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Amperometric biosensor based on denatured DNA for the study of heavy metals complexing with DNA and their determination in biological, water and food samples

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Abstract

Amperometric biosensor (BS) has been elaborated based on the stationary mercury-film electrode (SMFE) with silver support and cellulose nitrate (CN) membrane containing immobilized single-stranded DNA (ssIDNA). The sorption isotherms and ssDNA–heavy metal binding constants have been obtained with the BS. According to these data, the chosen heavy metals form the following series of binding strength with ssIDNA: Pb(II)>Fe(III)>Cd(II). It has been found that upon the competitive adsorption, there exists practically simultaneous sorption of different ions at ssIDNA containing membrane. The method of the determination of heavy metals based on preconcentration of metal ions on the BS followed by the destruction of DNA–metal complexes with ethylenediamine tetraacetate (EDTA) and voltammogram recording has been proposed. The lower limits of detectable contents are 1.0×10^{-10}, 1.0×10^{-9} and 1.0×10^{-7} mol l^{-1} for Pb(II), Cd(II) and Fe(III), respectively. Heavy metals have been assayed in natural and drinking water, milk and blood serum samples even under simultaneous presence with a selectivity factor of 1:10. The effect of matrix components has been estimated.

Keywords: Amperometric DNA biosensor; Heavy metals analysis; Adsorption

1. Introduction

Studying the process of complexing of DNA molecules and their parts with metals is an actual and interesting problem because it enables establishing features of DNA behaviour in the organism. Heavy metals are known to have a great affinity for DNA and cause muta- and cancerogenesis [1,2], the formation of malignant tumours being attended by an increase of heavy metals' concentration in the DNA of tumour cells [3]. Electrochemical biosensors (BS) based on immobilized DNA and its parts integrate sensitivity of detection with a high specificity of biomolecules, reduce the consumption of DNA and give rise to the development of modern methods of analysis of DNA effectors, including toxic ones, in environmental and biological objects [4–9].

The aim of this investigation is to perform voltammetric study of heavy metals (Fe(III), Pb(II), Cd(II)) complexing with denatured single-stranded DNA (ssDNA), and to use this complexing process for determining heavy metals in blood serum and in environmental objects with the developed amperometric BS based on ssDNA.

2. Experimental

2.1. Apparatus and reagents

Voltammetric measurements were performed with a SVA-1B-M-01 voltammetric system (Analytic, Bulgaria). The DNA-based BS constructed on the basis of a stationary mercury-film electrode (SMFE) with silver support ($d = 0.5$ mm) [10] or SMFE itself served as a working electrode. The reference electrode was a saturated calomel electrode (SCE). The solutions were deaerated with argon. An atomic-absorption spectrometer Z-6100 (Hitachi) was used. All measurements were performed at 298 ± 2 K. Chicken erythrocyte DNA (Reanal) at a concentration of 0.01 mg ml^{-1} in 0.9% NaCl was used. Medium-nitrogen cellulose nitrate (CN) films were employed. Highly purified organic solvents

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(acetone, toluene, hexane), 25%-glutaraldehyde solution (Reanal), phosphate buffer saline (PBS) pH 7.3, ammonia mixture $NH_4OH + NH_4Cl$ pH 7.3, 0.1 mol l^{-1} sodium ethylenediamine tetraacetate (EDTA) were used. Initial solutions of metal salts of 1×10^{-3} mol l^{-1} were prepared by dissolving Fe(III), Pb(II), Cd(II) nitrates in tridistilled water. Exact concentrations of the solutions were established by titration with EDTA.

2.2. Preparation of the sensor part of the amperometric DNA-based BS

CN was dissolved in a mixture of acetone and toluene, and then 0.2 ml of a solution containing 0.01 mg ml^{-1} denatured DNA, obtained by heating on a boiling water bath for 20 min followed by fast cooling, was added. The solution was stirred, and 0.06 ml of 25%-glutaraldehyde solution and hexane as a coagulator was added. The resulting mixture was cast on a glass plate and dried, and a membrane was obtained. The film was cut into pieces (2.5×6.5 cm^2). Washed with PBS (pH 7.3), a crimped film was fixed with clamping rings on the surface of the body of SMFE with silver support [10].

2.3. Plotting Pb(II) and Cd(II) adsorption isotherms with amperometric DNA-based BS

To plot adsorption isotherms, the set of ammonia mixture solutions of different initial concentrations (from 1.0×10^{-7} to 0.5×10^{-5} mol l^{-1}) of Pb(II) or Cd(II) was prepared, voltammograms of these solutions were taken within the potential range from -0.02 to -1.5 V, $E_{start} = -0.02$ V, $v = 0.5$ V s^{-1} at SMFE. Peak heights at -0.4 V for Pb(II) and at -0.65 V for Cd(II) were measured. The BS based on immobilized ssDNA (ssIDNA) was then immersed in every initial solution for 15 min., the biosensitive part of the BS being renewed before every immersion using a new part of the membrane. Then voltammograms of the solutions were taken again and the corresponding peaks were measured. The obtained peaks' values in this case were determined by the concentration of heavy metal ions left in the solution after binding with ssIDNA-based BS. The difference between initial and left concentrations gives a moles number of the metal bound on the BS and it was obtained for each initial concentration of metal ions. The left concentrations were calculated using earlier plotted calibration curves for Pb(II) and Cd(II) ions.

2.4. Determination of heavy metal ions in analysing solutions with amperometric DNA-based BS

Amperometric ssIDNA-based BS was immersed for 15 min in electrochemical cell containing supporting electrolyte (ammonia mixture pH 7.3 for Pb(II) and Cd(II) or aqueous HNO_3 pH 2.5 for Fe(III) determination) and heavy metal ions of various concentrations. Then the analysing solution was removed, the cell was washed out with supporting electrolyte. Five milliliters of the 2.5×10^{-2} mol l^{-1} EDTA solution in PBS pH 7.3 was placed into the cell, the solution was being deaerated with argon for 20 min and voltammograms were recorded in the range of -0.02 to -1.5 V, $E_{start} = -0.02$ V, $v = 0.5$ V s^{-1}. Cathodic peak heights at -0.7, -0.9 or -0.2 V were measured for Pb(II), Cd(II) or Fe(III) complexes with EDTA, respectively. The calibration graph of peak current (I_p) for the reduction of the metal complex with EDTA vs. metal's concentration was plotted based on the obtained data. The unknown concentrations of heavy metal in analysing solutions were obtained using these calibration graphs.

3. Results and discussion

3.1. Study of the specific adsorption of heavy metals on BS and determination of their binding constants with ssDNA

Amperometric BS was elaborated based on the SMFE with silver support and CN membrane containing immobilized molecules of ssDNA (see Experimental). The form of ssDNA as a most effective ligand for heavy metal complexing was selected due to the lack of interstrand hydrogen bonds [1,11,12], so DNA molecules in this form are in greater extent untwisted, fixed on the CN-matrix and their binding centres are most available.

Specific adsorption of the heavy metals on ssDNA-modified CN matrix was studied to establish the possibility of application of ssIDNA containing CN membrane as a biosensitive part of a new affinity amperometric BS for determination of heavy metals in biological and environmental objects. Pb(II) and Cd(II) ions being among the most dangerous environmental toxicants were chosen as a model. They are also characterized with a high affinity for DNA and, according to Eichhorn, they are at the beginning of the series of decreasing ability of two-charged ions to bind with DNA [3,13]. Chemical adsorption of Pb(II) and Cd(II) at the biosensitive part of the BS takes place in our case due to the formation of covalent bonds during complexing with ssIDNA which enables us to obtain more accurate and reproducible characteristics of the adsorption process.

A blank run was performed. The CN membrane without ssIDNA was immersed in the solutions of Pb(II) and Cd(II) after the registration of their voltammograms. This procedure did not lead to decay of the analytical signal thus confirming specificity of heavy metals adsorption at ssIDNA containing membrane.

The adsorption value is a number of moles of a compound adsorbed by 1 m^2 of a surface and is measured in mol m^{-2}. The process of adsorption of compounds from solution at the solid and uniform surface can be described by Langmuir's equation [14]: $a = a_\infty Kc/(1 + Kc)$, where a and a_∞—are adsorption values at given filling of the surface and in the saturated monomolecular layer (maximum adsorption

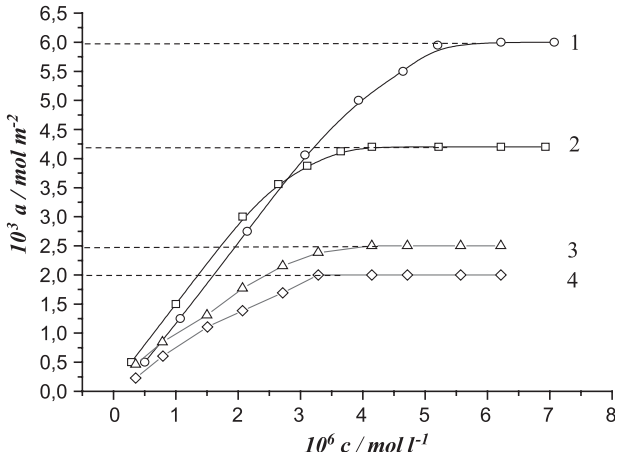

Fig. 1. Adsorption isotherms of the heavy metal ions on ssIDNA-containing membrane of amperometric BS: (1) for Pb(II) ; (2) for Cd(II); (3), (4) isotherms for Pb(II) and Cd(II) obtained in 1:1 [Pb(II)]/[Cd(II)] solution under competitive adsorption.

value), respectively; K—temperature dependent constant of adsorption equilibrium; c—concentration of a compound in solution. Upon competitive adsorption of a number of adsorbates [14], the adsorption equation appears as $a_i = a_{\infty,i}(K_i c_i/(1+\sum_j K_j c_j))$.

According to adsorption isotherms (see Experimental) (Fig. 1), the limiting adsorption (a_∞) of Pb(II), indicating maximum adsorption capacity of the membrane, was determined to be 6.0×10^{-3} mol m^{-2} (see Fig. 1, curve 1). It means that the maximum number of moles to be determined by the BS in the case of Pb(II) determination equals 4.8×10^{-6} mol (taking into account that the surface of the membrane is 7.95 cm^2). For Cd(II), a_∞ was found to be 4.2×10^{-3} mol m^{-2} (see Fig. 1, curve 2) and the maximum determinable Cd(II) value was 3.3×10^{-6} mol. The obtained data suggest that Pb(II) ions have greater affinity for ssIDNA than Cd(II) and re-confirm the literature data concerning Pb(II) affinity not only for purine bases but also for pyrimidine ones [1,13,15]. Joint adsorption isotherms for Pb(II) and Cd(II) were obtained for the solutions containing these ions in 1:1 ratio. It was possible due to the fact that both Pb(II) and Cd(II) reduction peaks current are observable owing to sufficient (250 mV) difference between the peak potentials (see Fig. 1, curves 3 and 4). This experiment shows that upon competitive adsorption, there exists practically simultaneous sorption of either ions at ssIDNA containing membrane, Pb(II) sorption ability being larger than Cd(II) again confirming their greater affinity for ssIDNA molecules. So, it is possible to determine selectively these two ions simultaneously presenting in the solution with ssIDNA-based BS.

The Scatchard graph [1,16] was used for the constants of binding (K_{bind}) of heavy metals Pb(II), Cd(II), Fe(III) with ssIDNA to be evaluated. This method is based on plotting $c_{[Me\text{-}DNA]}/c_{Me(Eq.)}$ ratio vs. $c_{[Me\text{-}DNA]}$, complex composition being assumed 1:1 in this model. The concentration of Me-DNA complex formed on BS was obtained from the difference between c_{Me} before and after immersing of BS using graduation graphs plotted similarly to the method described in Experimental for plotting adsorption isotherms. The values of K_{bind} of the metals with ssIDNA were calculated from tangent slope of obtained linear Scatchard graphs ($n=5$, $P=0.95$):

$$K_{bind}(\mathrm{Cd(II)-ssIDNA}) = (0.6\pm0.2)\times10^5 \text{ l mol}^{-1}$$

$$K_{bind}(\mathrm{Pb(II)-ssIDNA}) = (12.0\pm0.3)\times10^5 \text{ l mol}^{-1}$$

$$K_{bind}(\mathrm{Fe(II)-ssIDNA}) = (1.4\pm0.3)\times10^5 \text{ l mol}^{-1}$$

According to these data, the chosen heavy metals can be grouped in descending order of binding strength with ssIDNA: Pb(II)>Fe(III)>Cd(II). On the basis of literary data and obtained experimental results, the following main variants of the metal ions binding with DNA molecules under chosen conditions can be proposed [1–3,13]: chelating through purine N(7) atom and oxygen atom of phosphate group; formation of the coordination bond with N(7) of guanine (rarely of adenine). Two additional variants of metal binding with ssIDNA leading to a greater stability of the complex can be proposed: intrastrand chelating between N(7) and O(6) atoms of guanine and intrastrand bindings formation with N(7) guanine atoms of ssDNA.

3.2. The use of DNA based BS in environmental analysis and blood serum assay for heavy metals

The method was proposed for the determination of heavy metals in various objects with amperometric BS (see Ex-

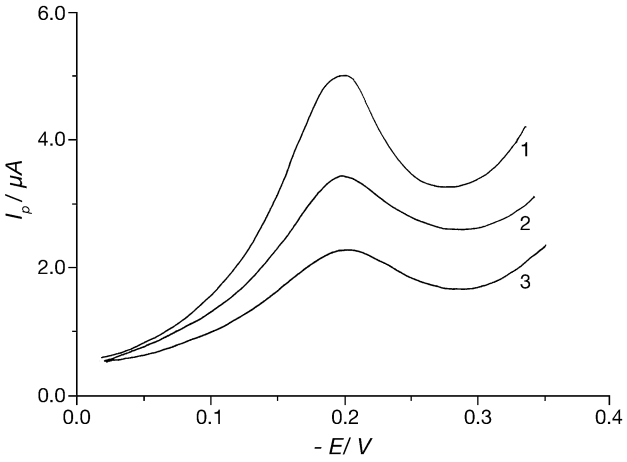

Fig. 2. Voltammograms of Fe(III)–EDTA complex solutions: (1) 2.50×10^{-5} mol l^{-1} Fe(III); (2) 1.25×10^{-5} mol l^{-1} Fe(III); (3) 0.55×10^{-5} mol l^{-1} Fe(III). [EDTA]$=2.5\times10^{-2}$ mol l^{-1}, PBS pH 7.3, $v=0.5$ V s^{-1}, DNA-based BS.

Table 1
Results of determination of Pb(II) and Cd(II) ions under simultaneous presence in model solutions with DNA-based biosensor ($n=5$, $P=0.95$)

Inserted, 10^8 c, mol l^{-1}		Found, 10^8 ($c \pm \delta$), mol l^{-1}		RSD	
Pb(II)	Cd(II)	Pb(II)	Cd(II)	Pb(II)	Cd(II)
0.20	0.20	0.19 ± 0.05	0.18 ± 0.05	0.21	0.22
0.20	0.40	0.23 ± 0.07	0.34 ± 0.09	0.24	0.21
0.40	0.40	0.37 ± 0.06	0.46 ± 0.08	0.13	0.14
0.50	2.50	0.43 ± 0.09	2.7 ± 0.8	0.17	0.24
2.50	25.00	2.3 ± 0.6	25 ± 6	0.21	0.19

perimental). This method is based, on one hand, on the found high heavy metals affinity for ssIDNA molecules which allows effective concentrating of these ions from solutions of low concentration at ssIDNA containing membrane of BS. On the other hand, the method uses metal removing from BS by its treatment with EDTA based on greater stability of heavy metal–EDTA complexes [17] than the stability of the complexes with ssIDNA estimated by us. Complexing with EDTA enables also to determine smaller concentrations of the metal ions in analysing solutions and to reactivate biosensitive part of BS for its repeated use.

As an example, voltammograms of Fe(III)–EDTA complex are shown in Fig. 2. The appearance of the reduction peak of metal–EDTA complex confirms the destruction of metal–ssIDNA complex with EDTA. This signal value depends on the concentration of heavy metal ions (see Fig. 2) and on the biological activity of DNA. ssDNA was shown to remain its biological activity not less than 30 days after immobilization. There is no washing out of ssDNA from CN membrane within that period.

The calibration graphs for heavy metal assay with ssIDNA-based BS were plotted as shown in Experimental. Their I_p (μA) vs. c (mol l^{-1}) equations are following: $I_p=(1.23 \pm 0.11) \times 10^9 c+(0.12 \pm 0.06)$, $r=0.9992$, for Pb(II); $I_p=(1.25 \pm 0.02) \times 10^9 c-(0.02 \pm 0.01)$, $r=0.9996$, for Cd(II); $I_p=(1.60 \pm 0.02) \times 10^5 c+(0.32 \pm 0.04)$, $r=0.9996$, for Fe(III).

Table 3
Results of determination of 2.0×10^{-5} mol l^{-1} Fe(III) ions with DNA-based biosensor in model solutions in the presence of matrix elements ($n=5$, $P=0.95$)

[Fe(III)]/[M] ratio	[Fe(III)] found, 10^5 ($c \pm \delta$), mol l^{-1}	RSD
1:1		
M/Mg(II), Zn(II), Cu(II), Al(III)	2.1 ± 0.3	0.12
1:5		
M/Mg(II), Zn(II), Cu(II)	1.8 ± 0.5	0.22
1:10		
M/Mg(II),Zn(II)	1.9 ± 0.4	0.17
1:100		
M/Mg(II)	2.3 ± 0.6	0.21

The linear dependence of I_p vs. c plot is observed in the range of 1.0×10^{-5}–1.0×10^{-10} mol l^{-1} for Pb(II) and 1.0×10^{-5}–1.0×10^{-9} mol l^{-1} for Cd(II). The range of determinable concentrations for Fe(III) was found to be 6.0×10^{-5}–1.0×10^{-7} mol l^{-1}. This range is useful for the determination of total amount of Fe(III) in blood serum as the Fe(III) content in healthy human's blood serum fluctuates within the range 1.0×10^{-5}–3.1×10^{-5} mol l^{-1}. The lower limits of detectable content were found to be 1.0×10^{-10}, 1.0×10^{-9} and 1.0×10^{-7} mol l^{-1} (RSD 0.33) for Pb(II), Cd(II) and Fe(III), respectively. A sufficient difference between reduction potentials of Pb(II) and Cd(II) complexes with EDTA enables their determination under simultaneous presence in the solution (Table 1). The data suggest that the determination is possible with a selectivity factor of 1:10. Such analysis with the DNA-based BS is very important because heavy metals even at low concentration can bind with DNA molecules causing its malfunction that affects the health of further generations.

Table 2
Results of heavy metals (Pb(II), Cd(II) and Fe(III)) determination in real objects with DNA-based biosensor ($n=5$, $P=0.95$, $t_{table}=2.78$; $F_{table}=6.39$)

Analysing object	M	Voltammetry with BS		Reference method		t_{calc}	F_{calc}
		Found, $c \pm \delta$	RSD	Found, $c \pm \delta$	RSD		
River water	Cd	$(2.8 \pm 0.1) \times 10^{-3}$ mg ml^{-1}	0.03	$(2.7 \pm 0.2) \times 10^{-3}$ mg ml^{-1}[a]	0.06	1.22	3.72
Lake water	Cd	$(4.1 \pm 0.3) \times 10^{-3}$ mg ml^{-1}	0.06	$(3.8 \pm 0.4) \times 10^{-3}$ mg ml^{-1}[a]	0.08	1.53	1.72
Drinking water before filter	Pb	$(3.8 \pm 0.5) \times 10^{-7}$ mol l^{-1}	0.11	$(3.6 \pm 0.4) \times 10^{-7}$ mol l^{-1}[a]	0.10	1.41	1.33
Drinking water after filter	Pb	$(4.2 \pm 0.7) \times 10^{-8}$ mol l^{-1}	0.13	$(3.8 \pm 0.5) \times 10^{-8}$ mol l^{-1}[a]	0.12	1.37	1.02
Milk sample 1	Pb	0.021 ± 0.002 mg l^{-1}	0.08	0.019 ± 0.001 mg l^{-1}[b]	0.04	2.5	1.75
Milk sample 2	Pb	0.052 ± 0.003 mg l^{-1}	0.05	0.047 ± 0.002 mg l^{-1}[b]	0.03	2.8	1.25
Milk sample 3	Pb	0.043 ± 0.003 mg l^{-1}	0.06	0.040 ± 0.002 mg l^{-1}[b]	0.04	2.1	1.65
Blood serum 1	Fe	$(0.9 \pm 0.2) \times 10^{-5}$ mol l^{-1}	0.18	$(0.7 \pm 0.1) \times 10^{-5}$ mol l^{-1}[c]	0.10	2.31	5.4
Blood serum 2	Fe	$(1.2 \pm 0.3) \times 10^{-5}$ mol l^{-1}	0.17	$(1.4 \pm 0.2) \times 10^{-5}$ mol l^{-1}[c]	0.09	1.87	2.6
Blood serum 3	Fe	$(2.2 \pm 0.4) \times 10^{-5}$ mol l^{-1}	0.15	$(2.3 \pm 0.2) \times 10^{-5}$ mol l^{-1}[c]	0.08	0.58	3.2
Blood serum 4	Pb	0.006 ± 0.001 mg ml^{-1}	0.13	0.007 ± 0.002 mg ml^{-1}[a]	0.23	1.25	4.19
Blood serum 5	Pb	0.011 ± 0.002 mg ml^{-1}	0.14	0.013 ± 0.003 mg ml^{-1}[a]	0.18	1.60	2.31
Blood serum 5	Cd	0.005 ± 0.002 mg ml^{-1}	0.32	0.004 ± 0.001 mg ml^{-1}[a]	0.20	0.79	4.00
Blood serum 6	Cd	0.010 ± 0.002 mg ml^{-1}	0.16	0.012 ± 0.004 mg ml^{-1}[a]	0.27	1.24	4.09

[a] Atomic-absorption spectrometry.
[b] Inversion voltammetry.
[c] Spectrophotometry.

The elaborated DNA-based BS was involved in environmental and food analysis (Table 2). The deviations between average values of the concentrations of heavy metal ions in various objects obtained by two independent methods are negligible and are in good agreement according to *t*- and *F*-criteria (see Table 2). The presence of heavy metal ions such as Ni(II), Co(II), Zn(II) in 10-fold excess does not interfere Pb(II) and Cd(II) assay due to their reduction potentials differ by more that 0.2 V. In some cases, for the analysis of the objects bearing high content of interfering components, an additional sample treatment may be required.

The developed BS was employed in biological assay of human's blood serum for heavy metals. For example, precise data on the concentration of unbound iron in blood serum are needed for anaemia diagnosing and upon establishing the extent of intoxication with Fe(III) entered from environment or industrial pollutions [18]. The determination of 2×10^{-5} mol l^{-1} Fe(III) with the BS was carried out in model solution in the presence of Mg(II), Zn(II), Cu(II) and Al(III) to study the effect of these matrix elements on Fe(III) analysis (Table 3). The choice of matrix elements is governed by the fact that these ions may exist in blood serum of healthy man in the same order of quantities as Fe(III) and have sufficient affinity for the DNA molecules [1,18]. The detailed procedure of the determination is described in Experimental, 3 μl of blood serum being placed into the electrochemical cell; pH 2.5 was chosen for Fe(III)-transferrine complex in blood serum to be destroyed leaving Fe(III) in nonhydrolysed form. Spectrophotometry was used as a reference method for establishing the reliability of the proposed method of blood serum analysis [19]. The results are presented in Table 2. The proposed fast method of Fe(III) determination in blood serum with the BS features selectivity, good reproducibility and easy sample preparation. This method can supplement known methods of Fe(III) control in human's organism and can be used for monitoring of antitumour drugs based on Fe(III)-complex in biological liquors [18,20].

The determination of Pb(II) and Cd(II) in blood serum with the BS is an important task in the diagnosing of acute poisoning with heavy metals and in the diagnosing of the professional diseases [18,19]. Some of the results of determination using 3 μl of sick human's blood serum are presented in Table 2. As a reference method, the assayed samples were also studied in a clinical laboratory with atomic-absorption spectroscopy (see Table 2).

It can be summarized that the study of the interaction of ssIDNA with the heavy metal ions (which is of independent interest) with the developed amperometric DNA-based BS enables to confirm the hypothesis about the mechanism of these metals binding to ssDNA and their specificity to ssDNA and their possible mutagenic properties. The preferential binding of Pb(II) with ssIDNA over Cd(II) and Fe(III) was quantitatively stated. Moreover, this study provided the choice of the optimal conditions for the functioning of the BS and for highly sensitive and selective assay of biological and environmental objects with this BS.

Acknowledgements

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