New potentiometric sensor based on molecularly imprinted nanoparticles for cocaine detection

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ABSTRACT

Here we present a potentiometric sensor for cocaine detection based on molecularly imprinted polymer nanoparticles (nanoMIPs) produced by the solid-phase imprinting method. The composition of polymers with high affinity for cocaine was optimised using molecular modelling. Four compositions were selected and polymers prepared using two protocols: chemical polymerisation in water and UV-initiated polymerisation in organic solvent. All synthesised nanoparticles had very good affinity to cocaine with dissociation constants between 0.6 nM and 5.3 nM. Imprinted polymers produced in organic solvent using acrylamide as a functional monomer demonstrated the highest yield and affinity, and so were selected for further sensor development. For this, nanoparticles were incorporated within a PVC matrix which was then used to prepare an ion-selective membrane integrated with a potentiometric transducer. It was demonstrated that the sensor was able to quantify cocaine in blood serum samples in the range of concentrations between 1 nM and 1 mM.

Keywords: cocaine, molecular modelling, nanoMIPs, solid-phase imprinting, potentiometric sensor

1. Introduction

Cocaine is one of the most widely-used recreational drugs in the world with the number of its users estimated between 13.8 - 20.7 million for the population aged between 15 to 64 (World Drug Report, 2015). It is known that cocaine addiction can cause serious side-effects in users, including anxiety, organ damage and cardiac arrest, in addition to wider societal and economic impacts (Wren et al., 2014; Emrani et al., 2016). Therefore, the development of fast, simple

and sensitive methods for cocaine detection is of vital importance for medical diagnostics and law enforcement purposes.

Although a wide range of sensors have been developed to date (Gupta et al., 2015; Gupta et al., 2015, Gupta et al., 2015 , Gupta et al., 2014) the analytical techniques that are used for cocaine detection and quantification are still mostly based on HPLC, mass-spectrometry, Raman spectroscopy, IR spectroscopy, and ion mobility spectrometry (Wren et al., 2014). The major drawback of these techniques is that they are time-consuming, rely on complex and expensive instruments and are difficult to miniaturise (Daemes et al., 2015). There is an urgent need to develop portable stand-alone cocaine-specific sensors that can be used for *in situ* testing and that should therefore be small, light, inexpensive, robust and compatible with multiplexing. There have been few attempts to produce such sensors based on various recognition elements such as antibodies, molecularly imprinted polymers (MIPs) and aptamers (Stubbs et al., 2015; Hien et al., 2012; Stojanovic et al., 2001).

MIPs are synthetic materials possessing specific binding sites able to recognise a target molecule (Piletska et al., 2008; Piletska et al., 2009), a useful feature for their application as recognition elements in sensor devices (Irshad et al., 2013; Verma et al., 2013; Gupta et al., 2013; Gupta et al., 2014). MIPs are prepared by co-polymerisation of functional monomers and a cross-linker in the presence of a template molecule. A self-assembly of the functional monomers around the template is responsible for the formation of specific binding sites in the resulting polymer. It is necessary to highlight that despite the remarkable recognition properties demonstrated by the first generation of MIPs that were either used as micro-sized particles or polymerised as a film directly on the sensor surface, their integration with transducers was very problematic (Wren et al., 2015). However, this issue has been resolved by using nano-sized MIPs or ´nanoMIPs´ which were produced using the solid-phase synthesis approach (Piletsky et al., 2011; Piletsky et al., 2013; Poma et al., 2013; Poma et al., 2014; Canfarotta et al., 2016). Among the practical benefits of the solid-phase synthesis of nanoMIPs include the absence of residual template molecules and the possibility to re-use the immobilised template. Another important point is that soluble nanoMIPs can be used easily in standard antibody-based manufacturing protocols (Poma et al., 2014; Piletsky et al., 1998; Piletsky et al., 2000; Piletsky et al., 2001; Moczko et al., 2013; Chianella et al., 2013; Guerreiro et al., 2014, Smolinska-Kempisty 2016). The reported nanoMIPs possess superior stability at low and high pH, temperature and pressure conditions (Poma et al., 2013; Chianella et al., 2013). The first successful examples of the application of the nanoMIPs in electrochemical sensors were described recently (Gutierrez-Climente et al., 2016; Basozabal et al., 2014). Here we present a

new potentiometric sensor for the detection of cocaine, which employs cocaine-specific nanoMIPs produced using a solid-phase approach.

2. Experimental

2.1. Materials

Benzoylecgonine (BE), norcocaine, cocaethylene, anhydroecgonine methyl ester, cocaine, histamine, acrylamide (ACRYL), *N*,*N*-diethylamino ethyl metacrylate (DEAEM), *N,N'*-methylenebisacrylamide (BIS), *N*-tert-butylacrylamide (TBAm), ammonium persulfate (APS), tetramethylethylene-diamine (TEMED), 3-aminopropyltrimethyloxysilane (APTMS), sodium hydroxide (NaOH), glutaraldehyde (GA), *N*-hydroxy-succinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), ethylene glycol dimethacrylate (EGDMA), trimethylolpropane trimethacrylate (TRIM), pentaerythritol tetrakis(3-mercaptopropionate) (PETMP), low molecular weight poly(vinyl chloride) (PVC), nitrophenyl octyl ether (NPOE), potassium tetrakis(4-chlorophenyl)borate (kTpBCl), sterile-filtered human serum (from male clotted whole blood of AB group, USA), potassium chloride (KCl) were from Sigma-Aldrich, UK. Dimethylformamide (DMF), acetonitrile (ACN), tetrahydrofuran (THF) and acetone were from Fisher Scientific, UK. *N*, *N*-diethyldithiocarbamic acid benzyl ester (iniferter) from TCI Europe. N-*(*3-aminopropyl*)*methacrylamide hydrochloride>98% (NAPMA) was from Polyscience Inc, UK. Phosphate buffered saline (PBS), consisted of phosphate buffer (0.01 M), potassium chloride (0.00268 M), and sodium chloride (0.14 M), pH 7.4 (Gibco Life Technologies Ltd, UK) were prepared from buffer tablets. All solvents were of HPLC quality grade. Glass beads with diameters in the range of 53-106 μm were purchased from Potters Industries (UK). Human blood serum (male AB clotted whole blood, sterile-filtered, USA origin) was purchased from Sigma, UK. All experiments were made using Ultrapure Milli-Q water (Millipore, UK). All chemicals and solvents were used without further purification.

2.2 Molecular modelling

The molecular modelling was performed as described in previous papers (Piletska et al., 2005; Breton et al., 2006; Tsyrulneva et al., 2014). The screening of a virtual library of monomers for their interactions with the template was carried out using LEAPFROG™ algorithm (SYBYL® 7.3 software package, Tripos International, USA). The library of functional monomers used for this project contained 20 functional monomers commonly used

in molecular imprinting which possessed polymerisable residues and functional groups able to interact with a template through ionic and hydrogen bonds, van der Waals' and dipole–dipole interactions (Piletsky et al., 2001; Piletska et al., 2015). Energy minimisation for each monomer was conducted to a minimum of 0.001 kcal mol^{-1} using the following parameters of molecular mechanics: method - Powell, force field - Tripos and charges - Gasteiger-Huckel. In order to screen the monomers in the database and select the functional monomers possessing the highest affinity towards the cocaine, the LEAPFROG algorithm was applied for 60,000 iterations. The library was sorted according to the binding energies; the monomers which formed the lowest energy complexes with the template were selected for polymer preparation.

2.3. Solid-phase synthesis of nanoMIPs

Glass beads, which were used as a solid phase support, were activated by boiling in NaOH for 15 min, washed with double-distilled water followed by acetone, and then thoroughly dried. The beads were then incubated overnight in a solution of 2% APTMS in dry toluene followed by washing with acetone.

In order to immobilise the BE template on the solid phase support, 0.1 mg mL^{-1} BE, 0.6 mg mL^{-1} of EDC and 0.6 mg mL^{-1} of NHS in PBS were added to the glass beads modified with APTMS, and incubated overnight at 4 °C. The glass beads with immobilised template were washed with Milli-Q water, dried, and stored at 4 °C.

In order to prepare the control non-cocaine-specific nanoparticles (nanoNIPs), histamine was used as a template. To immobilise the histamine, glass beads were modified with APTMS and incubated for 2 h at 4 °C in a solution of GA in PBS (pH 7.4). The beads were rinsed with water to remove the excess of GA. Then, the histamine solution in PBS (1 mg mL^{-1}) was added and incubated overnight at 4 °C.

The cocaine-specific nanoparticles were prepared in water and in organic solvent (DMF). In both cases the components were dissolved in the corresponding solvent, sonicated for 10 min, and deoxygenated by purging with nitrogen for 30 min. The polymerisation mixture was added to the glass beads functionalised with corresponding template (Tables 1, 2). Glass beads with immobilised BE were used to produce the nanoMIPs possessing affinity to cocaine, and glass beads functionalised with histamine were used to make control nanoparticles (which in relation with cocaine can be considered as a non-imprinted polymer, or NIP). Polymerisation in water was initiated by adding 30 mg mL^{-1} solution of APS and 15 μL of TEMED and conducted for 90 min. Polymerisation in DMF was initiated by UV radiation using a UV lamp system produced by Philips, UK (Philips Original Home Solaria, HB175, 4x15W lamps) and

conducted for 1.5 min. After the polymerisation reaction unreacted monomers and low affinity-polymeric particles were removed by washing with Milli-Q water at ambient temperature or cold DMF and acetonitrile at 0 °C, as shown in Tables 1 and 2. The high affinity-nanoMIPs were eluted using the solvent heated to 60 °C.

Table 1

Composition of monomer mixture and conditions for polymerisation in water.

Components	**Polymers prepared in water**			
	1	**2**	**3**	**4**
Glass beads, g	30	30	30	30
Water, g	50	50	50	50
ACRYL, mg	13.2	10.5	6.6	8.6
DEAEM, mg	-	6.9	8.6	8.6
BIS, mg	1	1	8.2	1
NAPMA, mg	2.2	2.2	2.2	2.2
TBAm, mg	16.5	16.5	16.5	16.5
APS, mg	30	30	30	30
TEMED, µL	15	15	15	15
Conditions				
Time, min	90			
Cold wash, mL	15 mL of water			
Elution, mL	4 x 20 mL of hot water, 60 °C			

Table 2

Composition of monomer mixture and conditions for polymerisation in DMF.

Components	**Polymers prepared in DMF**			
	1	**2**	**3**	**4**
Glass beads, g	30	30	30	30
DMF, g	25	25	25	25
ACRYL, g	2.4	1.9	1.2	1.8
DEAEM, g	-	1.2	1.6	1.6
BIS, g	-	-	1.3	-
NAPMA, g	0.11	0.11	0.11	0.11
EGDMA, g	3.2	3.2	3.2	3.2
TRIM, g	30	30	30	30
PETMP, g	0.18	0.18	0.18	0.18
Iniferter, g	0.75	0.75	0.75	0.75
Conditions				
UV irradiation, min	1.5			
Cold wash, mL	2 x 10 mL of DMF followed by 10x 10 mL of ACN, 4 °C			
Elution, mL	10 x 10 mL of ACN, 60 °C			

2.4. Characterisation of physical and chemical properties of nanoMIPs

The size of the nanoparticles was determined using the dynamic light scattering (DLS) technique executed by Zetasizer Nano-S instrument (Malvern Instruments Ltd, UK).

The interactions between cocaine, cocaine analogues (BE, norcocaine, cocaethylene, anhydroecgonine methyl ester), histamine and nanoMIPs were tested using a Biacore 3000 SPR instrument (GE Healthcare Life Sciences, UK). Au-coated chips (SIA Kit Au, GE

Healthcare Life Sciences, UK) were cleaned using hydrogen plasma treatment (RF plasma, 50 W on a K1050X plasma etcher, Emitech, UK). The sensor chips were washed with ethanol and dried, then coated with lipoic acid by incubating them in a solution of 0.3 mg mL^{-1} of lipoic acid and 5 % acetic acid (v/v) in absolute ethanol for 24 h. Chip surface activation was performed on-line by injecting at a flow rate of 15 µL min^{-1} 0.1 mL of aqueous solution of EDC and NHS at 0.6 and 0.4 mg mL^{-1}, respectively. Immediately after activation, the nanoMIPs (suspended in PBS, pH 7.4) were injected on the sensor chip at a flow rate of 15 µL min^{-1}. All binding affinity experiments were performed in PBS, pH 7.4 at 25 °C and a flow rate of 25 µL min^{-1}. For this, 100 µL-aliquots of the various analytes were injected using the KINJECT mode, which was programmed to analyse the dissociation and the sensor response for duration of 2 min. Kinetic data was fitted using BIAevaluation software v. 4.1 (GE Healthcare Life Sciences, UK).

2.5. Preparation of sensor membranes containing nanoMIPs

The immobilisation solution was prepared by dissolving 0.1 g of PVC in 3 mL of THF followed by the addition of 5 and 10 mg of nanoparticles, 0.2 g of plasticiser NPOE and 0.2 g of kTpBCl. The kTpBCl was added to increase membrane conductivity and reduce anion interference. The mixture was sonicated for 25 minutes and placed in 10 mL beaker, where it was left to dry for 48 h at room temperature, in order to obtain a membrane with 0.2 mm thickness. A disk of 7 mm diameter was punched from the membrane and placed inside of ion selective electrode (ISE) (Sigma-Aldrich), which was then internally filled with PBS spiked with 1 mM of cocaine.

2.6. Potentiometric measurements using model samples

Sensor performance was evaluated potentiometrically using a MeterLab PHM220 millivoltmeter (Radiometer, Denmark). Measurements were carried out at room temperature using an Ag/AgCl electrode (model 5241) as a reference electrode (Crison, Barcelona, Spain). The assembly of the potentiometric cell was as follows: Ag/AgCl(s), KCl (3 mM)//test solution/MIP membrane/AgCl(s), 1 mM of cocaine and 0.1 M KCl/Ag. A membrane containing NIP nanoparticles was used as control. Sensor responses for the solutions of cocaine in the concentration range between 1 nM and 1 mM were recorded.

2.7. Analysis of cocaine in biological samples

Human blood serum was spiked with cocaine in the concentration range of 1 nM to 1 mM. The detection of cocaine in human blood serum was conducted potentiometrically, accordingly to the protocol described above.

3. Results and discussion

3.1. Rational design of polymer compositions

Since cocaine structure has no functional groups suitable for immobilisation on the solid phase, its close structural analogue benzoylecgonine (BE) (Fig. 1) was selected as a ´dummy template´ for imprinting (Fig. 1).

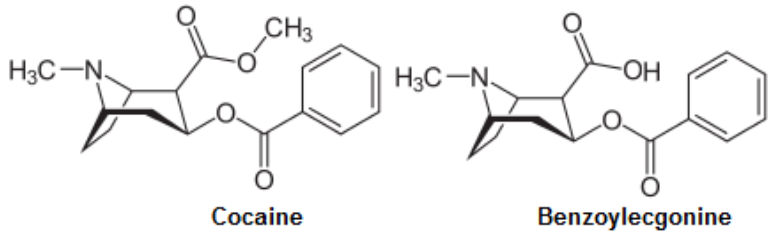

Fig. 1. Molecular structures of the cocaine and benzoylecgonine.

The results of computational modelling suggested that acrylamide (ACRYL: -32.43 kcal mol^{-1}), bisacrylamide (BIS: -30.11 kcal mol^{-1}) and 2-(diethylamino)ethyl methacrylate (DEAEM: -29.77 kcal mol^{-1}) were monomers with the best affinity for the recognition of BE and cocaine. In order to identify other potential interactions between monomers and template, a complex of acrylamide and BE was used as a new template in the screening test against the monomer database. This allowed us to find monomers capable of interacting with different parts of the template other than the acrylamide binding area. In total four different complexes were identified and used for synthesis and testing (Table 3). All nanoparticles were synthesised under aqueous conditions (water) as well as in organic solvent (DMF). HIS-imprinted nanoparticles that did not possess specificity towards cocaine were used as control polymers. In order to facilitate the immobilisation of the nanoparticles on the surface of a Biacore chip by amine coupling, a small amount of *N*-(3-aminopropyl) methacrylamide (containing a primary amine) was added to all monomeric compositions.

3.2 Nanoparticles characterisation

It was observed that the yield of nanoparticles polymerised in organic solvent was about 20 times higher than that obtained in water (see Table 3). This could be explained by the formation of more stable complexes between monomer-template in organics than in water, therefore higher probability of forming particles with high affinity binding sites and, correspondingly, their yield. Another advantage was a shorter time required for the UV-initiated polymerisation than for the chemical polymerisation in water (1.5 min and 90 min, respectively).

Table 3
Polymerisation yield (all measurements were made in triplicates).

No.	Monomers composition (molar ratio)	Polymerisation yield, mg			
		in water		in DMF	
		nanoMIPs	nanoNIPs	nanoMIPs	nanoNIPs*
1	BE:ACRYL – 1:3	3.84±0.53	1.28±0.58	22±8.48	8.8±1.55
2	BE:ACRYL:DEAEM – 1:4:1	4.16±1.40	1.36±0.17	20±12.72	11±1.41
3	BE:ACRYL:DEAEM:BISAC – 1:2:1:1	4.32±0.13	0.80±0.14	23±7.07	11±1.14
4	BE:ACRYL:DEAM – 1:3:1	3.20±0.71	0.88±0.16	25±2.82	10±1.24

*HIS was used as template for all NIPs

It was observed that the population of all synthesised nanoparticles was homogenous; with particles size in the range between 90 and 130 nm (see Table 4).

Table 4
Nanoparticles size (all measurements were made in triplicates).

No.	Nanoparticles size, nm			
	Polymerised in water		Polymerised in DMF	
	MIPs	NIPs	MIPs	NIPs
1	111 ±10	93 ±2.4	117 ±1.5	97 ±4.7
2	108 ±4.5	96 ±1.3	110 ±4.2	95 ±7.5
3	104 ±4.2	99 ±5.8	125 ±8.9	96 ±8.7
4	102 ±7.5	107 ±9.4	129 ±2.0	89 ±3.4

3.3. Affinity of nanoMIPs

The affinity of synthesised nanoparticles to BE and cocaine was determined using the SPR experiments. All tested nanoMIPs had very high affinity to benzoylecgonine and cocaine (Table 5 and Fig. 2). The affinity of nanoMIPs polymerised in DMF was higher than

nanoMIPs made in water. There was no detectable binding of HIS-specific nanoNIPs either to BE or cocaine in the same concentration range (the SPR sensorgrams for the tested nanoparticles are presented in the Supplementary Information section, Fig. 1S and Fig. 2S). Interestingly, the composition of nanoparticles had relatively little impact on their affinity. In order to assess the cross-reactivity of the cocaine-specific nanoparticles, the nanoMIPs3 (composition 3) prepared in organics were tested for the binding of various analogues of cocaine.

Table 5
Dissociation constants for nanoMIPs polymerised in water and DMF towards cocaine and BE.

No.	K_d, nM (Chi^2)			
	Cocaine		Benzoylecgonine	
	water	DMF	water	DMF
1	1.89 (0.158)	0.90 (0.264)	4.75 (0.150)	0.22 (0.130)
2	2.32 (0.104)	2.52 (0.157)	5.27 (0.046)	0.17 (0.169)
3	2.87 (0.08)	2.45 (0.109)	0.69 (0.052)	0.18 (0.270)
4	2.28 (0.105)	0.89 (0.229)	3.00 (0.120)	0.60 (0.129)

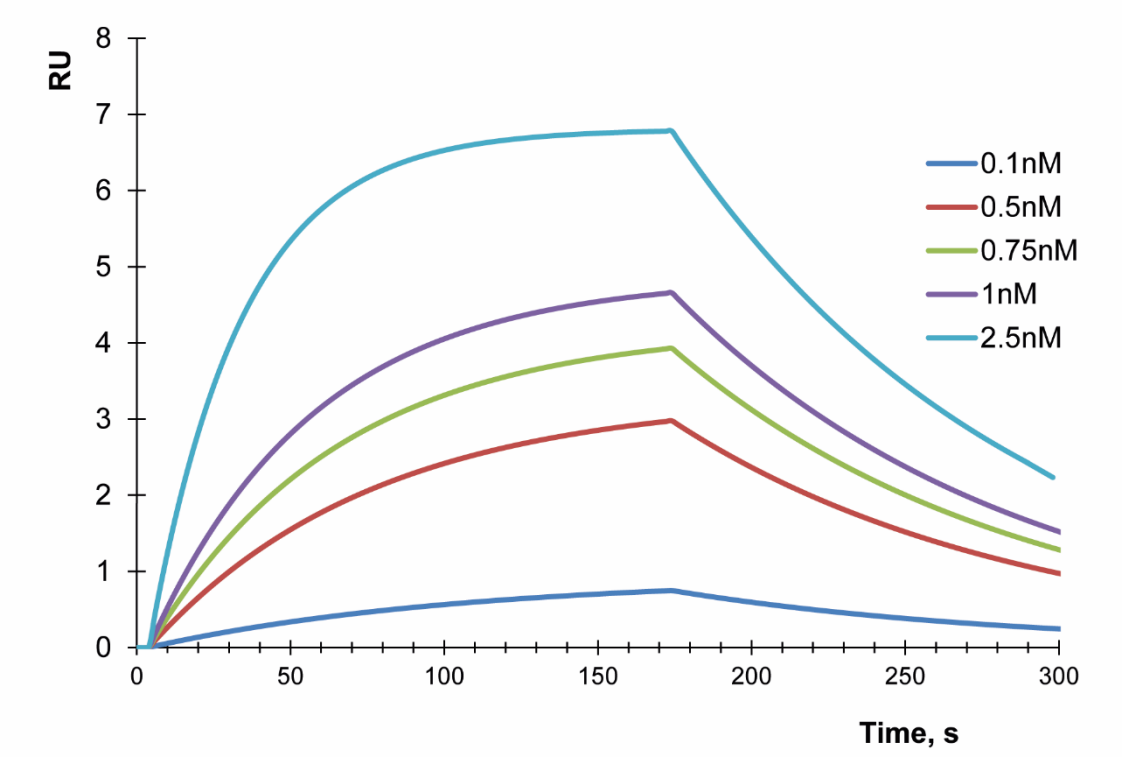

Fig. 2. A typical sensorgram showing the binding between cocaine and nanoMIPs.

It is known that the half-life of cocaine in the body is roughly 20-90 min (Fleming et al., 1990). After this time the drug is transformed into analogues such as norcocaine, BE, cocaethylene

and anhydroecgonine (Fig. 3). The results of binding of nanoMIPs3 to the cocaine metabolites in SPR experiments are as follows: dissociation constant for norcocaine was about 2.45 nM, for cocaethylene was 2.45 nM and for anhydroecgonine methyl ester was 1.79 nM. The low dissociation constants of nanoMIPs and metabolites demonstrate the efficiency of MIP binding to cocaine and its metabolites, which is very important for potential analysis of this drug in bodily fluids (see Fig. 3S, Supplementary Information section).

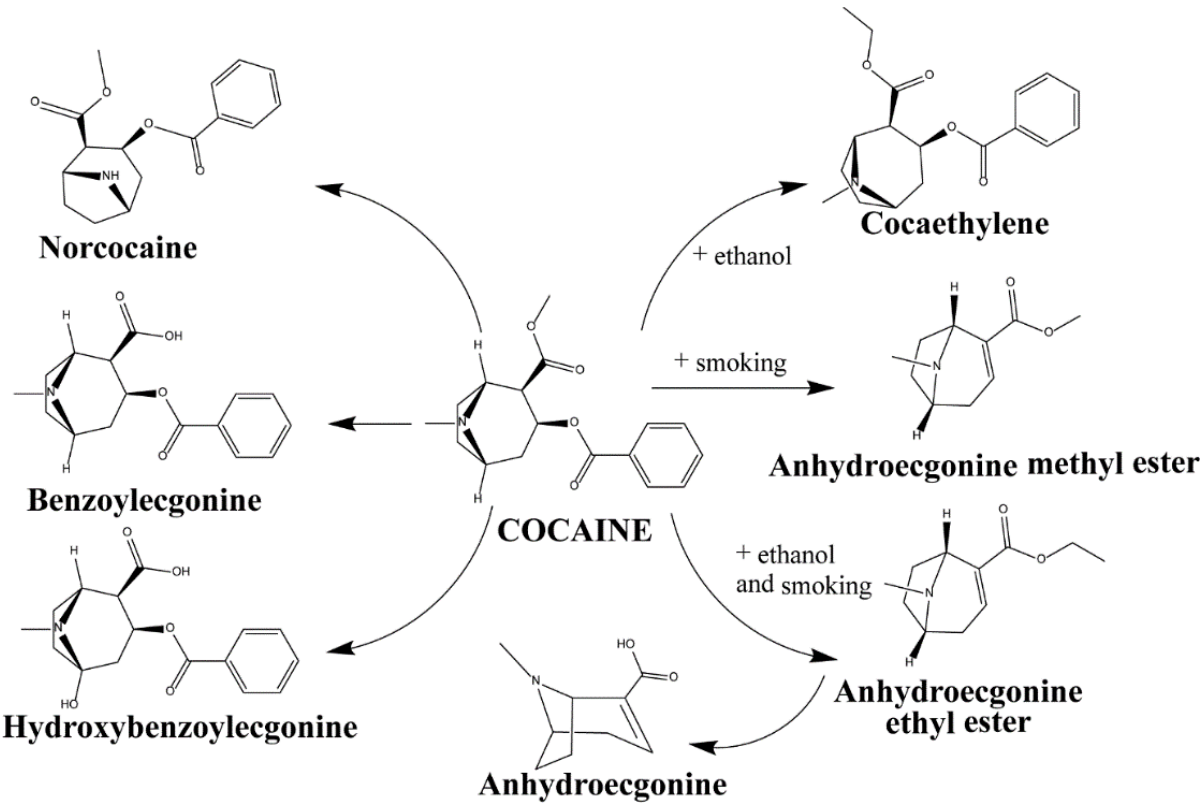

Fig. 3. Metabolism of cocaine in the body.

3.4. Development of a potentiometric cocaine sensor

The potentiometric sensor for cocaine was developed following a protocol described earlier (Gutierrez-Climente et al., 2016; Basozabal et al., 2014). Ion-selective membranes containing nanoMIPs were used as the recognition element of the sensor. The membrane composition in relation to the amount of either nanoMIPs or kTpBCl were optimised as described in Table 6. The sensor response was based on the specific recognition and binding of charged cocaine species to the membrane receptors which then resulted in generation of a potential difference across the membrane. It is also important to highlight that there was no sensor response for compounds such as galantamine (Fig. 5).

Table 6
Composition of the membrane used for potentiometric measurements.

No.	Nanoparticles, mg	PVC, g	NPOE, g	kTpBCl, g	Slope, mV decade^{-1}	Working conc. range
1	5 (MIPs)	0.1	0.2	0.15	7.2	1 nM - 1 mM
2	10 (MIPs)	0.1	0.2	0.2	22.3	1 nM- 1 mM
3	10 (NIPs)	0.1	0.2	0.2	-	-

The potentiometric response for cocaine was measured in the concentration range between 1 nM and 1 mM (Fig. 4). This is in line with data reported for optical cocaine sensors which exhibited a similar working range (Nguyen et al., 2012). Others include sensors based on optical fibres for cocaine detection, these demonstrated good sensitivity with a detection range of 1-100 μM (Wren et al., 2014). A fluorescent sensor based on a structure-switching aptamer, gold nanoparticles and graphene oxide was used to measure cocaine in purified water in the range between 0.1 μM and 50 μM (Shi et al., 2013). It was also reported that a fluorescent sensor based on molecularly imprinted polymer-coated quantum dots was able to detect 1 mg L^{-1} (3.3 μM) of cocaine in urine (Chantada-Vázquez et al., 2016). However, the study presented here constitutes the first example of a potentiometric sensor for cocaine which has nanoMIPs as its recognition elements.

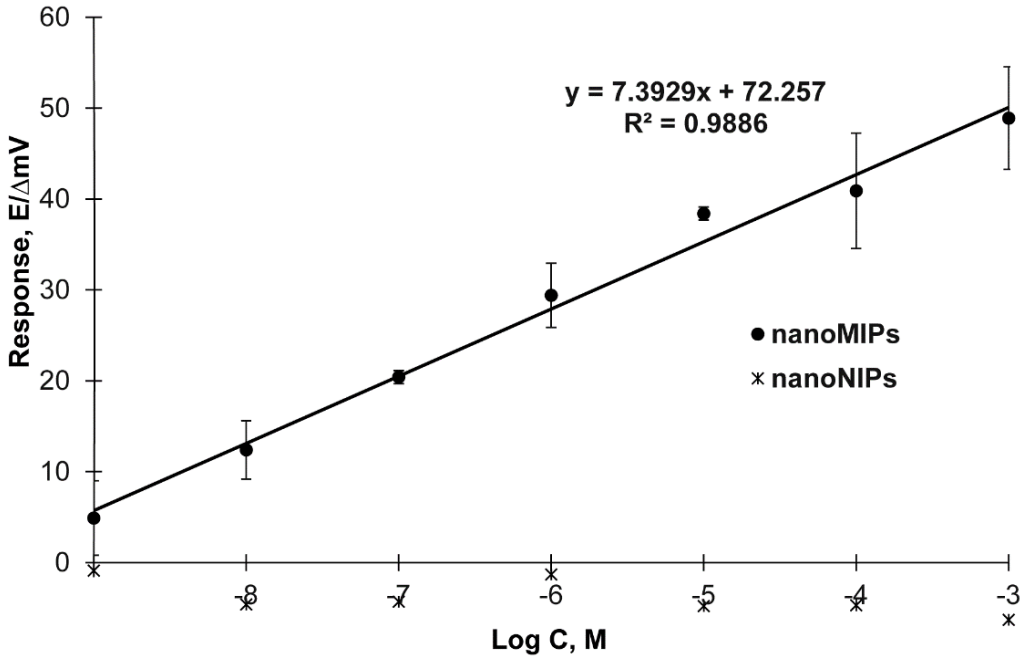

Fig. 4. Sensor response for cocaine demonstrated by the membranes containing nanoMIPs and nanoNIPs in the concentration range between $1 \cdot 10^{-9}$ and $1 \cdot 10^{-3}$ M in PBS. All experiments were repeated in triplicate.

In contrast to nanoMIPs, no response was detected from the membrane containing nanoNIPs. Similarly, no sensor response was detected towards galantamine, which was selected as one example of a functional analogue of cocaine (Fig. 5).

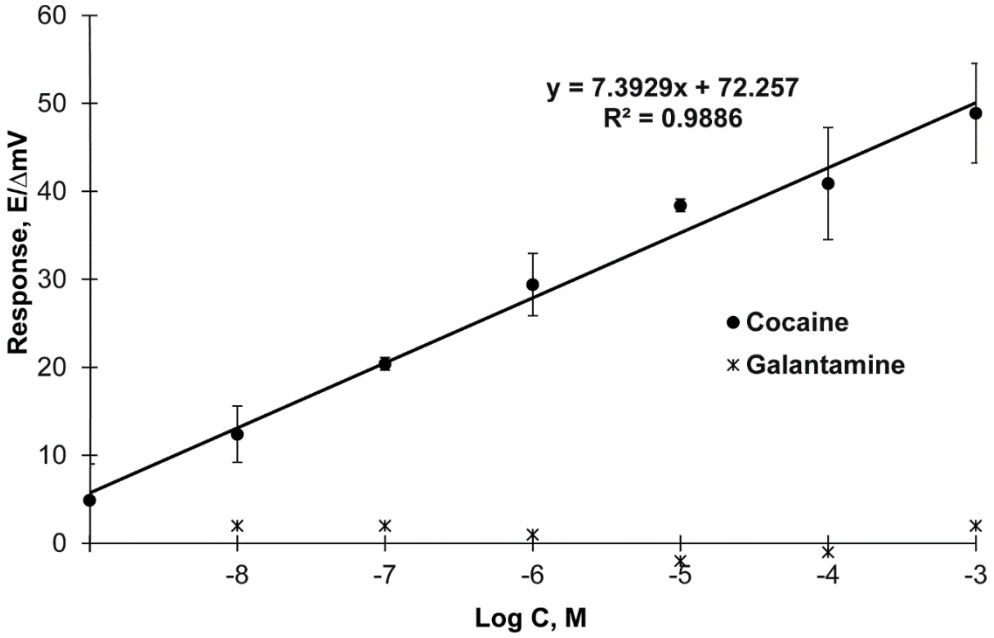

Fig. 5. Comparison of the sensor responses towards cocaine and galantamine. Experiments were repeated in triplicate.

Finally, the sensor was used to detect and measure the concentration of cocaine in human blood serum. Serum samples were spiked with varying concentration of cocaine and measured as described in the Materials and Methods section. It was found that the complex serum matrix had practically no effect on the linearity of sensor response and its sensitivity (Fig. 6). The possibility of regenerating the sensor by adding a short washing step using PBS suggests that the sensor has the potential to easily be re-used. The logical conclusion is that the electrochemical sensor can be used successfully for the accurate measurement of cocaine in biological samples, particularly in blood serum.

The stability of similar nanoMIPs has been assessed in the previous studies. It was found that their high stability (especially when compared with natural receptors like antibodies) allows for the development of robust assays which do not require cold storage (Basozabal et al., 2014; Smolinska-Kempisty et al., 2016).

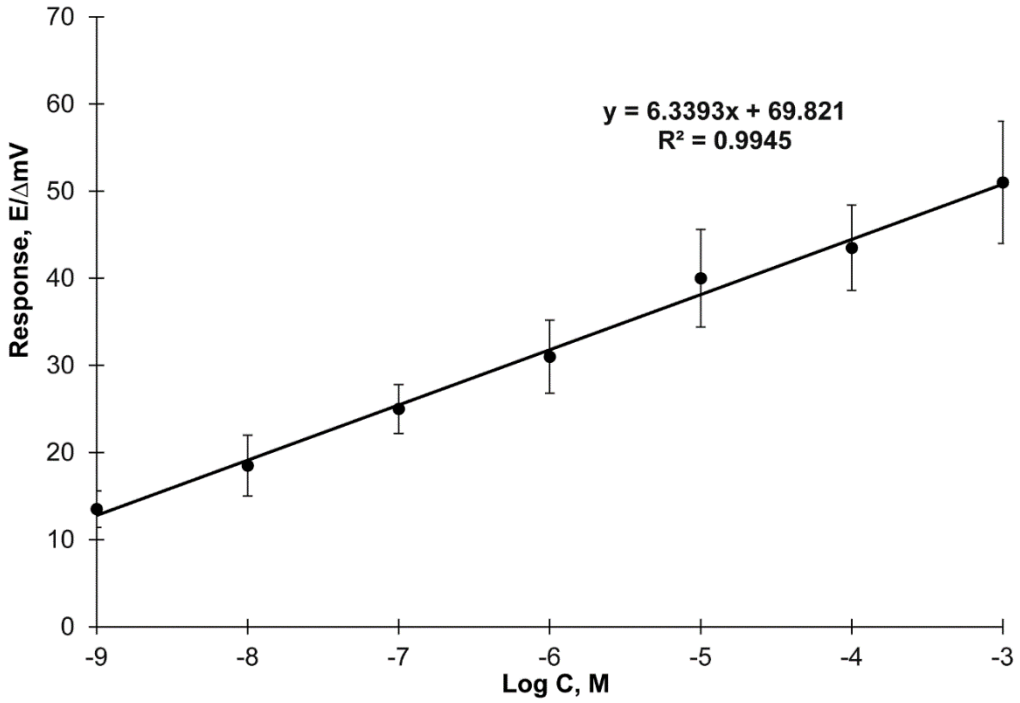

Fig. 6. Sensor response to cocaine in blood serum samples. The experiment was performed in triplicate.

4. Conclusion

This is the first report in which molecularly imprinted nanoparticles with high affinity to cocaine were prepared and used as synthetic recognition elements of a potentiometric sensor. Results demonstrate that the developed sensor is capable of accurate measurement of drug content in blood serum and within a wide range of concentrations (linear range between 1 nM and 1 mM). Therefore, it can be concluded that the sensor as developed is a good proof-of-concept for future development of field sensors for use by the police and medical personnel.

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