A novel DNA biosensor using a ferrocenyl intercalator applied to the potential detection of human population biomarkers in wastewater

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Abstract: A new label-free electrochemical DNA (E-DNA) biosensor using a custom synthesized ferrocenyl (Fc) double-stranded DNA (dsDNA) intercalator as a redox marker is presented. Single-stranded DNA (ssDNA) was co-immobilized on gold electrodes with 6-mecarpto-hexanol (MCH) to control the surface density of the ssDNA probe, and hybridized with complementary DNA. The binding of the Fc intercalator to dsDNA was measured by differential pulse voltammetry (DPV). This new biosensor was optimized to allow the detection of single base pair mismatched sequences and, able to detect as low as 10 pM target ssDNA with a dynamic range from 10 pM to 100 nM. DNA extracted from wastewater was analysed by quantitative polymerase chain reaction (qPCR) targeting human-specific mitochondrial DNA (mtDNA). The aim of this approach is to enable the analysis of population biomarkers in wastewater for the evaluation of public health using wastewater-based epidemiology (WBE). The E-DNA biosensor was employed to detect human-specific mtDNA from wastewater before and after PCR amplification. The results demonstrate the feasibility of detecting DNA biomarkers in wastewater using the developed biosensor, which may allow the further development of DNA population biomarkers for public health using WBE.

Keywords: population biomarkers, wastewater-based epidemiology, DNA biosensor, intercalator,

1. Introduction

Wastewater-based epidemiology (WBE) has shown to be a powerful tool for the evaluation of community-wide drug[1-3], alcohol[4] and tobacco use[5], and has the potential to be utilized for the evaluation of public health by assessing disease biomarkers[6]. The approach is based on the analysis of specific urinary biomarkers such as drug residues and/or their metabolites in wastewater following their excretion by humans, using urban wastewater collected at a treatment plant selected for sampling. WBE is a cost-effective, fast and near real-time tool for the evaluation of population health compared with traditional questionnaire survey approaches[7]. Until now, the quantitative measurement of specific drug biomarkers in wastewater has been performed to study the drug use habits of numerous communities[1, 3]. As an example, illicit drug use trends were recently evaluated in 21 countries (42 cities, total population 24.74 million) across Europe[3]. Furthermore, some drugs and their metabolites have also been proposed as a potential population biomarker for WBE such as creatinine[8], cholesterol and coprostanol[9], as well as caffeine and nicotine metabolites[10]. Chen *et al* [11] outlined criteria for a candidate population biomarker: (1) must be quantifiable; (2) have little affinity to particulate matter in wastewater or to filter paper; (3) be stable in wastewater; (4) be constantly excreted and (5) the total excretion should correlate with census population, meaning there should be no contribution other than human metabolism. The potential population biomarkers reported in the literature were evaluated by Chen *et al*[11] and few of them could strictly meet the proposed criteria. The major limitation of these chemical biomarkers is that they rely on the stability and the contribution from non-human metabolism.

Completion of sequencing the human genome in 2003 has shown that up to 1% of the human coding sequences are associated to cancer by mutation[12]. This valuable information is increasingly being utilized for diagnostic purposes by using known cancer associated mutations as biomarkers[13-16]. DNA is a surprisingly resilient biomolecule capable of persisting in the environment for many centuries, as shown by the complete genome sequencing of a Neanderthal individual from a bone sample[17]. DNA is also naturally shed into the environment by virtually all living organisms through urine, faeces, exudates or tissue residues as demonstrated by the advent of environmental DNA studies[18]. These robust characteristics have allowed the association of human specific mitochondrial DNA (mtDNA) to human faecal contamination to assess water quality[19, 20]. Since mtDNA also has mutations known to be associated to cancer[21, 22], with a high copy number per cell[23], it has the potential to be suitable for evaluating the feasibility of using novel analytical tools like biosensors to

detect human specific DNA from wastewater samples, with the perspective of further developing the system to identify population biomarkers for WBE studies. Consequently, mtDNA is clearly a potential population biomarker for WBE because it meets the outlined criteria such as stability, being associated to cancer, presenting human specific sequences and being quantifiable.

Electrochemical biosensors have great promise for the detection of disease biomarkers in body fluids, in particular due to being cost-effective, offering fast response times and being easily integrated with other devices[24]. The advantages over conventional analytical techniques are the possibilities of portability, miniaturisation and ability to measure complex matrices with minimal sample preparation. Typically, E-DNA biosensors are designed with the following elements: immobilization of the single-stranded DNA (ssDNA) probe, hybridisation with complementary target sequence, introduction of an electroactive indicator, and the electrochemical investigation of the surface[25]. Differential pulse voltammetry (DPV) is a convenient electrochemical tool for the detection of DNA, and the signal transducer may consist of either labelled sequences with an active redox maker, or a double-stranded DNA (dsDNA) intercalator such as metal ion complexes with iron, cobalt, osmium or ruthenium, and organic compounds like methylene blue, daunomycin, Hoechst 33258, or anthraquinone derivates[26-28]. Amongst these compounds, threading intercalators that carry bulky substituents on the periphery of the intercalating moiety, are reported to be very efficient and stable to interact with the DNA duplex[28]. Naphthalene diimide derivatives (like ferrocenyl derivates) have been proven to be active candidates as dsDNA intercalators and the constructed biosensors demonstrate excellent analytical performance for the detection of DNA[28, 29].

Here we present an electrochemical biosensor using a custom synthesized ferrocenyl (Fc) dsDNA intercalator derived from naphthalene diimide as a redox marker for amperometric detection. This biosensor is able to detect as low as 10 pM complementary DNA and is shown to be suitable for single mismatched base pair detection, potentially enabling the detection of single-nucleotide polymorphism (SNP) cancer markers. Human-specific mtDNA was successfully detected from wastewater using this E-DNA sensor with and without prior qPCR amplification. To the best of our knowledge, this is the first time a E-DNA biosensor has been reported for the detection of a mtDNA biomarker in wastewater. We hope this technique may be further improved for the purpose of population DNA biomarkers study to evaluate public health by means of a WBE approach.

2. Experimental

2.1 Materials

Gold disc working electrodes with a radius of 1.0 mm were purchased from IJ Cambria Scientific Ltd (Cambridge, UK). If not stated otherwise, all chemicals were purchased from Sigma-Aldrich (UK) and used as received.

Single stranded DNA, used as reverse and forward primers for PCR amplification, was synthesized by Eurofins MWG (Ebersberg, Germany). The oligonucleotides used as DNA probes for the biosensors were modified with a thiol group at the 5' end to form HS-$(CH_2)_6$-ssDNA to assemble on gold electrodes. The thiolated DNA sequences for elaboration of biosensors were synthesized by Sigma-Aldrich (UK). The sequence 5'-HS-$(CH_2)_6$-CCA CGT CGA GCG ATG-3' (15 bps) was used as capture probe, and one fully complementary and three mismatched sequences (5'- CAT CGC TCG ACG TGG-3', 5'- CAT CGC TC*A* ACG TGG-3', 5'- CAT CGC T*AA* ACG TGG-3' and 5'- CAT CGC *AAA* ACG TGG-3', respectively) (Sigma-Aldrich) were employed to hybridize with the DNA probe. The human specific mtDNA forward and reverse primers[20], respectively 5'-CAG CAG CCA TTC AAG CAA TGC-3 and 5'- GGT GGA GAC CTA ATT GGG CTG ATT AG-3' were used for PCR amplification targeting the human mitochondrial gene NADH dehydrogenase subunit 5, and the modified forms with a HS-$(CH_2)_6$ group on the 5'-end of forward and reverse primers were used for the elaboration of biosensors. Additionally, two thiolated random sequences, which have the same number of base pairs with either the forward or reverse primer (5'-HS-$(CH_2)_6$-AGA AGA AAC GGA GGA AGG GAA-3', 21 bps and 5'- HS-$(CH_2)_6$-AAG AAC TGA AGG GCC AAA TGA GCC GA-3', 26 bps), were also synthesized for specificity studies. The HPLC DNA powders were aliquoted in 10 mM Tris-HCl, 1mM ethylenediaminetetraacetic acid (EDTA) upon receipt and stored in a freezer at -20 ^{o}C for long-term usage.

The buffer for DNA probe immobilisation (IB) consisted of 0.8 M phosphate buffer (PB) + 1.0 M NaCl + 5 mM $MgCl_2$ + 1 mM ethylenediamine tetraacetic acid (EDTA) pH 7.0. The DNA hybridisation and measurement buffer was composed of 50 mM PB and 100 mM K_2SO_4 (pH 7.4).

The influent wastewater was collected from the VEAS wastewater treatment plant (WWTP; Oslo, Norway), and a sample of pooled urine was collected from portable public toilets from Oslo during July, 2014. The collected samples were immediately frozen (-20 °C) and stored frozen until analysis.

129 *2.2. Synthesis of Fc DNA intercalator*

130 The synthesis of dsDNA intercalators *N,N'*-((((((1,3,6,8-tetraoxo-1,3,6,8-
131 tetrahydrobenzo[*lmn*][3,8]phenanthroline-2,7-diyl)bis(ethane-2,1-diyl))bis(oxy)) bis (ethane-
132 2,1-diyl)) bis(oxy)) bis(ethane-2,1-diyl)) diferrocenamide (**1**) was generated by *N*-(2-(2-(2-
133 aminoethoxy)ethoxy)ethyl)-2-ferrocenamide (**2**) and 1,4,5,8-Naphthalenetetracarboxylic
134 dianhydride (**3**). The synthesis procedure is briefly illustrated in Scheme 1, and the detailed
135 protocol of synthesis and characterization **2** and **1** is described in the supporting information.

136

137

138 **Scheme 1**

139

140 *2.3. Elaboration of biosensors*

141 Gold electrodes were cleaned following a procedure described elsewhere[30]. In brief,
142 they were polished with 50 nm aluminium oxide particles (Buehler, USA) on a polishing pad
143 (Buehler) for 5 min, followed by sonication in ultrapure water, polishing on a blank polishing
144 pad, and sonication in ultrapure water to remove any particles. Gold electrodes were rinsed
145 with fresh piranha solution (H_2SO_4/H_2O_2, v/v 7/3) followed by rinsing thoroughly with
146 deionized water, and readily used for electrochemical cleaning. Electrodes were
147 electrochemically cleaned in a classical three-electrode cell by immersing them into H_2SO_4
148 (0.5 M) solution and the potential scanned between the oxidation and reduction potentials of
149 gold, 0 V and +1.5 V *versus* an Ag/AgCl reference electrode, with a scanning rate at 0.2 V/s
150 for 60 cycles until there was no further change in the voltammogram.

151 Gold electrodes then were rinsed with deionised water, dried in a stream of nitrogen
152 and incubated with mixed ssDNA/6-mercapto-1-haxanol (MCH) immobilization solution for
153 16 h in a humidity chamber at 4 ºC. The molar ratio between ssDNA and MCH was 1:3, with
154 1 µM ssDNA in immobilization buffer. After immobilization, electrodes were rinsed in 50
155 mM PB + 100 mM K_2SO_4 + 10 mM EDTA (pH 7.0) to remove any remaining Mg^{2+}. In order
156 to ensure complete thiol coverage of the gold surface and make favourable ssDNA
157 conformation for hybridization, the electrodes were backfilled with MCH (1 mM, H_2O) for 1
158 h, followed by rinsing with ultra-pure water and slightly drying with N_2 stream. The
159 electrodes was electrochemically characterized and then incubated with Fc intercalator at

desired concentration for 1 h. The electrodes were extensively rinsed with 0.1 % Tween-PB buffer with vortex for 30 s, followed by rinsing with DI water and drying in N_2 stream.

2.4. Isolation and characterization of DNA from wastewater

The DNA in wastewater from Oslo (sample 'WW1') and in the pooled urine (sample 'WW2') was isolated with a PowerWater® DNA Isolation Kit (14900-50-NF, (MO BIO laboratories, Inc), without previous concentration according to manufacturer's instructions to obtain 100 µL DNA sample. Sterilized distilled water was included as a negative extraction control. The concentration of the isolated DNA was determined with a Nanodrop® spectrophotometer (ND-1000, Nanodrop Technologies, Wilminton, Delaware, USA).

2.5. qPCR analysis of human-specific DNA in wastewater

Quantitative real-time PCR (qPCR) was performed using a Bio-Rad CFX96 instrument (Bio-Rad Laboratories, Hercules, CA, USA). The human specific mtDNA ND5 primers were used for PCR amplification from wastewater DNA samples[20]. The final PCR reaction volume was 15 µL, consisting of 7.5 µL SsoFast mastermix (Bio-Rad) 0.6 µL reverse and forward primers (0.4 µM final concentration), 2 µL DNA sample and completed with 4.3 µL ddH_2O. The amplification conditions were modified as following: 2min hot start step at 98 ^oC, followed by 35 cycles of 98 ^oC for 5 s and 60 ^oC for 5 s. The amplification was followed by a melt curve analysis with a temperature incrementation of 0.2 ^oC from 65 ^oC to 95 ^oC. A positive control consisting of human blood DNA sample and two negative controls (one from sterilized water during DNA isolation control and one is non-nucleotide water) were added in each run of amplification. The amplification was considered to be valid if only one unique product peak was identified by melting curve analysis.

Human mitochondrial gene ND5 nucleotide sequences were retrieved from GeneBank (http://www3.ncbi.nlm.nih.gov), and the PCR amplicon as part of AY972053 was: 5'-CAGCAGCCATTCAAGCAATCCTATACAACCGTATCGGCGATATCGGTTTCATC CTCGCCTTAGCATGATTTATCCTACACTCCAACTCATGAGACCCACAACAAATAG CCCTTCTAAACGCTAATCCAAGCCTCACCCCACTACTAGGCCTCCTCCTAGCAGC AGCAGGCAAATCAGCCCAATTAGGTCTCCACC (195 bps)-3'. An aliquot (3 µL) of each amplification reaction was analysed on 1% (w/v) agarose gel in TAE 1x buffer (pH 8). The image was recorded by Bio-Rad reader (Image Lab 4.1 software). In addition, an 182bp non-complementary PCR product amplified from the sequence of fibroblast growth factor

receptor 3 FGFR3 S249C) as described previously[31], was randomly used to evaluate the selectivity of the biosensors.

2.6. Electrochemical characterization of biosensors

All electrochemical measurements were performed on a three-electrode cell, with an Ag/AgCl reference electrode (Radiometer Analytical, Lyon, France) against which all potentials are quoted, and a Pt counter electrode (BASi, USA). Before measurement, electrodes were placed into measurement buffer for 1 h to stabilize the electrodes. PCR products were heated at 95 ^{o}C for 10 min and then cooled down to room temperature before the incubation with ssDNA probes.

Differential pulse voltammetry (DPV) measurements were performed by placing electrodes in 100 mM PB with Ag/AgCl as reference electrode. DPV scans run between -0.2 V and 0.7 V *vs* Ag/AgCl (scan rate 0.05 V/s, step potential 0.005 V, modulation amplitude 0.05 V, modulation time 0.05 s, interval time 0.1 s). The schematic illustration of elaboration of DNA sensors with Fc intercalator as redox marker is presented in Scheme 2.

Scheme 2

3. Results and discussion

3.1 Development of biosensors based on Fc intercalators

A crucial control parameter for electrochemical DNA biosensors, is the surface density of the immobilized ssDNA probe onto the electrode surface in order to effectively hybridize with complementary DNA. Many studies have shown that a low density of ssDNA (1-3 x 10^{12} molecules/cm^2) on the surface of the electrodes could achieve high hybridization efficiency for DNA biosensors[30, 32, 33]. In our early study[30], we optimised the surface density to maintain high hybridization efficiency by controlling the molar fraction of ssDNA with MCH on the electrode surface. Here, we used the optimal molar ratio between ssDNA and MCH (1:3) (ssDNA surface density of 1.6 x 10^{12} molecules/cm^2) in order to allow complementary DNA to effectively hybridize with the DNA probe. The concentration of Fc intercalators was optimized to 1 µM for binding with dsDNA. As shown in Figure 1A, the current peak at around 0.49 V *versus* Ag/AgCl was associated with the oxidation of ferrocene on the electrodes. The peak current increases proportionally to complementary DNA hybridization to the ssDNA probe, enabling target complementary DNA concentration

measurement. The synthesized dsDNA intercalator can specifically bind with matched bases in-between the double-helix of DNA; the detailed synthesis and characterization is described in the supporting information. The number of paired bases on the electrode surface increases with increasing concentration of complementary DNA, leading to more intercalators to bind with dsDNA and a current value increase. The signal response to the complementary DNA hybridization shows a good dynamic range spanning from 10 pM to 100 nM (R^2=0.98), and the limit of detection was determined to be 10 pM (3σ). These results suggest that our custom synthesized intercalator displays effective and specific binding with the dsDNA.

Several groups have developed voltammetric biosensors to detect DNA using different surface modification to improve sensitivity and analytical performances as presented in Table S1 on the Supplementary Information[34-38]. As an example, Bo *et al*[35] reported a voltammetric biosensor based on the chemically modified graphene paste with a limit of detection of 0.02 nM and a dynamic range of 0.02 - 21.2 nM. Liu *et al* [36] developed a sensitive DNA biosensor with a limit of detection at 0.001 nM using a hollow gold nanosphere to enhance the immobilized DNA probe amount. However, the elaboration of biosensors required complicated surface coating procedures and allowed non-specific adsorption of the analyte onto the surface. Compared to those biosensors, the sensor described here could effectively control the surface density by the molar ratio between the MCH and thiolated ssDNA. The elaboration procedure was robust, easy-to-operate, time-saving whilst still sensitive enough for complementary DNA detection at pM range. The stability of the DNA biosensors was also investigated by storage of the immobilized DNA probes at 4 ^{o}C. The results show that the peak current value from hybridization with the 1 nM complementary DNA only reduced to 88%, 79.3 % and 68 % following storage for 1, 3 and 7 days, respectively (See Figure 1). This is due to the robust co-immobilized SAMs layer on the gold electrodes.

Figure 1

In order to further investigate the specific interaction of the Fc intercalator, fully and partially complementary DNA containing 1, 2 and 3 mismatched base pairs were employed to hybridize with the ssDNA probe on the electrode. As shown in Figure 1B, the peak current decreased with the increasing number of mismatched base pairs, suggesting that our Fc intercalator is able to distinguish a single mismatched base pair, and therefore potentially be employed for the detection of single-nucleotide polymorphisms (SNPs) disease biomarkers.

The naphthalene diimide moiety in the Fc intercalator is able to insert or intercalate between adjacent base pairs of dsDNA. The intercalator carries bulky ferrocene on the periphery of intercalating moiety, and it becomes placed in the major and minor grooves simultaneously when intercalated to the DNA duplex[39]. This kind of intercalator has a strong affinity with dsDNA because of this peculiar binding mode, and enables to easily discriminate between double- and single-stranded DNA with a large margin. More importantly, due to its highly specific affinity to well matched base pairs it can even discriminate a single mismatched base pair. SNPs are the most common type of genetic variation among people, and each SNP represents a difference in a single DNA nucleotide. In this study, we investigate feasibility for SNPs detection in wastewater samples for the purpose of public health epidemiology. Pänke *et al*[25] electrochemically detected single base-pair mismatch using a competitive binding assay between un-labelled and labelled DNA with an electroactive redox-marker methylene blue, and methylene blue was also demonstrated to be useful for the detection of point mutation in DNA voltammetric biosensor[40]. In our study, we simply use the affinity between Fc intercalator and fully/partially matched base pairs to transduce the signal, which is label-free and rapid for single base-pair mismatch detection [41]. Besides, this intercalator also shows an enhancement of impedimetric signal not only for DNA hybridization (see Figure S9) but also for aptamer sensors for the detection of protein[41]. Furthermore, to evaluate the feasibility of E-DNA biosensor for the detection of complex matrix, 1 μM fully complementary DNA (5'- CAT CGC TCG ACG TGG-3', 15 bps) was spiked into wastewater and deionized water for assay, respectively, the peak values from all the blanks are negligible (0.02 μA in buffer, 0.04 μA in wastewater and 0.03 I deionized water), indicating that our sensors are specific for complex matrices detection. The peak value (3.95 μA) from DNA spiked in wastewater is only slightly higher than that (3.72 μA) in buffer, and the current value (3.61 μA) from DNA spiked in deionized water is a bit lower than that in buffer. However, in general the responses of the spiked DNA samples in three matrixes are not significant.

3.2 qPCR analysis of human-specific mitochondrial DNA from wastewater

In addition to mtDNA mutations associated to cancer[21, 22], mutations in the ND5 subunit of complex I of the mitochondrial DNA are a frequent cause of oxidative phosphorylation disease[42]. Therefore, the ND5 sequence could potentially be used as a population biomarker candidate. DNA was extracted from the Oslo wastewater sample WW1) and the pooled urine sample WW2; human-specific mtDNA was amplified by qPCR and the product specificity was checked by melting curves analysis and gel electrophoresis. The

qPCR protocol[20] was modified for the optimal use of the qPCR instrument and the reagents used in this study. A human DNA sample was included as a positive control (PC), and two negative controls were amplified (one from sterilized water during DNA isolation control NC1, another from non-nucleoid water NC2). As shown in Figure 2A, the PC sample yielded the highest signal, followed by WW2 and then WW1 samples. This correlated well with the fact that the mtDNA template content in WW1 was less than that in WW2 due to around 1-10 dilution factor for WW1 collected from the wastewater treatment plant. Figure 2B presents the melting curve of the PCR amplicons. A single well defined melting peak at around 83 ^{o}C was observed for both the samples studied and the human positive control sample confirming the specificity of the PCR amplification. Furthermore, specificity was also confirmed by gel electrophoresis showing a product size corresponding to the expected 195 bp (see Fig 2C). The two negative controls show no or negligible amplification, confirming that the tested positive samples are true positives. We can therefore conclude that the DNA sample preparation and qPCR assay produced specific and quantitative results from urine and wastewater samples.

Figure 2

3.3. Detection of human specific mtDNA from wastewater with developed biosensors

The thiolated forward and reverse primers were separately immobilized onto electrodes for the detection of mtDNA qPCR products from the three tested samples: PC, WW1 and WW2. The measurements were performed using the same procedure as applied to the standard samples and the developed biosensor. As shown in Figure 3, three PCR samples (PC, WW1 and WW2) diluted 1 in 50, were hybridized with two different probes on the sensor and the current peak at around 0.49 V *versus* Ag/AgCl, attributed to the oxidation peak of the Fc intercalator, was obtained from both probes each binding with each of the three samples. The peak current value, proportional to the concentration of mtDNA PCR products, is the highest for PC and it shifts to lower values in the urine-pool WW2 and wastewater WW1 due to the decreased concentration of mtDNA amplicons. The results are in good agreement with the qPCR results (Figure 2). However, the peak current value (0.17 μA) from the forward primer probe (21 bp) is lower than that (0.20 μA) from the reverse primer probe (26 bp) when detecting the same WW2 sample. This is due to the fact that the reverse primer contains 5 more bases than the forward one, therefore resulting in more Fc

intercalators interacting with dsDNA on the electrodes. The highest peak current was achieved using the reverse primer as probe and therefore this probe was selected for further detection.

Figure 3

In order to evaluate the specificity of the binding between the ssDNA probes and the PCR products, two thiolated ssDNA that contain the same number of bases as either the forward or the reverse primers and are non-complementary to the mtDNA PCR product sequence, were immobilized and named probe F(non) and probe R(non). Figure 4A presents the peak current value obtained from the 4 different probes with WW1 PCR products diluted 1 in 50. The peak currents from non-complementary probes are low compared to those from complementary ones, indicating that the intercalator could specifically bind with matched base pairs. The same experiment was performed in the analysis of the WW2 sample, which shows similar results (Figure 4B). Though there are small peaks from the non-complementary probes, it is likely that the long-sequence mtDNA may foul the electrode surface introducing spurious effects. To further determine the non-specific adsorption of probes, a non-complementary PCR product (182 bps) diluted 1 in 50 was introduced to hybridize on a probe R electrode under the same conditions. As shown in Figure 4C, the current signal from non-complementary sample gives a negligible signal. These results demonstrate that the biosensors can specifically detect human mtDNA PCR products extracted from wastewater using the custom synthesized ferrocenyl intercalator.

Figure 4

The optimized biosensor was used to detect mtDNA of PCR products from WW1 and WW2 with various dilution factors. As shown in Figure 5, the peak current signal decreased with the increasing dilution-factor for WW1 and WW2, displaying a good dynamic range spanning from dilution-fraction 1/5 to 1/100, with a LOD determined to be 7.1 nM from WW1 and 4.4 nM from WW2 (1/100 and 1/200 dilution respectively; the concentration was measured with a spectrophotometer). The threshold value was determined from the average signal of blanks (“PCR non-com”) plus 3 times standard deviation. However, to obtain a

more reliable threshold value, a cohort of non-complementary PCR products may need to be evaluated. On the basis of these promising results, we then attempted to directly detect target DNA from the WW1 (12.2 μg/mL) and WW2 (33.7 μg/mL) samples (extracted from 100 mL wastewater) without PCR amplification. Although the peak current values obtained were quite close to that from non-complementary PCR products (Figure 5C), WW2 was above the threshold while WW1 scored negative. This difference is quite understandable since WW2 is undiluted urine, while WW1 is approximately 1% urine. Wastewaters are complex matrices with a high bacterial content along with diverse cell residues from plants and animals aside of human shed material. Such a dilution of the target human DNA, along with the interference of non-target DNA is expected to be challenging for direct detection as signal may also be associated to non-specific adsorption from self-fouling of long DNA sequences. However, the concentrated DNA extracted from pooled urine (WW2) produced a stronger signal than that obtained with WW1, as expected as it is likely to contain proportionally more target human DNA, as already shown by qPCR. To our knowledge this is the first time that an E-DNA biosensor is used for direct detection of target DNA from a wastewater sample. The

Figure 5

Although direct detection and quantification of human mtDNA from extracted wastewater samples remains challenging, we believe that this proof-of-concept study will accelerate the development of ultrasensitive biosensing technology for the analysis of biomarkers in wastewater for epidemiology studies. For example, this system may be further improved by using nanomaterials for signal enhancement[43] for a more sensitive DNA biomarker analysis in wastewater without PCR amplification.

In summary, a sensitive E-DNA biosensor using a custom synthesized Fc intercalator as the electrochemical signal transducer was developed. The optimized biosensor could detect complementary DNA at concentrations as low as 10 pM with a dynamic range spanning from 10 pM to 100 nM, also enabling the detection of single nucleotide mismatches. To further evaluate the feasibility of using this technology for analysing human population DNA biomarkers from wastewaters, primers designed to amplify human-specific mtDNA were used for qPCR from wastewater and pooled urine samples. Serial dilutions of the human mtDNA PCR products were specifically detected by the customized biosensors down to a

200-fraction dilution (4.4 nM). Furthermore, E-DNA biosensors appear well suited for implementation in portable PCR microdevices directed at the rapid detection of DNA biomarkers [44], including wastewater for the purpose of WBE. Finally, our study demonstrates the feasibility of analysing human specific DNA biomarkers directly in wastewater by using an E-DNA biosensor. This shows that E-DNA biosensors have a strong development potential for the monitoring of DNA-disease biomarkers in wastewater, such as SNPs, for the evaluation of public health by WBE.

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Captions:

Scheme 1 Synthesis of Fc dsDNA intercalator

Scheme 2 Illustration of detection of human mitochondrial DNA from wastewater with electrochemical biosensor based on Fc intercalator.

Figure 1 DPV curve of 1 mM ferrocenyl interacalator for the detection of various concentrations of complementary DNA and the current as the function of the complementary DNA concentration (insert) (A), and of DPV curve from effects of the number of mismatched base pairs and the current value as the function of the number of mismatched base pairs (insert) (B).

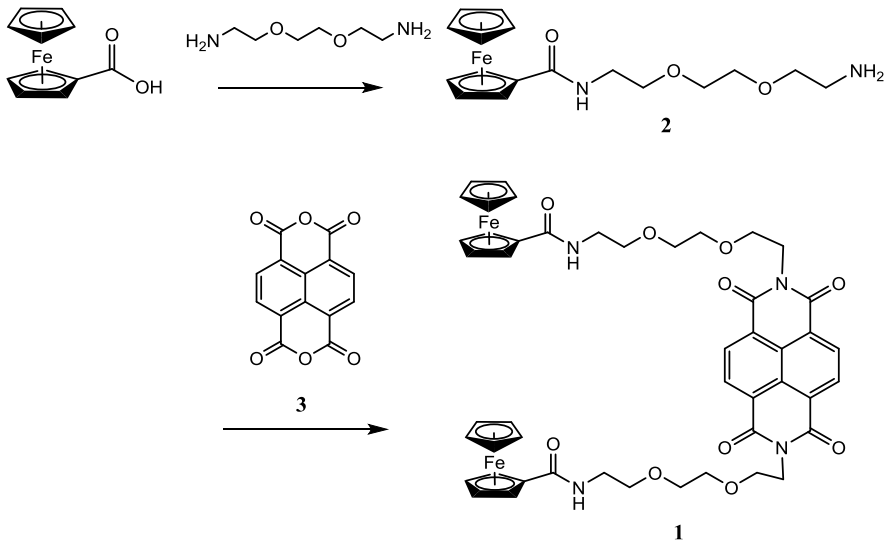
Figure 2 Amplified cycles (A) and melting curve (B) of qPCR analysis of mtDNA in wastewater sample using prime expected products 195 bps, and image (C) of electrophoresis gel analysis of PCR products with double replicates from each sample. PC: human positive control, WW1: wastewater treatment plant, WW2: urine pool, NC1: extraction negative control, NC2: qPCR mastermix negative control.

Figure 3 DPV curve of detection of human sample (PC), WW1 and WW2 samples amplified with PCR using thiolated forward (A) and reverse (B) primers as probes

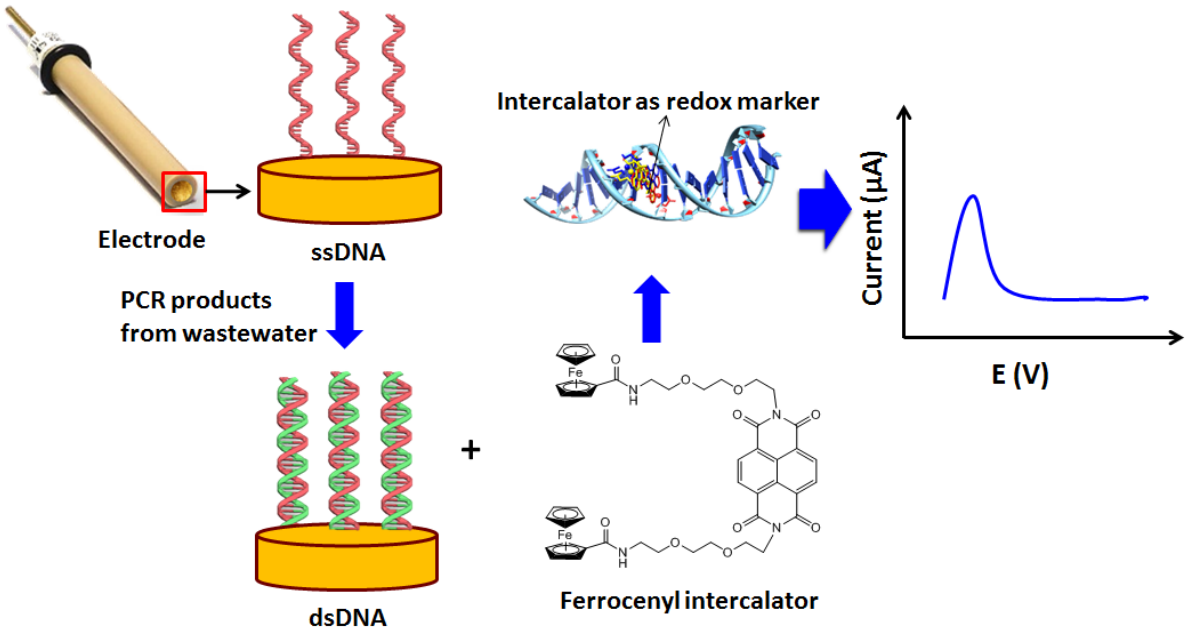
Figure 4 Detection of PCR products (1/50 dilutions) of WW1 (A) and WW2 (B) with different thiolated probes (probe F: thiolated forward primer; probe R: thiolated reverse primer probe; F/R (non): 21/26 bps non-complementary to the mtDNA PCR product), and (C) current signal response to the complementary and non-complementary PCR products hybridisation to probe R.

Figure 5 Detection of WW1 (A) and WW2 (B) PCR product serial dilution, and (C) the current response from the detection of mtDNA extracted from wastewater with/without PCR amplification, reference PCR products of WW1 and WW2 were diluted 1 in 50.

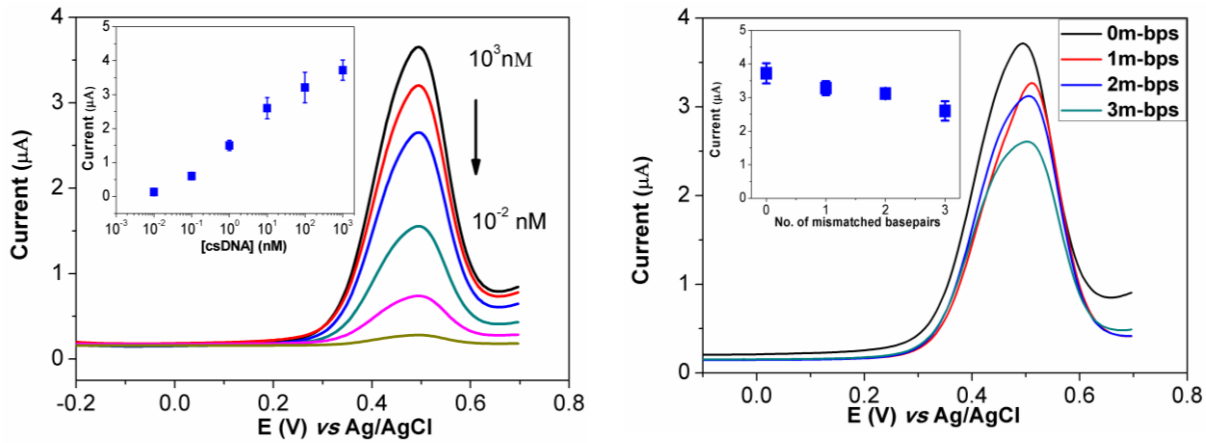
Schemes and Figures



Scheme 1 Synthesis of Fc dsDNA intercalator



Scheme 2 Illustration of detection of human mitochondrial DNA from wastewater with electrochemical biosensor based on Fc intercalator.



A B

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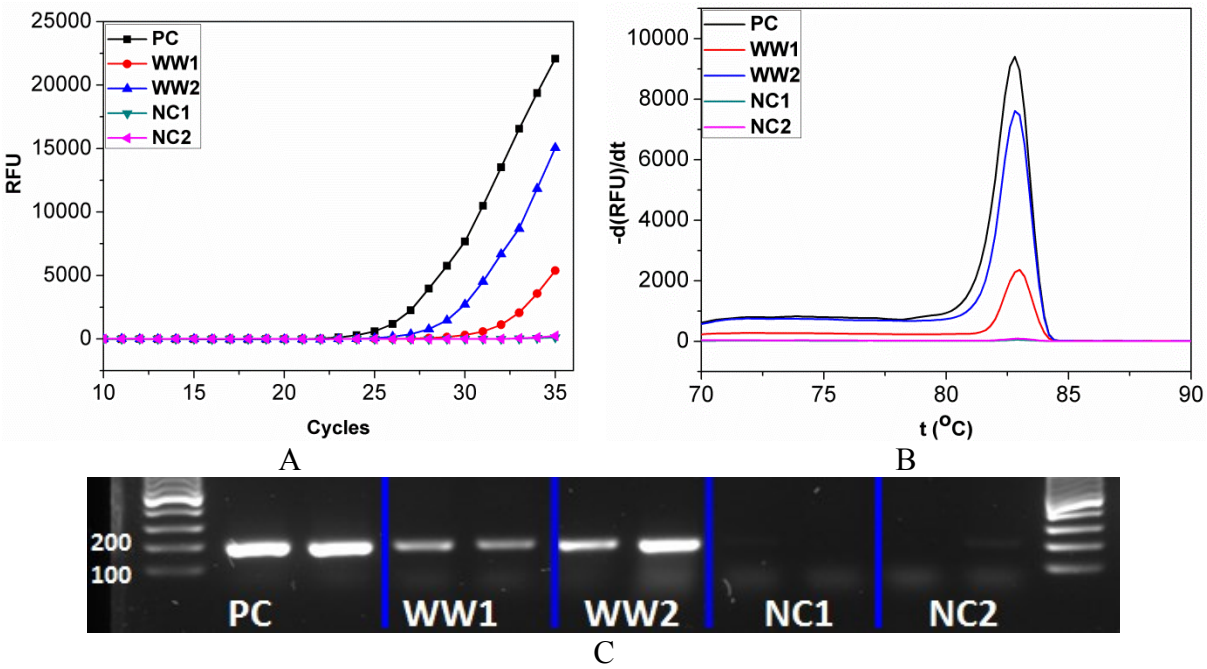

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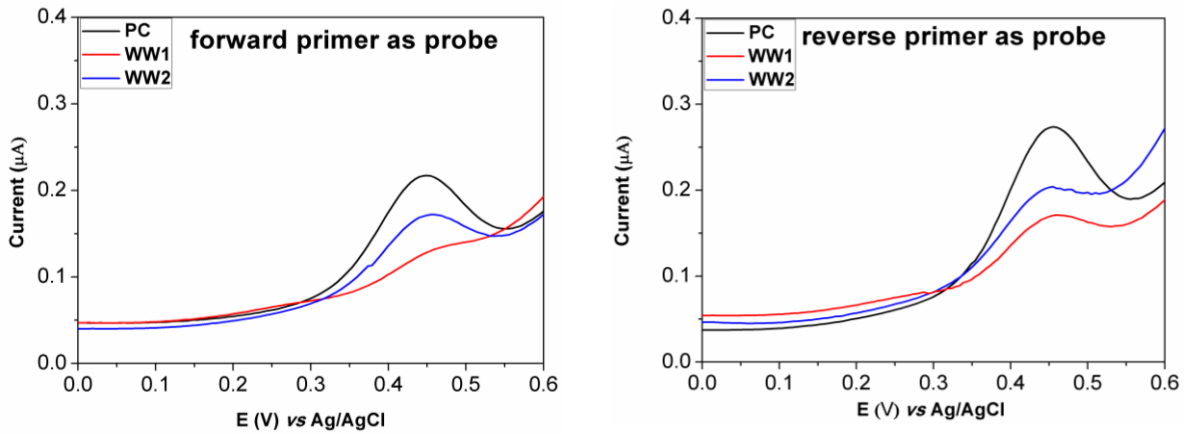

A B

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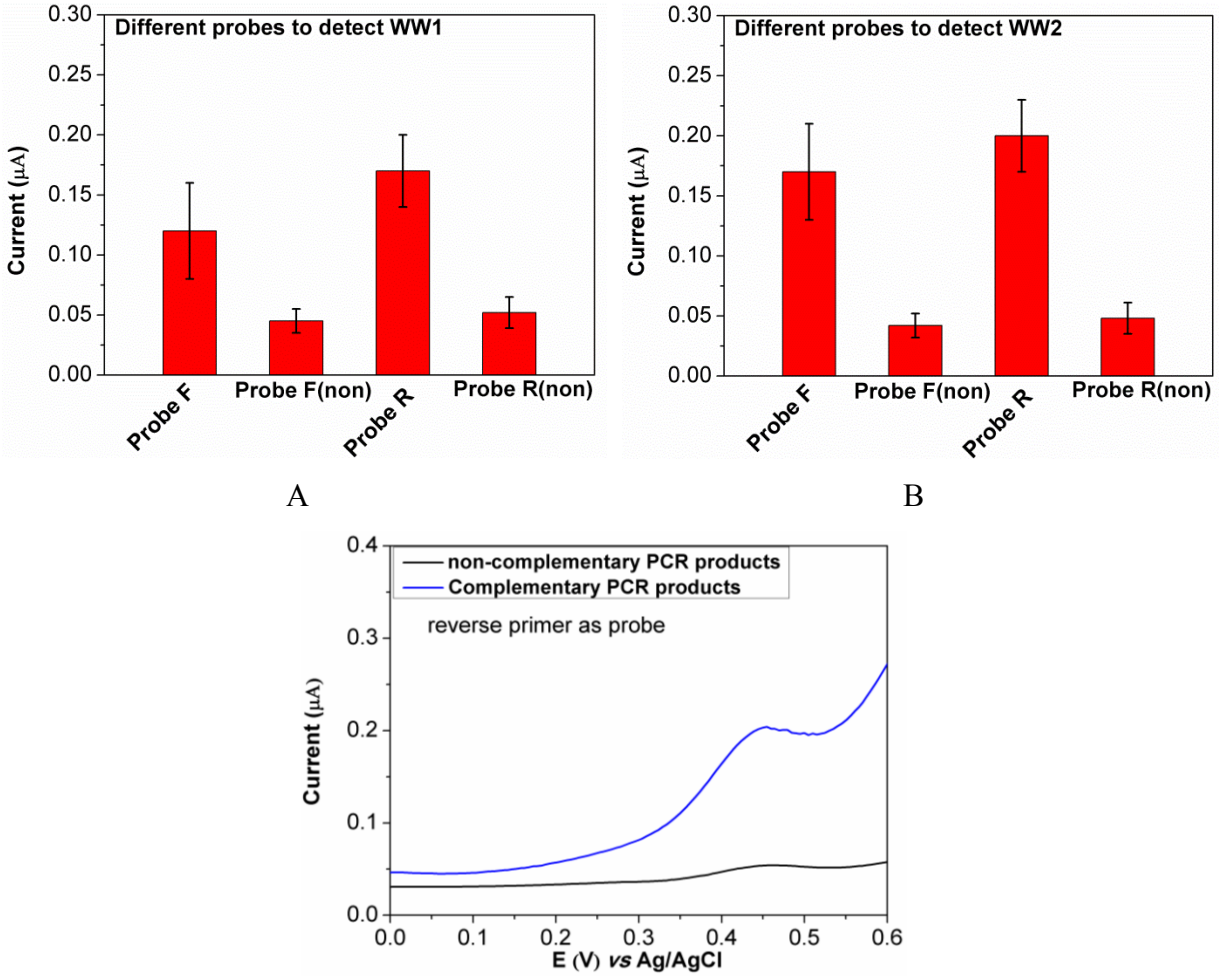

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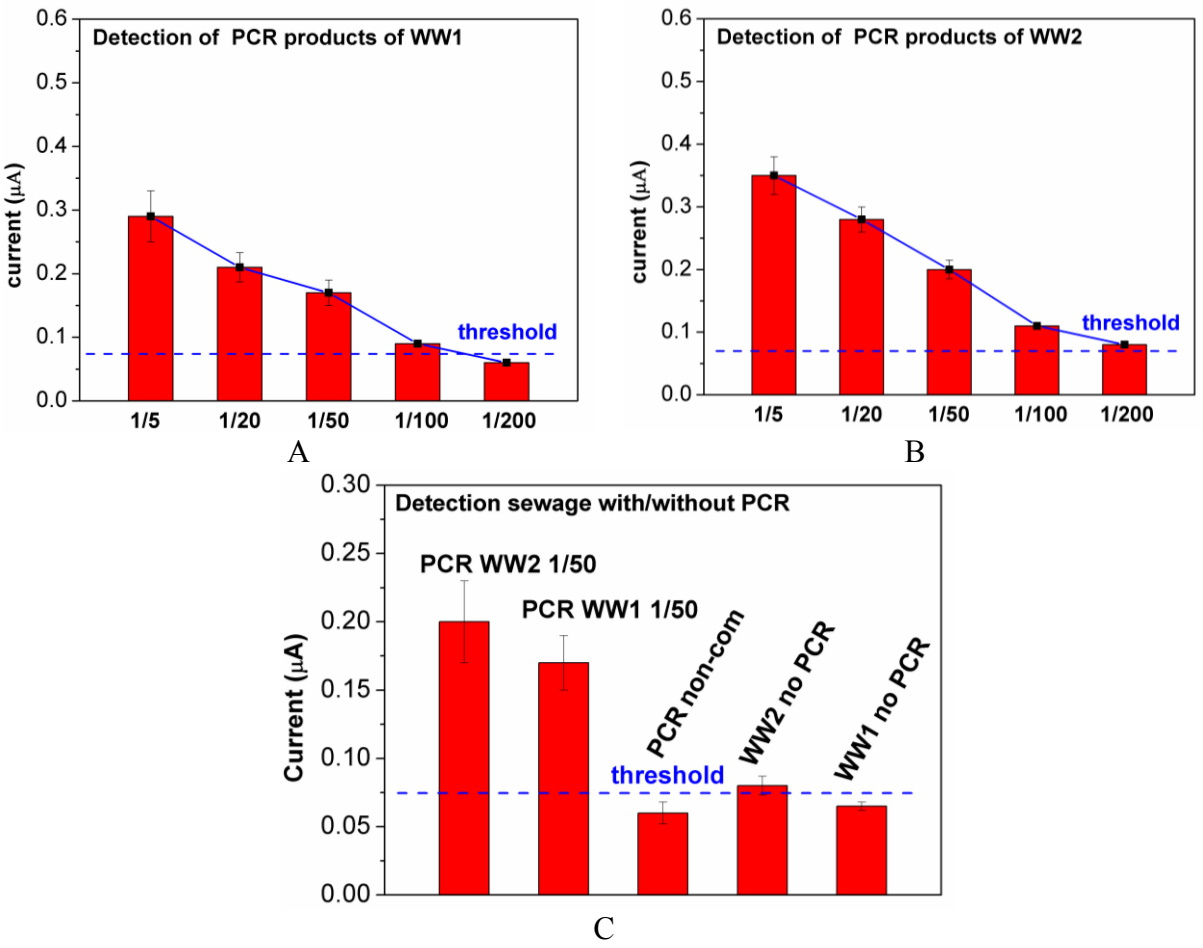

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690 Figure 5 Detection of WW1 (A) and WW2 (B) PCR product serial dilution, and (C) the current
691 response from the detection of mtDNA extracted from wastewater with/without PCR amplification,
692 reference PCR products of WW1 and WW2 were diluted 1 in 50.

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