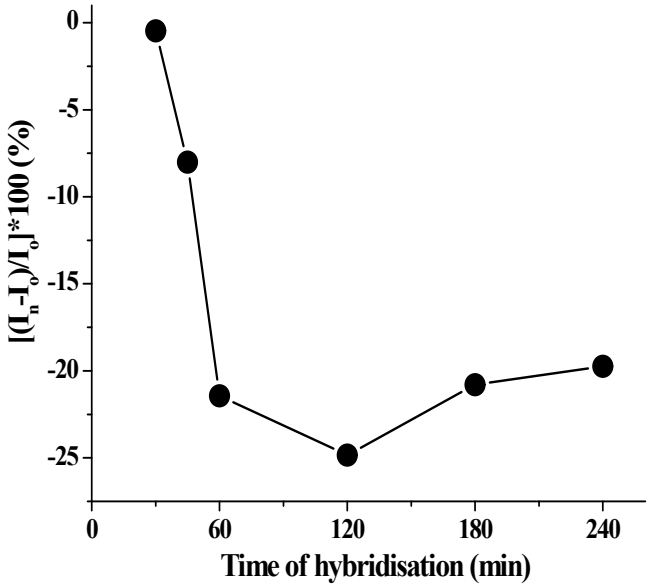

Figure S8. Dependence of relative changes in current intensity of the redox couple Co (II)/Co (III) $[(I_n-I_0)/I_0]\times100\%$ as a function of the hybridisation time measured in the presence of 0.5 fM c-ssDNA target.

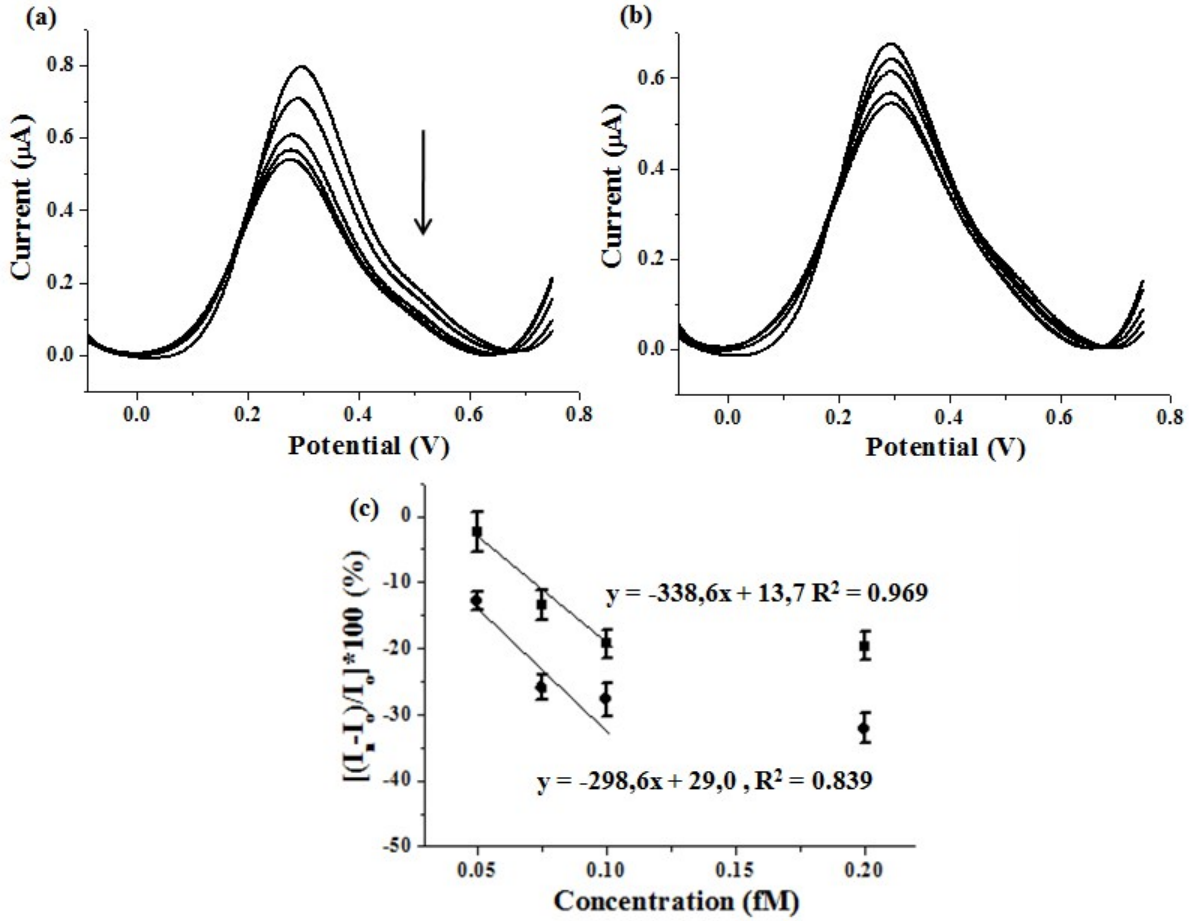

Figure S9. Representative Osteryoung square-wave voltammograms recorded using gold electrode modified with AuNPs-CoP-ssDNA (**1:10**) layer after hybridisation with 20-mer (a) complementary and (b) non-complementary ssDNA at concentrations of (i) 0, (ii) 0.05, (iii) 0.075, (iv) 0.1, and (v) 0.2 fM (in PB buffer). Buffer composition: 2.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4 and 50 mM NaCl, pH 7.0. (c) Relative intensity $[(I_n-I_0)/I_0]\times100\%$ of redox Co(II)/Co(III) current vs. concentration of (●) complementary c-ssDNA and (■) non-complementary nc-ssDNA.

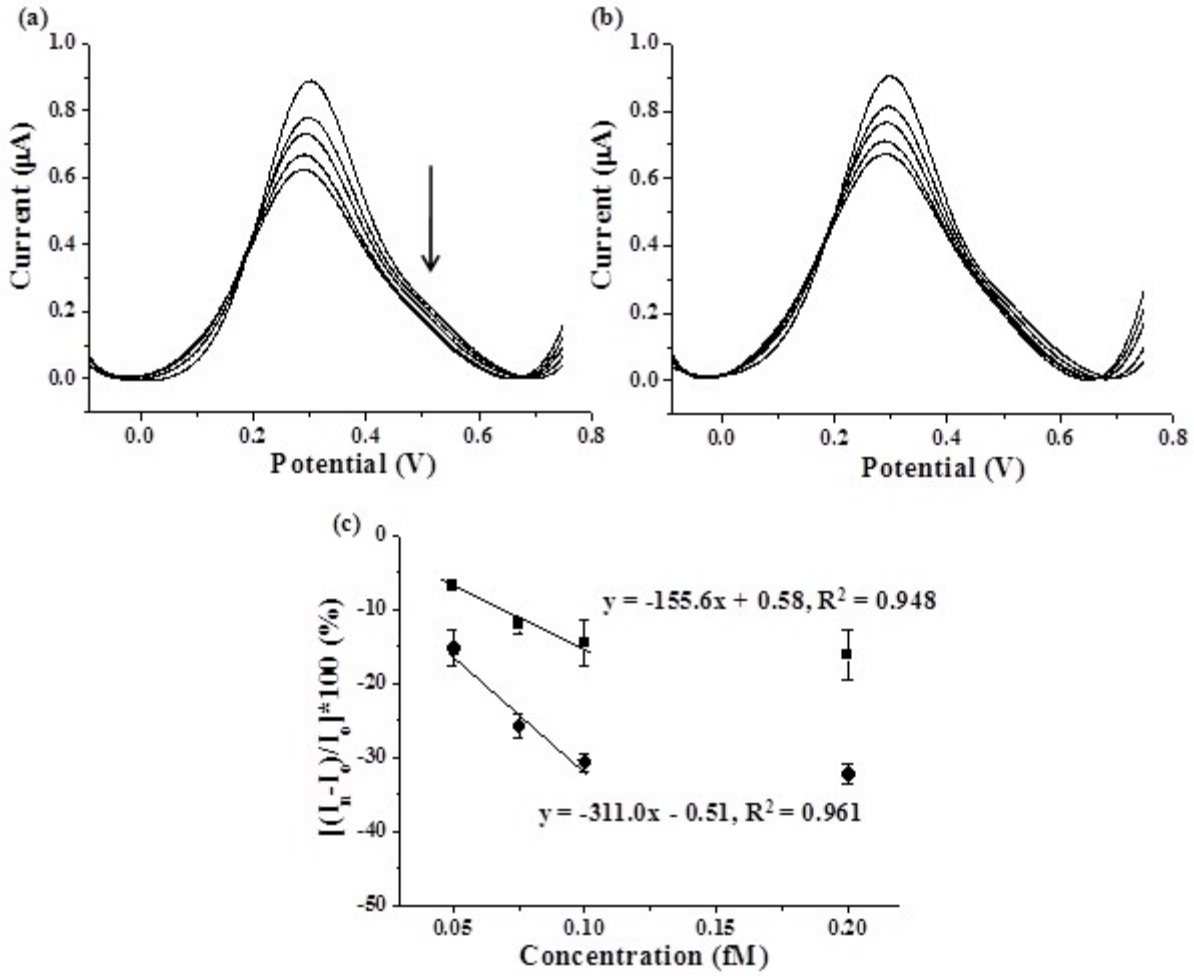

Figure S10. Representative Osteryoung square-wave voltammograms recorded at the gold electrode modified with AuNPs-CoP-ssDNA (**1:100**) after hybridisation with 20-mer (a) complementary and (b) non-complementary ssDNA at concentrations of (i) 0, (ii) 0.05, (iii) 0.075, (iv) 0.1, and (v) 0.2 fM (buffer composition: 2.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4 and 50 mM NaCl, pH 7.0). (c) Relative intensity of redox Co(II)/Co(III) current vs. concentration of (●) c-ssDNA and (■) nc-ssDNA.

Table S1. Electrochemical parameters (oxidation and reduction peak positions (E) and current intensities (I)) of various modified gold electrodes (n=8). For the measuring conditions see Fig.S1 and Fig.S2.

Technique	Parameters	AuNPs-CoP-ssDNA (1:10)	AuNPs-CoP-ssDNA (1:100)	AuNPs-CoP-ssDNA (1:200)
CV	E_{ox} (V)	0.379±0.018	0.386±0.011	0.368±0.013
	I_{ox} (μA)	0.17±0.02	0.12±0.03	0.11±0.01
	E_{red} (V)	0.238±0.008	0.247±0.045	0.238±0.011
	I_{red} (μA)	0.19±0.02	0.18±0.01	0.17±0.02
OSWV	E (V)	0.291±0.008	0.300±0.004	0.297±0.005
	I (μA)	0.66±0.32	0.83±0.06	0.86±0.09
DPV	E_{ox} (V)	0.301±0.009	0.308±0.003	0.297±0.004
	I_{ox} (μA)	0.12±0.02	0.13±0.01	0.12±0.01
	E_{red} (V)	0.283±0.020	0.285±0.007	0.269±0.001
	I_{red} (μA)	0.11±0.02	0.12±0.01	0.11±0.01

Table S2. The electrochemical parameters calculated using modified gold electrodes: density of redox-active layers (Γ), electron transfer coefficient (α) and the electrode reaction standard rate constant (k_s). (n=4)

Modified gold electrode	Γ (mol/cm²)	α	k_s (s^{-1})
AuNPs-CoP-ssDNA (1:10)	3.0 (±0.2) × 10^{-11}	0.74 ± 0.15	0.70 ± 0.13
AuNPs-CoP-ssDNA (1:100)	2.8 (±0.3) × 10^{-11}	0.66 ± 0.08	0.71 ± 0.06
AuNPs-CoP-ssDNA (1:200)	2.9 (±0.5) × 10^{-11}	0.68 ± 0.04	0.71 ± 0.02

Table S3. Selectivity of sensor for complementary c-ssDNA sequence: slope S_j of the calibration curve for c-ssDNA, slope S_i of the calibration curve for nc-ssDNA sequence, response ration $R_{i,j}$.

Sensing layer	Slope [% / fM]	$R_{i,j} = S_j / S_i$
AuNPs-CoP-ssDNA (1:10)	S_j =-338,6 S_i =-298,6	1.13
AuNPs-CoP-ssDNA (1:100)	S_j =-155.6 S_i =-311.0	0.50
AuNPs-CoP-ssDNA (1:200)	S_j =-116.0 S_i =-276.8	0.42

Table S4. Comparison of the developed genosensor with those already reported in literature.

Electrode Modification	Analytical signal source	Linear Range [M]	Limit of Detection [M]	Ref.
AuNPs+ssDNA /Pt-UME	H	1×10^{-12} - 100×10^{-9}	1×10^{-12}	[2]
AuNPs+ssDNA/ITOE	H	1×10^{-15} - 10×10^{-12}	1×10^{-15}	[3]
ssDNA-AuNPs/ aDNA/(+)AuNPs/Nf/GCE	MB	1×10^{-12} - 100×10^{-12} 100×10^{-12} - 1×10^{-9}	372×10^{-15}	[4]
AuNPs-(c-ssDNA)/SH-ssDNA + MCH/AuE	$[Co(phen)_3]^{3+/2+}$	0.51×10^{-12} - 8.58×10^{-12}	0.51×10^{-12}	[5]
AuNPs-ssDNA+MCH/PSS+PAH/MPA/AuE	$Ru(NH_3)_6^{3+}$	1.0×10^{-11} - 10×10^{-5}	1×10^{-11}	[6]
hpDNA/AuNPs/rpDNA)/tDNA/cpDNA/AuE	$[Ru(NH_3)_5L]^{2+}$	-	1×10^{-15}	[7]
AuNPs/TGA/MCH- hpDNA/AuE	$[Fe(CN)_6]^{3-/4-}$	1.0×10^{-17} – 1.0×10^{-11}	1.7×10^{-18}	[8]
ssDNA/Av/PAMAM/MPA/AuE	$Ru(NH_3)_6^{3+}$	1.4×10^{-11} – 2.7×10^{-14}	1.4×10^{-14}	[9]
AuNPs-CoP-(ssDNA) (1:200)/SH-ssDNA + MCH/AuE	CoP	5×10^{-17} - 1×10^{-16}	3.8×10^{-18}	This study
AuNPs-CoP-(ssDNA) (1:100)//SH-ssDNA +MCH/AuE			2.6×10^{-17}	
AuNPs-CoP-(ssDNA) (1:10)//SH-ssDNA + MCH/AuE		7.5×10^{-17} – 2.0×10^{-16}	4.8×10^{-17}	

Abbreviations: Pt UME: Platinum ultramicroelectrode; AuNPs: Gold nanoparticles, ssDNA: single stranded DNA; ITOE: Indium Tin oxide electrode, Nf: nafion, GCE: Glassy carbon electrode, hpDNA: Hairpin DNA, rpDNA: reporter DNA, tDNA: target DNA, cpDNA: capture DNA, aDNA: DNA aptamer, TGA: thioglycolic acid, MCH: 6-Mercapto-1-hexanol, AuE: Gold electrode, PAMAM: polyamidoamine, MPA: 3-mercaptopropionic acid, Av: avidin, MB: methylene blue, H: hydrazine, PSS: poly(styrene sulfonate), PAH: poly(allylamine hydrochloride), CoP – cobalt (II) porphyrin

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