Biosensors and Bioelectronics ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

DNA-based biosensors for Hg^{2+} determination by polythymine–methylene blue modified electrodes

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ARTICLE INFO

Article history:
Received 6 June 2014
Received in revised form
12 September 2014
Accepted 15 September 2014

Keywords:
DNA-based biosensor
Mercury
Polythymine

ABSTRACT

In this work we have developed a new electrochemical DNA-based biosensor for the selective determination of the Hg^{2+} ion by the use of different electrodes modified with polythymine, bearing methylene blue, as redox probe, in 3′ position. The determination of Hg^{2+} can be employed with an excellent degree of selectivity by the use of DNA biosensors through the formation of the complex Thymine–Hg–Thymine (T–Hg–T): in fact, Hg^{2+} tends to bind two thymines, generating a T–Hg–T complex with a formation constant higher than that one of the coupling Adenine–Thymine, which can be employed for a selective, fast and cost-effective Hg^{2+} detection.

The presence of the Hg^{2+} in solution leads to the formation of T–Hg–T complex thus causing the "hairpin-like" folding of oligonucleotide, leading to an improved electronic exchange of methylene blue with the electrode surface due to the reduced distance and thus to an increase of the faradic current which is detected by means of square wave voltammetry (SWV). To test the feasibility of this kind of biosensor to be applied to the analysis of Hg^{2+} we have developed several biosensors configuration by modifying the electrochemical sensor transducer: (a) Au electrode; (b) Au screen-printed electrode (SPE). The proposed system, allows the determination of Hg^{2+} in the range 0.2–100 nM (0.05–20 ppb), with a sensitivity 0.327 μA/nM, LOD 0.1 nM (0.02 ppb), LOQ 0.2 nM (0.05 ppb) and RSD $\leq$4.3% when Au electrode is used as electrochemical transducer; on the other hand, in the case of Au SPE the linear range is 0.2–50 nM (0.05–10 ppb), with a sensitivity 0.285 μA/nM, while LOD and LOQ are the same as previously and RSD is $\leq$3.8%. This enabled the detection of mercury in real samples (waters and fishes) with good accuracy (recoveries 92–101% on waters and 92–107% on fishes, respectively) and reproducibility (RSD $\leq$9.6% for measurements on waters and $\leq$8.8% on fishes, respectively).

1. Introduction

Heavy metal ions contamination may cause risks on human health and the environment. The main threats to human health from heavy metals are associated with exposure to lead, cadmium, mercury and arsenic. These metals have been extensively studied and also their effects on human beings (Sardar et al., 2013).

Development of new metal ion-sensing strategies for detecting and quantifying metal ions in many different fields (Agasti et al., 2010; Aragay et al., 2011) continues to be the focus of numerous researches.

Mercury together with other heavy metals is one of the most worrisome environmental contaminant: mainly because of its metabolic inertia it remains permanently in the body procuring metabolic and structural damage to the cells for its ability to interact with proteins. In particular, mercury contamination is widespread and arises from a variety of natural sources, such as oceanic and volcanic emissions (Renzoni et al., 1998) as well as anthropogenic sources (Pirrone et al., 2010; Stracher et al., 2010) such as gold mining and the combustion of solid waste and fuels.

The transformations and cycles of mercury within the environment have been thoroughly studied (Hammerschmidt and Fitzgerald, 2001). Once introduced into the marine environment, bacteria convert inorganic mercury into methylmercury, which enters the food chain and accumulates in higher organisms, such as large edible fish (Harris et al., 2003). Methylmercury is neurotoxic and has been implicated as a cause of mercury-pollution-related diseases (Grandjean et al., 1998).

Several methods have been developed for the determination of mercury in environmental samples, but the problems related to

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http://dx.doi.org/10.1016/j.bios.2014.09.031
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the selectivity and interference of other metals still remain. Among the various forms thereof, for mercury (II) ion Hg^{2+} the Italian and European Environmental Protection Agency (EPA) have set the limit at 1 ppb and 2 ppb in drinking water, respectively. Nevertheless, the electrochemical methods can be hardly applied due to low sensitivity.

A wide variety of instrumental methods has been developed for the determination of environmental mercury. Sophisticated analytical techniques include atomic absorption spectrometry, atomic emission spectrometry, inductively coupled plasma/mass spectrometry and cold vapor atomic absorption spectrometry (Mondal et al., 2001; Segade and Tyson, 2003; Han et al., 2006; Leermarkers et al., 2005; Li et al., 2006). These methods are time-consuming and laborious, and they lack the procedural simplicity for analysis in situ.

To avoid this, in recent years electrochemical sensors have proven to be effective tools for mercury monitoring due to their characteristics as good portability, low cost, simple instrumentation and high sensitivity (Lost and Crespilho, 2012).

Numerous reports on optical Hg^{2+} detection by using Hg^{2+}-sensitive fluorophores or chromophores (Yoon et al., 2007; Nolan and Lippard, 2008) have been proposed. However, most of these fluorophores or chromophore-based Hg^{2+} sensors only work in organic media, which cannot be directly used to detect mercury contaminants in aqueous media.

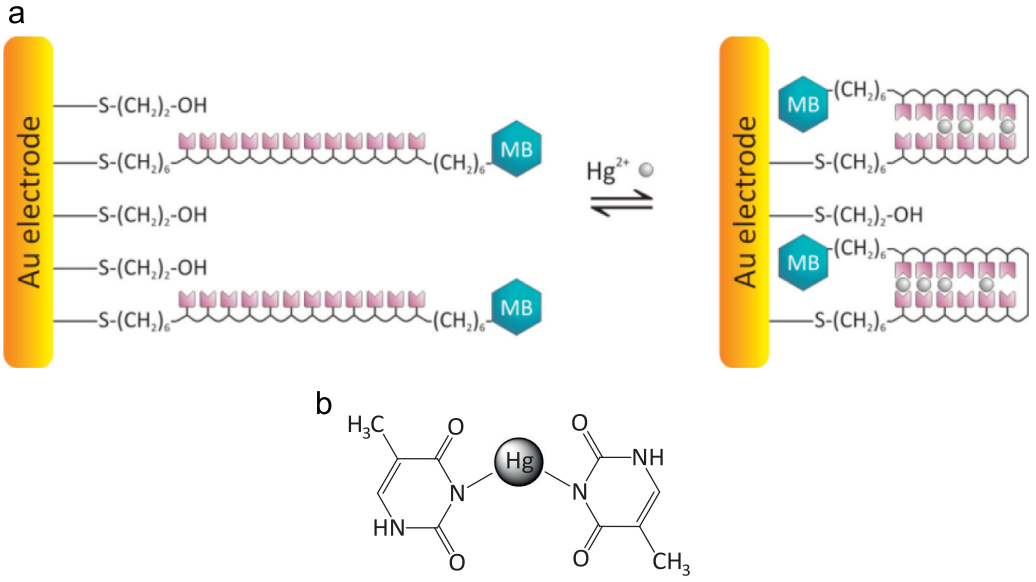
Recently, the coordinate interaction between Hg^{2+} and bis-thymine has attracted significant interest (Clever et al., 2007; Miyake et al., 2006). It was found that within a double-stranded DNA structure, the mercuric ion Hg^{2+} tends to bind two thymines, replacing the imino protons and inducing a conformational change that depends on the sequence of the base pairs. In addition, analysis with other metal ions, have shown that this interaction is specific for mercury.

The determination of Hg^{2+} can be employed with an excellent degree of selectivity by the use of DNA biosensors through the formation of the complex Thymine–Hg–Thymine (T–Hg–T) as depicted in Scheme 1(a). In fact, Hg^{2+} tends to bind two thymines, generating a T–Hg–T complex (see Scheme 1(b)) with a formation constant higher than that one of the coupling Adenine–Thymine, which can be employed for a selective, fast and cost-effective Hg^{2+} detection.

Really, only Hg^{2+} ions and no other metal ions have been found to bind with the Thymine–Thymine (T:T) mismatch in duplex DNA to form T–Hg–T. The interaction between Hg^{2+} pairs in duplex DNA or the corresponding perfectly matched DNA was analyzed by UV melting experiments, isothermal titration calorimetry (ITC) and circular dichroism spectroscopy (Torigoe et al., 2010). ITC analyzes demonstrate that the Hg^{2+} ion specifically binds with the T:T mismatched base pair at a molar ratio of 1:1 with a binding constant K_a of nearly $10^6\ M^{-1}$ (detected at pH 6.8). More recently (Stobiecka et al., 2012), the formation constants have been determined from fluorimetric measurements and results of ^1H NMR spectral analysis, for Hg^{2+} binding reversibly to a (T:T) mismatch in the stem of a molecular beacon, leading to $K_a = 8.92 \pm 0.42 \times 10^{17}\ M^{-1}$ (for bridge formation data). This confirms the results obtained by Torigoe et al., carried out for a T–Hg–T bridge in a 25-base-pair DNA duplex with a single T–T mismatch; in fact, whether their constant, $K_a = 5.61 \pm 1.35 \times 10^5\ M^{-1}$, is recalculated to account for the presence of $Hg(OH)_2$ in aqueous solutions and the respective pH dependence, it results that $K_a = 2.0 \times 10^{14}$ at pH 7.45. Hence, the value of K_a calculated by Stobiecka et al. for a molecular beacon is 3 orders of magnitude higher than that for a duplex dsDNA determined by Torigoe et al. This difference likely results from the reduced strain on the T–Hg–T bridge due to the short length of the molecular beacon stem; in any case, such high values of the formation constant, form the basis for a sensitive and selective determination of mercury using this system.

Novel Hg^{2+} detection assays in aqueous solutions have been reported, based on the principle above, but, most of these works rely on optical techniques (Lee and Mirkin, 2008; Lee et al., 2007; Liu and Lu, 2007; Ye and Yin, 2008).

In recent years electrochemical sensors have proven to be effective tools for mercury monitoring due to their features (Lost and Crespilho, 2012). Great efforts have been made to design electrochemical sensors for probing trace of mercury ions with the goal to develop portable and reliable analytical devices (Marcolino-Junior et al., 2007; Wu et al., 2010; Gao et al., 2008). Zhu and his coworkers proposed a highly sensitive electrochemical sensor for the detection of Hg^{2+} ion in aqueous solution by using a thymine rich, mercury-specific oligonucleotide probe and gold nanoparticles based signal amplification. They reached a detection limit of 0.5 nM. Their sensor also exhibited an excellent selectivity over a spectrum of interference metal ions (Zhu et al., 2009). An ultrasensitive electrochemical sensor for Hg^{2+} based on target-induced structure-switching DNA has been reported by Wu team.



Scheme 1. (a) DNA-based Hg^{2+} biosensor functioning principle and (b) structure of complex Thymine–Hg–Thymine.

Please cite this article as: Tortolini, C., et al., Biosensors and Bioelectronics (2014), http://dx.doi.org/10.1016/j.bios.2014.09.031

The sensor was thoroughly investigated using cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS). The effect of the reaction temperature on the response of the sensor was also studied in detail. The results revealed that the device showed sensitive response to Hg^{2+} in a concentration range from 0.1 nM to 5 μM with a detection limit of 0.06 nM (Wu et al., 2010). Strategies for mercury detection based on T–Hg–T coordination chemistry have been recently reported also by Wang et al. (2014), obtaining a three orders wide dynamic range from 10 nM to 10 μM and by Zhang et al. (2013), basing on grafting DNA strands on modified graphene oxide. Furthermore, by employing suitable amplification procedures of the signal, very low detection limits were achieved: 2.5 pM (Yuan et al., 2014) and even 1 pM (Chen et al., 2014).

It should be pointed out that, most methods reported were based on the "signal-off" assay strategy (Wu et al., 2010; S. Liu et al., 2009), which entails a high background signal for the newly as-prepared sensors. On the other hand, only a few electrochemical sensors were constructed based on the "signal-on" assay mode, usually involving cumbersome procedures using nanomaterials (Guo et al., 2011), enzymatic amplification (Zhang et al., 2011), or auxiliary oligonucleotide strands (Li et al., 2010).

The Zhuang group described a simple and reusable electrochemical sensor for mercury determination based on "signal on" assay mode, by coupling target-induced conformational switch of DNA hairpins with T–Hg–T coordination chemistry. Each hairpin was labeled with ferrocene redox tag. They obtained a dynamic concentration range from 5.0 nM to 1.0 μM and a detection limit of 2.5 nM (Zhuang et al., 2013).

In this work we have developed a new electrochemical DNA biosensor based on "signal on" assay mode for the selective determination of the Hg^{2+} ion by the use of a gold electrode (either classical or screen printed) where polythymine, modified in 3′ position with methylene blue as redox probe, was immobilized as self assembled monolayer (SAM). Methylene blue is already known as electrochemical probe for DNA-based biosensors even if it is usually employed as intercalative probe to detect DNA strands hybridization (Kelley et al., 1997; Drummond et al., 2003; Li et al., 2007; Kerman et al., 2002; Zhu et al., 2004; Ozkan et al., 2002); in this case is covalently bound to polythymine strand and in the presence of the Hg^{2+} in solution, the formation of T–Hg–T complex causes the "hairpin-like" folding of oligonucleotide, thus approaching the methylene blue to the electrodic surface. This leads to an improved electronic exchange of methylene blue with the gold electrode due to the reduced distance and thus to an increase of the faradic current which is detected by means of square wave voltammetry (SWV) and is proportional to Hg^{2+} concentration.

2. Experimental

2.1. Chemicals and reagents

All reagents were of analytical grade and were used as received without further purification.

The following chemicals were supplied by Sigma-Aldrich (Sigma Chemical Company, St. Luis, MO, USA): $Hg(NO_3)_2$, $NaClO_4$, Na_2HPO_4, NaH_2PO_4, TRIS, H_2SO_4, $Zn(NO_3)_2 \cdot 6H_2O$, $Mn(NO_3)_2$, $Cu(NO_3)_2$, $FeCl_2$, $Pb(NO_3)_2$, 2-Mercaptoethanol ≥ 99%, ethanol ≥ 99.8%; NaCl and KCl were obtained by Merck (Merck, Darmstadt, Germania). The oligonucleotide 5′-HS-$(CH_2)_6$-TTTTTTTTTTT-$(CH_2)_6$–methylene blue-3′ employed in this research was synthesized ad hoc by Biomers (Ulm, Germania) with the structure indicated in Scheme SM1.

For Au classical electrode cleaning alumina slurry of different granulometry (0.3–0.05 μm) has been employed (SIEM, Bologna).

All the aqueous solutions were prepared with deionized water ($R = 18.2$ MΩ cm at 25 °C; TOC < 10 μg L^{-1}) obtained by a Millipore Milli-Q system (Millipore, Molsheim, Francia).

2.2. Au surfaces SAM modification

The considered Au surfaces (either the Au-slides for AFM analysis or the Au working electrodes for electrochemical investigation) were modified with a mixed SAM by immersing in a solution of 1.33 pM thiolated oligonucleotide and 0.2 nM 2-mercaptoethanol (ratio 1:1500). Since the amino groups of thymine may interact weakly with the electrode surface, forcing the oligonucleotide to be arranged horizontally (Leff et al., 1996) thus preventing a correct packing of the self assembled monolayer (SAM), a proper spacer (2-mercaptoethanol) was added to the modifying solution. Gold substrates have been incubated for 6 h in the solution of thiols, then rinsed with ethanol and dried in a stream of nitrogen.

2.3. Methods and measurements

The microscopic analysis was performed on a glass Au-coated slide modified as described above, by a MultiMode Atomic Force Microscope system (Veeco, Somerset, NJ, USA) with Nanoscope IIIa; the measurements were carried out in tapping mode and analyzed using Gwyddion 2.31 software.

The electrochemical experiments were performed in a borosilicate glass cell with the typical three-electrodes configuration using as electrochemical interfaces either the μ-Autolab (Eco-Chemie, Utrecht, The Netherlands) in the case of classical electrodes or the PalmSens (PalmSens, Utrecht, The Netherlands) in the case of screen-printed electrodes.

In the first case the working electrode was an Au microelectrode (model 6.1204.320, Metrohm, Herisau, Switzerland) with diameter $d = 3$ mm, the reference electrode was a saturated calomel electrode SCE (model 303/SCG/12, Amel, Milano, Italy) and the counter electrode a glassy carbon rod (model 6.1248.040, Metrohm, Herisau, Switzerland).

In the second case Au screen-printed electrode (Au-SPE) (model DPR-220AT, DropSens, Oviedo, Spain), constituted by an Au working electrode with a surface diameter of 4 mm, silver pseudo reference electrode and gold counter electrode, has been employed.

In the case of classical Au working microelectrode, before modification the surface of electrode is thoroughly cleaned both mechanically and electrochemically. A mechanical cleaning is firstly performed on appropriate support for lapping by an aqueous suspension of alumina decreasing grain size (0.3–0.05 μm) then a rinse with deionized water and the sonication for 10 min for eliminating any residual alumina, follows. Subsequently, the electrode is subjected to electrochemical cleaning, through cyclic voltammetry, effecting 30 scans at 100 mV s^{-1} potential in the range of 500–1600 mV (vs. SCE) in H_2SO_4 0.5 M in order to cause a series of successive oxidations and reductions of the surface electrode, thus favoring the release of any contaminants adsorbed. Conversely, the SPE electrodes were used as are, without any cleaning pretreatment.

The Au electrodes (either classical or SPE) were modified using the SAM procedure described above, before proceeding with electrochemical experiments.

The measurements are carried out in two steps: firstly, the sensor is dipped in the solution containing Hg^{2+} for 1 h in the dark (either standard solutions in Milli-Q water for analytical characterization or real samples solutions properly diluted) in

order to avoid possible degradation of methylene blue due to visible light (Umebayashi et al., 2003), then it is rinsed with Milli-Q water and placed in an electrochemical cell in a typical three-electrodes configuration (saturated calomel as reference electrode) and containing a buffer solution. Secondly, the electrochemical measurements are carried out using square wave voltammetry (SWV): taking into account that the formal potential of methylene blue falls normally in the range −0.1 to −0.4 V (vs. SCE) (Wang et al., 2009), the scan is performed from −0.7 to 0 V (amplitude=0.001 V, potential step=0.025 V, frequency=60 Hz).

The electrode is regenerated by immersion in a solution of 1 M KI and 1 M NaCl in 10 mM phosphate buffer (pH 7.4) for about 30 min (Zhuang et al., 2013). Finally, it is rinsed thoroughly with distilled water. In these conditions the formation of mercuric iodide is fostered through the following reaction:

$$Hg^{2+} + 2I^{-} \rightleftharpoons HgI_2\downarrow$$

It relies on the ability of iodide ion to subtract Hg^{2+} to the oligonucleotide structure, since the formation constant of the bond HgI_2 is of the order of 10^{29} (Marcus, 1957), much higher than that one of the complex Thymine–Hg–Thymine which is of the order of 10^{18} (Stobiecka et al., 2012).

In the case of Au screen-printed electrode, the SWV measurements for the calibration curve and the regeneration step were the same utilized for the Au microelectrode.

For real samples analysis, samples were prepared according to published procedures (Lamble and Hill, 1998; LamLeung et al., 1991; Wu et al., 2011): briefly, specimens of fish tissue (0.2 g) were dissected from frozen whole specimens after scale removal and digested in nitric acid (5 mL) at 180 °C for 5–10 min. The resulting solution was neutralized with NaOH and HEPES to pH 7. Then fish samples, properly diluted, were spiked with 5 nM (1 ppb) of mercury (II) and the analytical determination was performed with the standard additions method.

3. Results and discussion

The mechanism that regulates the biosensor functioning is based on the characteristic affinity that the ion mercury (II) displays for the nitrogenous base thymine. Over the past decade, studies by capillary electrophoresis and atomic absorption spectrometry, infrared spectroscopy and circular dichroism showed a high binding affinity to mercury and its complexes towards the DNA (Selid et al., 2009). In particular, it was found that within a structure of double-stranded DNA, the mercuric ion Hg^{2+} tends to bind to two thymine (see Scheme 1(b)), by replacing the protons on the nitrogen and inducing a conformational change that depends on the sequence of the base pairs (Ono and Togashi, 2004).

Some studies have confirmed that the thymine has an highest affinity towards the mercuric ion that the Adenine (Zhu et al., 2009) and that the formation of the complex Thymine–Hg–Thymine leads to an increase of stability of the double-stranded structure of DNA (Miyake et al., 2006). Furthermore, analysis with other metal ions, particularly heavy metals with negative effects on human health, has shown that this interaction is specific for mercury (Wu et al., 2010; Miao et al., 2009).

The T–Hg–T bridge is accounted for very high stability since its formation constant ranges from 10^{14} in the case of dsDNA (Torigoe et al., 2010) to 10^{18} in the case of molecular beacons (Stobiecka et al., 2012), paving the way for a reliable and sensitive detection of mercury based on it.

First of all, a microscopic characterization of the SAM-modified Au surface was carried out by AFM; to this end two mixed SAM of

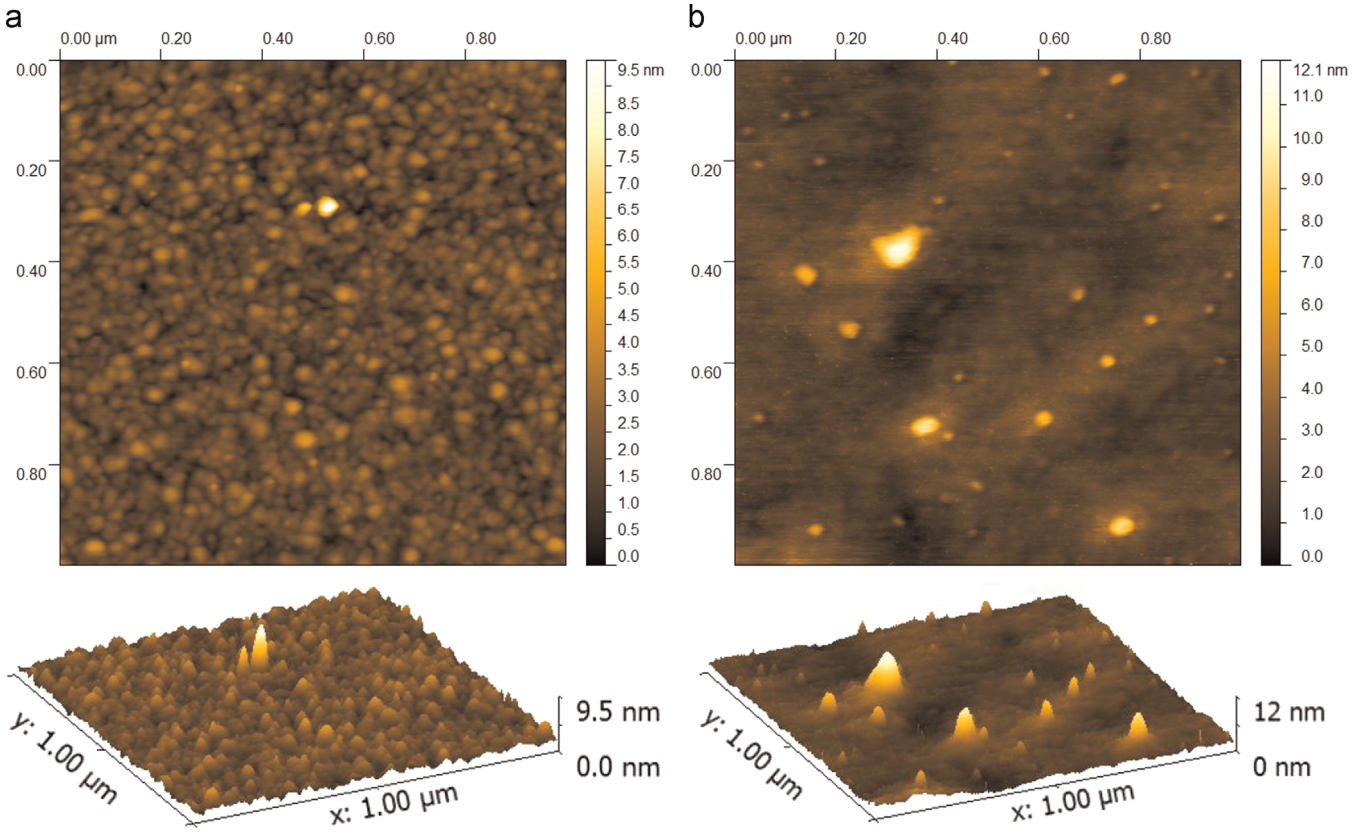


Fig. 1. AFM images for Au surface modified with SAM of different compositions: (a) 1:100 and (b) 1:1500 thiolated oligonucleotide/2-mercaptoethanol, respectively.

different density were prepared on Au-covered glass slide: 1:100 and 1:1500, thiolated oligonucleotide/2-mercaptoethanol respectively. From the comparison of the results obtained by AFM on SAM of different polythymine density reported in Fig. 1 it is possible to state that in both cases the Au surface is not homogenous but dotted with structures about 8–10 nm tall that confer a certain roughness. These structures could be attributed to polythymine clusters interspersed with a continuum of mercaptoethanol, in fact it is known that in the case of mixed-SAM some degree of phase segregation can occur (Love et al., 2005) suggesting that the homogeneous molecules generally tend to organize themselves. In the case of the polythymine richer SAM (see Fig. 1(a)), the cluster structures are very close together while in the other case (see Fig. 1(b)) they have the appearance of well-separated islands and relatively distant one from each other. A series of preliminary tests conducted on electrodes modified with SAMs of different compositions and put in contact with a solution of mercury 50 nM, have shown that the SAM 1:100, did not give rise to any electrochemical signal relating to the methylene blue, that conversely was clearly visible in the case of SAM 1:1500. It is possible to attribute this behavior to the fact that in the case of a excessively dense SAM, it proves impossible to polythymine chains to fold into the shape of hairpin thus allowing the methylene blue to approach the surface and produce the electrochemical signal. Conversely, in the case of a less crowded SAM folding is possible and the electrochemical signal ready visible. Hence, the 1:1500 thiolated oligonucleotide/2-mercaptoethanol SAM composition was chosen throughout the electrochemical measurements and by integration of faradic peak an estimation of chain density was also performed (vide infra).

As far as the electrochemical measurements are concerned, the influence of solution composition contained in the measuring cell on the obtained signal was studied by varying the concentration of either buffer (phosphate or TRIS) or supporting electrolyte ($NaClO_4$) while keeping constant the Hg^{2+} concentration in contact with the biosensor during the first step. To this end, a classical Au–polythymine–MB modified electrode was first immersed in a solution of Hg^{2+} 50 nM (10 ppb) in Milli-Q water, for 1 h, in the dark at room temperature as described in Section 2, then the signal obtained by SWV dipping the electrode in a measuring solution at different concentrations of phosphate buffer in the range of 10–50 mM in the absence of $NaClO_4$, was registered; the results are shown in Fig. SM1.

It can be observed that increasing the phosphate concentration leads to a decrease of peak intensity (see Fig. SM1(b)); it is known that the high phosphate concentration could affect the detection of divalent metal ions using DNA-based sensors (C.-W. Liu et al., 2009), even if taking into account that the stability constant of T–Hg–T bridge is around $10^{18}\ M^{-1}$ (Stobiecka et al., 2012) a possible competition with phosphate–mercury complex is unlikely. Anyway, since the increasing phosphate concentration seems to negatively affect the signal intensity and an elevated phosphate concentration is not indispensable in order to maintain the pH buffered to the wished value, is chosen to adopt the concentration of 10 mM for all the successive measurements thus avoiding to reduce the sensitivity of the proposed method.

In addition, we evaluated the influence on the signal of the supporting electrolyte ($NaClO_4$); the latter was chosen for its poor ability to form complexes with divalent metal ions (Criscenti and Sverjensky, 1999) thus introducing errors in the quantitative analysis. We studied the response of the system by varying the concentration of $NaClO_4$ in a range 0–0.5 M, keeping constant the concentration of Hg^{2+} (50 nM; 10 ppb) in phosphate buffer 10 mM, pH 7.4. The corresponding voltammograms obtained are shown in Fig. SM2 and evidence that the increase of electrolyte concentration fosters the signal relative to methylene blue reduction.

Furthermore, in order to investigate whether the nature of the substance buffer had an influence on the signal eventually due to partial subtraction of Hg^{2+} ions from the oligonucleotide structure thus leading to a stiffening of the chain and preventing methylene blue to approach the electrode surface and then to exchange electrons, the obtained response of the system in phosphate/$NaClO_4$ solution was compared to that one obtained in TRIS/$NaClO_4$ solution. To this end, a set of measurements were performed in TRIS buffer 10 mM, pH = 7.4 and $NaClO_4$ 0.5 M after having put in contact the biosensor with the testing concentration of Hg^{2+} (50 nM; 10 ppb). The results reported in Supplementary material (Figs. SM3 and SM4) evidence that in TRIS buffer the signal, despite being greater with respect to that one in phosphate, does not remain constant but rather decreases exponentially until almost to zero after 10 min (Fig. SM3(b)); on the contrary, in phosphate the signal remains stable indefinitely and allows to repeat several times the measurement thus calculating an average value.

Basing on these findings, the measuring solution used in subsequent experiments is composed by $NaClO_4$ 0.5 M in phosphate buffer 10 mM, pH 7.4 and each measurement reported is calculated as the mean value of three independent experiments.

The response of the biosensor to different concentrations of Hg^{2+} has been studied; to this end, the modified working electrode was immersed in a $Hg(NO_3)_2$ solution in Milli-Q water at a selected concentration in the range of 0.2–2000 nM (0.05–400 ppb) in water for 1 h, in the dark at room temperature. Subsequently, the electrode was rinsed with deionized water and introduced into the electrochemical cell containing the measuring solution (10 mM phosphate buffer and 0.5 M $NaClO_4$, pH 7.4). Between a measurement and the subsequent one, the biosensor was regenerated by immersion in a solution of 1 M KI and 1 M NaCl in 10 mM phosphate buffer (pH 7.4) for 30 min. Fig. 2 (a) shows the voltammograms obtained by SWV measurements and Fig. 2(b) the dependence of peak current with the Hg^{2+} concentration; it can be observed as the electrochemical response towards increasing Hg^{2+} concentration in the range 0.2–2000 nM (0.05–400 ppb) displays a linear dependence for about two and a half decades and satisfactory fits the requirements of both Italian (1 ppb) and European Environmental Protection Agency (2 ppb) regulation (Miao et al., 2009); then, the signal attains a plateau above 1000 nM (200 ppb). The plateau signal obtained for high concentration of mercury was considered in order to calculate the amount of polythymine strands immobilized on the electrode surface and their density. To this end, it can be stated that once the signal reaches the plateau this means that all the immobilized polythymine strands are engaged in forming the hairpins due to T–Hg–T bridges; hence, by integration of the faradic peak relative to 2000 nM (400 ppb) Hg^{2+} obtained using linear sweep voltammetry, the exchanged electric charge is obtained. The latter, considering a two-electron process for methylene blue (Sevcik and Dunford, 1991; Silber et al., 1996; Piskorski and Jaun, 2003), divided by the Faraday constant leads the number of electroactive molecules on the surface: the calculated density is 5.1×10^{13} molecules cm^{-2}. The limit of detection was calculated using the calibration graph and taking into account the baseline variation, as the concentration corresponding to the mean value plus three times its standard deviation: it results 0.1 nM (0.02 ppb). The reproducibility is $\leq 4.3\%$ as RDS on individual measurement. The full analytical characterization of the proposed biosensor is reported in Table SM1.

The obtained analytical performances are extremely encouraging if compared to those obtained with other DNA-based approaches for the determination of Hg^{2+} reported in recent

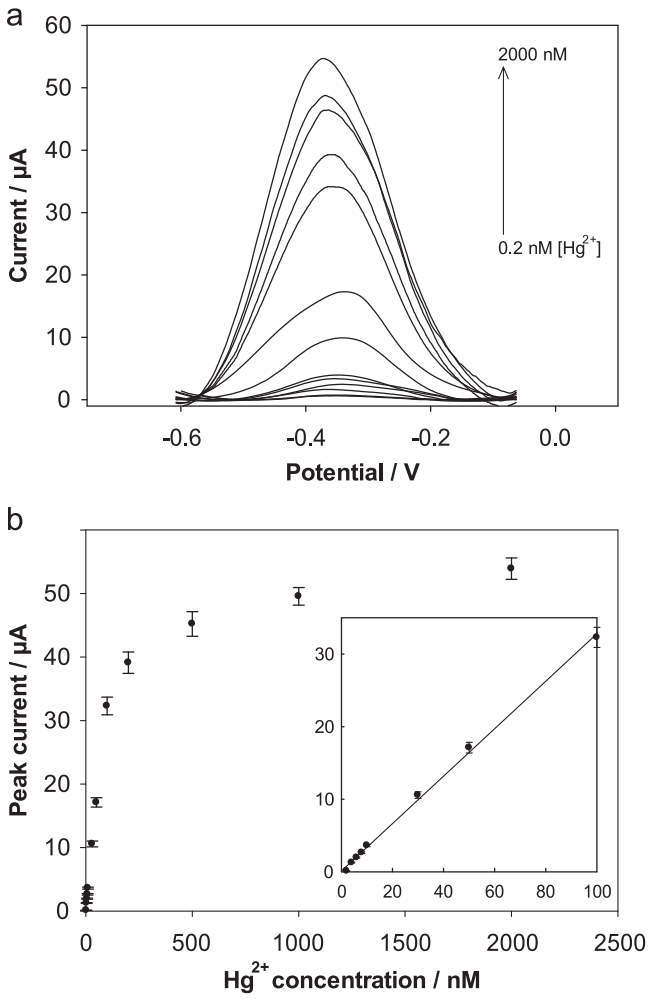


Fig. 2. Experimental SWV signal (scan from −0.7 to 0 V, amplitude=0.001 V, potential step=0.025 V, frequency=60 Hz) (a) and calibration curve (b) in the whole considered concentration range (inset: linearity range) for Au–polythymine–MB biosensor relative to different Hg^{2+} concentration values in phosphate buffer 10 mM and $NaClO_4$ 0.5 M, pH 7.4; error bars show the standard deviations of measurements taken from three independent experiments.

literature: Zhuang et al. (2013) using a similar hairpin system, claim a dynamic concentration range spanning from 5.0 nM to 1.0 μM and LOD=2.5 nM whereas we obtained a reliable detection of mercury at lower concentration level in the range 0.2–100 nM with LOD=0.1 nM. This could be explained taking into account that in our work a simpler polythymine strand was employed which allows the formation of a denser SAM (10^{14} molecules cm^{-2} vs. 10^{12} molecules cm^{-2} reported by Zhuang and coworkers) on the electrodic surface, thus enabling to obtain an analytically useful signal even for lower concentration values. In addition, the obtained results herein are better than those obtained with more cumbersome double-strand based systems reporting LOD 10 nM (Wang et al., 2014) and 5 nM (Zhang et al., 2013) respectively, while the limits of detection of the order of pM can be only pursued through complex amplification procedures (Yuan et al., 2014; Chen et al., 2014).

Selectivity of the sensor was evaluated by comparing the experimental signal obtained for several interfering metal ions one thousand-fold more concentrated than mercury; the obtained results are reported in Fig. 3. It can be highlighted a remarkable selectivity of the biosensor towards mercury ion (II), since a concentration of this metal 1000 times lower than that of other metals, corresponds to a voltammetric signal 5–10 times higher. On the other hand, the stability of the biosensor was investigated

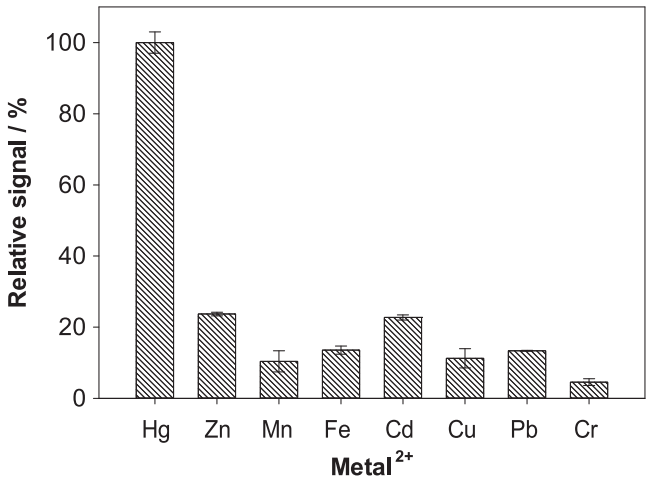


Fig. 3. Response for Au–polythymine–MB biosensor to different heavy metal ions 10 μM, relative to the signal of Hg^{2+} 10 nM; error bars show the standard deviations of measurements taken from five determinations obtained from repetitive SWV scans.

by studying the voltammetric response for Hg^{2+} at a concentration of 100 nM (20 ppb), subjecting the biosensor to repeated regeneration cycles, for 25 times over two weeks. As shown in Fig. SM5, in the first 10 cycles of regeneration, the signal remains unchanged throughout the first ten cycles within 10%, then by 10–15% until the twenty-fourth cycle after that it is lowered considerably (50%). This result is a confirmation of the stability that the redox indicator methylene blue gives to the realized biosensor in comparison to similar works employing different electrochemical probes.

Finally, the reliability of the method was estimated by evaluating the recovery of Hg^{2+} in suitably fortified real matrices. To this end, water samples of different origin (bottled water, tap water, river water) were spiked with 5 nM (1 ppb) of mercury (II) and the analytical determination was carried out with the standard additions method. The obtained concentration value was compared to the known value, calculating the percentage recoveries. Water samples were not previously treated, but used as such, in order to assess the matrix effect in its entirety, taking into account that the electrochemical biosensors should be used even in turbid media (Grieshaber et al., 2008).

From the recovery values reported in Table 1, it is possible to confirm a good performance of the method, especially for the sample of bottled water (101%), while the calculated recovery for river water is lower (92%), probably due to the presence of the natural occurring interferents able to sequester mercury ions; RSD on individual measurement is always $\leq 9.6\%$.

Once we confirmed that our proposed biosensor was able to detect Hg^{2+} ion with excellent analytical performances also in real samples, the same modification procedure was applied to Au-SPE and the resulting disposable biosensor was applied to the

Table 1
Recovery on water real samples spiked with Hg^{2+} 5 nM (1 ppb) for Au–polythymine–MB biosensor in phosphate buffer 10 mM and $NaClO_4$ 0.5 M, pH 7.4.

Real sample	Found value	Recovery (%)
Tap water	4.72 ± 0.45 nM 0.94 ± 0.09 ppb	94
Bottled water	5.05 ± 0.08 nM 1.01 ± 0.02 ppb	101
River water (Tevere)	4.59 ± 0.44 nM 0.92 ± 0.08 ppb	92

Please cite this article as: Tortolini, C., et al., Biosensors and Bioelectronics (2014), http://dx.doi.org/10.1016/j.bios.2014.09.031

Table 2
Recovery and accuracy on fish samples for Au–polythymine–MB SPE biosensor in phosphate buffer 10 mM and $NaClO_4$ 0.5 M, pH 7.4.

Real sample	Found value (ppm)	Reference value (ppm)	Accuracy (%)	Recovery (%)
Sample A	5.47 ± 0.21	5.78	5	95
Sample B	0.124 ± 0.009	0.135	8	92
Sample C	0.789 ± 0.051	0.74	−7	107
Sample D	0.99 ± 0.08	1.08	8	92

determination of mercury in fish samples. First of all, the SPE-based biosensor was analytically characterized with Hg^{2+} standard solutions, hence a curve of calibration was obtained, with a linear range between 0.2 and 50 nM (0.05–10 ppb) and a limit of detection of 0.1 nM (0.02 ppb) while the reproducibility is $\leq 3.8\%$ as RDS on individual measurement; the full list of analytical characterization parameters was reported in Table SM1.

As far the analysis on real samples are concerned, in Table 2 the obtained data on four different samples of fish tissue were reported and compared to the values from ICP taken as reference method, calculating the percentage recoveries. As it can be observed, the proposed method even when applied by employing a SPE electrode as electrochemical transducer, retains satisfactory analytical performances towards the analysis on real samples: the accuracy is always good and the recovery is in the range 92–107%, RSD on individual measurement is always $\leq 8.8\%$.

4. Conclusion

In this work a DNA biosensor with electrochemical transduction for the determination of mercury ion (II) was prepared, characterized and applied to the analysis of real samples. The biological component of molecular recognition on which the biosensor is based is an oligonucleotide consisting of 12 thymine, modified in 5′ with a –SH group which allows the immobilization on a Au surface by self assembly and in 3′ with methylene blue which serves as the electrochemical probe. The folding of the oligonucleotide that occurs in the presence of Hg^{2+} is revealed by means of square wave voltammetry, obtaining a reduction of peak for the methylene blue whose height is directly proportional to the Hg^{2+} concentration in the sample under test. The biosensor exhibits a linear response over the concentration range 0.2–100 nM (0.05–20 ppb) Hg^{2+} with a sensitivity of 0.327 μA/nM; the detection limit, is 0.1 nM (0.02 ppb) while the reproducibility is $\leq 4.3\%$ as RSD on individual measurement. The analytical performance obtained are particularly interesting in view of the limits allowed by the Hg^{2+} ion concentration according to the Italian and European legislation in the water (1 ppb) and fish (0.5 ppm for fresh edible product, increased to 1 ppm for some large size fishes). The recoveries obtained on real matrices (tap water, bottled water and river water), suitably fortified are in the range 92–101% and RSD $\leq 9.6\%$.

Subsequently, an Au screen printed electrode, modified with the same procedure, has been characterized. It shows a linear range over 0.2–50 nM (0.05–10 ppb) Hg^{2+} with a sensitivity of 0.285 μA/nM; the detection limit is 0.1 nM (0.020 ppb) while the reproducibility is 4.3% as RDS on individual measurement. The suitability of the proposed system as a biosensor for measurement in real samples has been checked by determining mercury ion in fish samples: obtained data compared to reference method show that recovery is in the range 92–107%, while RSD on individual measurement is always $\leq 8.8\%$.

The encouraging results obtained on real samples using an Au screen printed electrode as transducer open the way for further developments and applications such as the realization of new miniaturized biosensors and analysis mercuric ion in complex real matrices.

Acknowledgments

The authors are greatly indebted to Francesca Scaramuzzo, Ph. D. of the Department of Fundamental and Applied Sciences for Engineering, in Sapienza University of Rome, for her contribution to this research with the AFM characterization of surface.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.09.031.

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Please cite this article as: Tortolini, C., et al., Biosensors and Bioelectronics (2014), http://dx.doi.org/10.1016/j.bios.2014.09.031