

Development of competitive 'pseudo'-ELISA assay for measurement of cocaine and its metabolites using molecularly imprinted polymer nanoparticles

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The analytical test-system for cocaine, benzoylecgonine and norcocaine was developed in ELISA format using molecularly imprinted polymeric nanoparticles (nanoMIPs) as synthetic recognition elements that were produced using solid-phase synthesis approach. The experimental conditions of the assay were optimized using a Box-Behnken experimental design protocol. The detection of free cocaine and its metabolites was performed using a competitive binding assay in the model samples and in blood plasma. There was no cross-reactivity of the developed assay towards paracetamol and caffeine. The developed assay had a picomolar limit of detection of cocaine (LOD = 4.24 pM), which was almost three orders of magnitude lower than the LOD expected from commercial antibody-based ELISA (3.3 nM), other attractive features of a new assay included a long shelf-life, lower economic cost and a short production time. Therefore, it is possible to state that nanoMIPs have the potential to become the recognition elements of choice for the development of a new generation of test-systems and sensors.

Introduction

Cocaine is an alkaloid extracted from the leaves of *Erythroxylum coca* or *Erythroxylum novogranatense*. This alkaloid stimulates the central nervous system, increasing alertness and euphoria states. Currently, after cannabis cocaine is the most commonly consumed illicit drug in the world.¹⁻⁴ Furthermore, drug consumption is considered a major public health problem that impacts society in multiples areas, mainly associated with criminal, social and economic problems.⁵ After consumption, cocaine is mainly metabolised and excreted in urine as benzoylecgonine (BZE), ecgonine methyl ester, and minor metabolites, such as norcocaine, p-hydroxycocaine, m-hydroxycocaine, p-hydroxybenzoylecgonine, m-hydroxybenzoylecgonine and cocaethylene. Due to its abundance BZE is considered one of the most important metabolites. It is known that BZE is formed under physiological conditions through hydrolysis of the methyl ester of cocaine and that it does not have a significant biological activity in humans. On the other hand, norcocaine contributes to hepatotoxic effects observed in cocaine users.⁶⁻⁹

The ability to measure and quantify cocaine is important for such analytical applications as forensic toxicology, emergency toxicology and drug treatment.¹⁰ Currently, the most common techniques used for analysis of cocaine and their metabolites in biological matrices include gas chromatography with mass spectrometry (GC/MS) detection, liquid chromatography coupled various MS detectors (LC/MS). These instrumental methods are widely used due to their ability to detect and quantify very low concentrations of cocaine in complex biological samples.¹¹ Nevertheless, these methods are expensive and usually involve multiple steps of extraction, pre-concentration, cleaning and, sometimes, derivatisation.¹

Another popular technique for quantification of cocaine is enzyme-linked immunosorbent assay (ELISA). ELISA is a powerful tool used for the detection and quantification of specific antigens or antibodies in a sample. The competitive ELISA consists of a competitive reaction between the free analyte and an enzyme-labelled conjugate for binding to antibodies immobilised on the microplate wells. The chromogenic signal, which is obtained as a result of the reaction between the enzyme-linked conjugate and substrate, is inversely related to the concentration of the analyte in the sample, so the presence of colour indicates the absence of antigen in the sample.¹²⁻¹⁵ ELISA is easy for use, does not need sophisticated instrumentation and can be standardised; unfortunately the biological nature of antibodies used as recognition elements has a negative impact on its shelf-life, reliability and cost associated with analysis.¹⁶

To address these aforementioned drawbacks, many studies have been focused on the development of synthetic materials that can act as antibody mimics and could substitute them in ELISA.¹⁷⁻¹⁹ Among the most promising candidates for this role

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are molecularly imprinted polymers (MIPs). MIPs are cross-linked polymeric materials prepared in the presence of a template (analyte) that serves as a mould for the formation of template-complementary binding sites.²⁰⁻²⁵ As a result, polymeric materials with recognition properties toward the template molecule are produced. Furthermore, MIPs are known for their stability, robustness, resistance to a wide range of pH, solvents and temperature.^{8, 26} Further advances are coming from the development and applications of nano-sized MIPs¹⁸ that could be used as antibody mimics in numerous applications, particularly in catalysis, chemical sensors, solid phase extraction, liquid chromatography, drug delivery and remediation of environmental matrices.^{7, 27-29}

The aim of this work was to produce cocaine-specific molecular imprinted polymer nanoparticles (nanoMIPs) using solid phase synthesis approach followed by their integration into ELISA-style microplate-based system suitable for the detection of cocaine, BZE and norcocaine in water and blood serum.

Materials and methods

Materials

Cocaine (COC), benzoylecgonine (BZE), norcocaine (NOR), caffeine, paracetamol, acrylamide (AA), *N,N'*-diethylamino ethyl methacrylate (DEAEM), *N,N'*-methylene-bisacrylamide (MBAA), *N*-tert-butylacrylamide (TBAm), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), ethylene glycol dimethacrylate (EGDMA), trimethylolpropane trimethacrylate (TRIM), *N,N*-diethyldithiocarbamic acid benzyl ester, pentaerythritol tetrakis(3-mercaptopropionate) (PETMP), 3-aminopropyltrimethyloxysilane, sodium hydroxide, bovine serum albumin (BSA), ovalbumin, Tween 20, horseradish peroxidase (HRP), 3,3',5,5'-tetramethylbenzidine (TMB), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), phosphate buffered saline (PBS), polyethylene glycol (PEG) with molecular weight of 1100 and dimethylformamide (DMF) were obtained from Sigma-Aldrich, UK. *N,N*-diethyldithiocarbamic acid benzyl ester >98% (iniferter) was purchased from TCI Europe, UK. *N*-(3-aminopropyl)methacrylamide hydrochloride >98% (NAPMA) was obtained from Polyscience Inc., UK. Acetonitrile was purchased from Fisher Scientific, UK. All chemicals were of analytical or HPLC grade. Nunclon 96-wells flat-bottom microwell plates were purchased from Thermo Scientific, UK. Blood serum sample (male, AB type, clotted whole blood, sterile-filtered, USA origin) was purchased from Sigma, UK.

Synthesis of molecularly imprinted polymers nanoparticles specific for cocaine

Molecularly imprinted polymers nanoparticles (nanoMIPs) were obtained using solid-phase synthesis approach using BZE as template (Fig. 1) as it was described by Smolinska-Kempisty et al.³⁰ The protocol for immobilisation of BZE on the glass beads was adopted from the paper of Canfarotta and colleagues.³¹ The

template (BZE) was immobilised on the surface of glass beads using EDC/NHS coupling. Briefly, the coupling protocol consisted of three steps: 1) activation of carboxyl groups of BZE using EDC to form an active ester; 2) reaction between the active ester and NHS to form sulfo-NHS ester, and 3) the reaction between the sulfo-NHS esters on BZE and the amine groups the glass beads (see Supplementary Information, Fig. S1, S2).

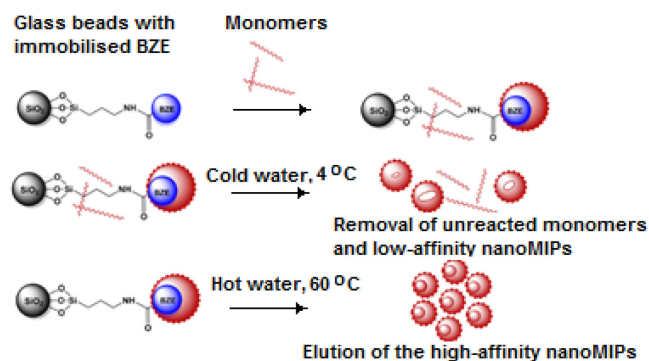


Fig. 1 Schematic representation of the solid-phase synthesis of nanoMIPs in water.

Synthesis of nanoMIPs in water

The monomeric mixture was prepared as follows: 6.6 mg AA, 8.6 mg DEAEM, 2.2 mg NAPMA, 15.6 mg TBAm and 8.2 mg MBAA; the components were dissolved in 50 mL of water, added to 30 g of glass beads with immobilised BZE and deoxygenated by purging with N₂ for 20 min. A solution of 30 mg of APS and 15 µL of TEMED in water was prepared and added to the polymerisation vessel. The polymerisation was carried out at room temperature for 1.5 h. After synthesis, the content was transferred to the plastic tube fitted with a frit. The non-polymerised monomers and low affinity nanoMIPs were removed by washing with eight volumes of cold water. The high-affinity nanoMIPs were eluted by adding consecutively 5 x 20 mL aliquots of hot water kept at 60 °C. In order to maintain the temperature and ensure the effectiveness of the elution the solid-phase-containing cartridge was kept in a water bath at 60 °C for 2 min before collecting the filtrate using a vacuum manifold (Supelco, UK) equipped with a vacuum pump. All eluted fractions of high-affinity nanoMIPs were combined and stored at 4 °C. For the application in ELISA the concentration of the nanoparticle solution was determined by weighing a freeze-dried aliquot, and adjusted at 0.06 mg mL⁻¹.

Synthesis of nanoMIPs in DMF

For the synthesis of nanoMIPs in organic solvent following monomeric composition was prepared: 1.19 g AA, 1.55 g of DEAEM, 0.112 g of NAPMA, 1.29 g of MBAA, 3.24 g EGDMA and 3.24 g of TRIM. The components were dissolved in 25 mL of DMF. The monomeric mixture was added to 30 g of glass beads with immobilised BZE and deoxygenated by purging it with N₂ for 20 min. In order to initiate the polymerisation 0.75 g of *N,N*-diethyldithiocarbamic acid benzyl ester and 0.18 g of PETMP were added, and the glass beads with the polymerisation mixture were placed between two UV light sources (Phillips, UK) for 1.5 min. After the polymerisation the unreacted

monomers and low affinity nanoMIPs were removed by washing with four volumes of cold acetonitrile (4 °C).

Post-synthesis modification of nanoMIPs using PEG

Eight millilitres of a solution of 9.4 mg mL⁻¹ of PEG in acetonitrile was added to the glass beads containing the high affinity nanoMIPs prepared using protocol in DMF and placed under UV light for 1 min. The excess of PEG was removed using cold acetonitrile. The PEG-coated nanoparticles were eluted using hot acetonitrile as described above. For the application in ELISA the concentration of the nanoparticle solution was determined by weighing a freeze-dried aliquot, and adjusted at 0.06 mg mL⁻¹.

Determination of the size of the nanoMIPs

The size of the nanoparticles was measured by dynamic light scattering (DLS) using a Zetasizer Nano-S (Malvern, UK). Prior the DLS analysis each aliquot of nanoMIPs solution was subjected to sonication for 3 min and the size was measured at 25 °C.

Preparation of the HRP-BZE conjugate

3 mg of BZE, 19 mg of EDC and 17 mg of NHS were dissolved in 10 mL of PBS buffer, pH 7.2, followed by the addition of 4.5 mg of HRP. The mixture was incubated for 2 h at 4 °C. The unreacted BZE was removed by washing the conjugate with ten volumes of PBS on the centrifuge at 2500 g for 20 min using Amicon Ultra centrifugal filter units, 30 kDa MWCO (Millipore, UK). Produced conjugate was reconstituted in 2 mL of HPLC water, aliquoted and stored in Eppendorf tubes at -20 °C.

Immobilisation of nanoMIPs on the surface of microplate wells

Before the immobilisation of the nanoparticles (40 µL per well, 0.06 mg mL⁻¹) on the microplate, the surface was treated with plasma using the plasma treatment (RF, 13.56 MHz, K1050X Emitech, UK). The plasma treatment conditions consisted of the following: the treatment was done for 5 min at 95 Watts under the pressure of 0.5 mBar. For the immobilisation the solution of nanoMIPs was added to the wells of the pre-treated microtiter plate and left to dry completely overnight at room temperature (see Supplementary Information, Fig. S3).

Optimisation of the blocking conditions in the pseudo-ELISA

In order to optimise the assay conditions three blocking solutions in PBS were tested: 1% ethanolamine and two solutions of BSA and ovalbumin. Additionally, Tween 20 was also added as surfactant to all solutions. The blocking solution that showed the lowest non-specific binding was chosen for further optimisation, which was made using Box-Behnken design experimental design (BBD). Optimisation of the blocking protocol was performed using response surface methodology for studying the correlation between response and factors. The aim of the experimental design was to maximise the signal of the analyte and minimise the non-specific binding of the assay. BBD was employed to evaluate the main effects, interaction effects, and quadratic effects of the protein, surfactant, and time of incubation of the

blocking solution in the ELISA assay. The second-order polynomial models were obtained using MODDE 7, version 7.0.0.1, through three-factor three-level design. Among the investigated parameters were the concentrations of BSA in the concentration range 0.1-0.5% (F₁), Tween 20 - 0.5-1% (F₂) and incubation time in the range between 60 and 120 min (F₃). The responses were analysed as ratios between the signal produced by nanoMIPs and nanoNIPs (R₁) and as a ratio between the signals produced by MIPs and without MIPs (R₂). The complete experimental design comprised of 15 runs, particularly the value ranges of each factor, the constraints for each response, and factors and response values are presented in Supplementary Information, Table S1.

Optimisation of the conjugate concentration (HRP-BZE)

The optimisation of the conjugate concentration was performed under the optimised blocking conditions. For this purpose, diluted solutions were prepared to different concentrations of HRP-BZE (1:100 to 1:800) by diluting the stock solution. After that, these solutions were incubated for 2 h in the microplate using a microtiter plate with immobilised nanoparticles. Later, the absorbance was measured at 450 nm using a microtiter plate reader Hidex Sense (LabLogic, UK). The highest ratio in absorbance between signal of the empty wells and wells with immobilised nanoMIPs was selected as the optimum concentration for the following experiments.

Development of pseudo-ELISA using nanoMIPs as recognition elements

The microplate wells containing immobilised nanoMIPs were conditioned using PBS (2 x 250 µL), then the blocking solution comprising of 0.1% of BSA and 1% Tween 20 in PBS was added and incubated for 2 h. After the incubation, the blocking solution was removed by washing each well 3 times using 250 µL of PBS. Then 100 µL of a solution that contained HRP-BZE (1:100) and different standard solutions of the free analyte (BZE, COC and NOR) in the range of concentrations between 10⁻¹³ and 10⁻⁷ M were added to each well, and incubated for 1 h at room temperature. After the incubation the reaction mixture was removed and a plate was washed three times using 300 µL of blocking solution and dabbed on a paper towel upside down to remove the remaining liquid. In order to develop the HRP reaction 100 µL of TMB solution was added and incubated for 10 min at room temperature. The reaction was stopped by adding of 50 µL of 5 M H₂SO₄ per well, and the absorbance was measured at 450 nm. COC, NOR and BZE were also tested in blood serum samples. All serum samples were diluted 1:10000.

Cross-reactivity of nanoMIPs in the blood serum sample

The cross-reactivity of the nanoMIPs was tested in the blood serum sample by using two commonly-used drugs, caffeine and paracetamol, in the range of concentration from 10⁻¹³ to 10⁻⁷ M.

Results and discussion

The nanoMIPs were polymerised using solid phase with immobilised BZE, as described in Section 2.2 and tested in 'pseudo'-ELISA for the detection of COC and its analogues. BZE was selected as dummy template because it has a high homology with cocaine and a functional carboxyl group which allowed immobilising it on the solid phase.³⁰ Two different formats of nanoparticles were produced, one using chemical polymerisation in water and second prepared using UV polymerisation in DMF followed by post-synthetic PEGylating treatment. Control non-imprinted polymer nanoparticles (NIPs nanoparticles) were synthesised using histamine as template.

The determination of the size of the nanoMIPs was made using DLS. The average hydrodynamic diameters of the nanoparticles polymerised using water and DMF was 234.9 and 235.3 nm, respectively. Furthermore, the polydispersity indexes which were obtained by repeating the measurements four times were lower than 0.149. The size distribution of nanoparticles prepared using water is shown in Supplementary Information section, Fig. S4. The morphology and shape of the nanoparticles polymerised using solid-phase method and also PEGylated nanoparticles are demonstrated in the earlier publications.^{24, 25}

All steps and conditions of ELISA were carefully optimised. In order to improve the coverage and distribution of the nanoparticles the polystyrene surface the microplate was treated with plasma for 5 min. The effectiveness of the plasma treatment was confirmed using immobilisation and binding of nanoparticles on the treated and untreated polystyrene surface. The 40 μL -aliquots of nanoMIPs solution (0.06 mg mL^{-1}) were added and left overnight to dry followed by binding to the BZE-HRP conjugate. The absorbance of the plasma-treated microtiter plate wells and untreated wells were measured and compared (Fig. 2).

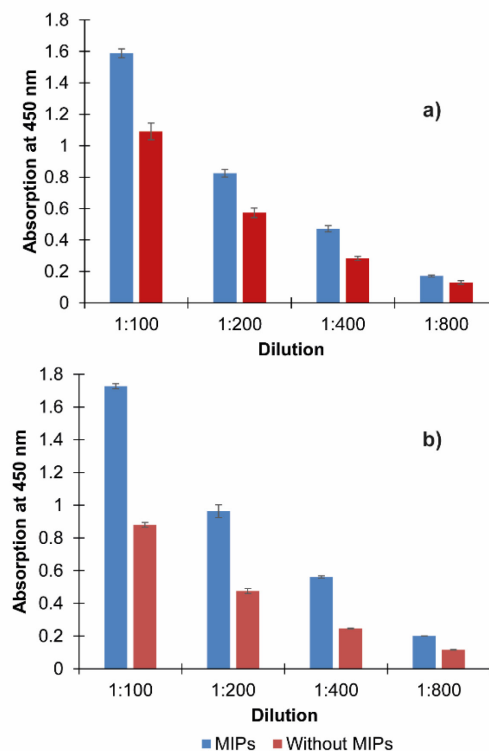


Fig. 2 Comparison between HRP-BZE conjugate binding to the nanoMIPs-coated and empty polystyrene microplates without (a) and with plasma treatment (b).

It was found that the difference in absorbance was always higher for the microplate treated with plasma for 5 min, in comparison with the results obtained without plasma treatment. It appeared that a treatment with plasma gradually changed the surface characteristics of the microplate improving the adhesion of the nanoparticles and, as consequence, improving the results (Fig. 2).³² The beneficial effect of the plasma treatment was also confirmed by the results obtained in ELISA.

In order to perform the competitive measurements of COC and analogues using pseudo-ELISA the standard solutions of free BZE and other cocaine analogues were prepared and tested in competition with BZE-HRP conjugate. In order to determine the optimum concentration of the conjugate used for the ELISA the conjugate was diluted from 100 to 800 times. As a ratio of the HRP conjugate absorption between MIPs and without MIPs for 1:100, 1:200 and 1:400 were not much different (≈ 2), the selection of the HRP conjugate dilution 1:100 was based on the higher absorption values for the wells containing MIPs ($1.728 \pm 0.016 \text{ a.u.}$) that allowed to achieve higher sensitivity of the assay (Suppl. Inf. Table S3). Therefore, 1:100 dilution of the conjugate was used in optimised ELISA protocol (Fig. 2). The TMB substrate was used to evaluate the presence of BZE-HRP conjugate and, therefore, to evaluate the concentration of free analyte.

In order to effectively reduce the non-specific binding three blocking solutions in PBS were tested (1% ethanolamine, 0.1% BSA and 1% of Tween 20, and 0.1% ovalbumin and 1% of Tween 20). To assess the effectiveness of the treatment the relative absorbance of the wells with and without MIPs was tested. It was found that BSA-containing blocking solution (No.

2) showed the best response and was effectively protecting the wells surface and decreasing the non-specific binding during the detection (Table 1).

Table 1 An optimisation of the blocking conditions

No.	Condition	With MIPs / without MIPs	Dilution
1	1% ethanolamine	0.611	1:400
		0.677	1:800
2	0.1% BSA, 1% of Tween 20	1.191	1:400
		1.195	1:800
3	0.1% ovalbumin, 1% of Tween 20	1.042	1:400
		0.979	1:800

Since the effective blocking is one of the most important conditions of the successful ELISA, some further optimisation of the composition of the blocking solution was conducted using Box-Behnken design protocol. All factors and responses generated using BDD, particularly analysis of variance for the response surface quadratic model for optimisation of the blocking solution and three-dimensional response surface contour plots of the signal between MIPs and wells without MIPs are included in the Supplementary Information section, Table S1, S2 and Fig. S5. The suitability of the model fit and the effect of each variable were checked by analysis of variance (ANOVA). The coefficients were determined for both responses as $R_2R_1 = 0.93$ and $R_2R_2 = 0.98$, which indicates that 93% and 98% of the variability of the response could be predicted by the model, respectively. Based on these results, all further ELISA tests were performed using the optimum blocking conditions, including a solution of 0.1% of BSA, 1% Tween 20 and 120 min of an incubation time.

To demonstrate that the BZE-imprinted nanoparticles possessed affinity towards the template, several ELISA experiments were made. The solutions of different concentrations of BZE in PBS were tested in the presence of nanoMIPs, nanoNIPs and without nanoparticles. It was shown that nanoMIPs had a linear response to the BZE in a wide concentration range from 10^{-13} M to 10^{-7} M with R-squared values of 0.96. The assay that was made with non-specific control nanoparticles (HIS-specific nanoMIPs) and without nanoparticles has not demonstrated any response for BZE (Fig. 3).

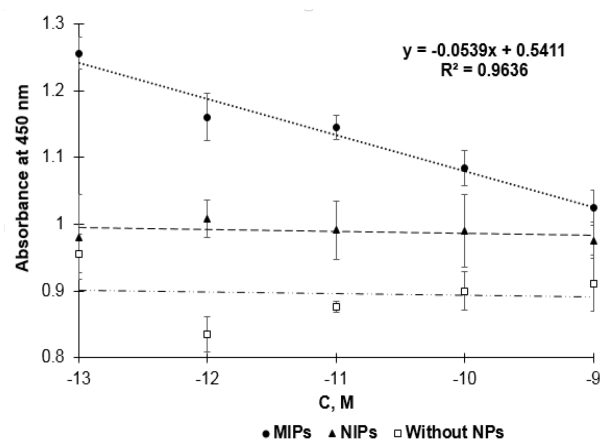


Fig. 3 Competitive pseudo-ELISA assay made in PBS solutions for detection of BZE using nanoMIPs, nanoNIPs and without nanoparticles.

The specificity of nanoMIPs towards COC and one of its metabolites NOR was also tested. It is known that average half-life of cocaine in the body is 20-90 min, depending on the dose,³³ then the drug is metabolised into BZE or NOR. Similarly to BZE, a linear range of the response was observed for COC and NOR in the concentrations range between 10^{-12} and 10^{-9} M (Fig. 4).

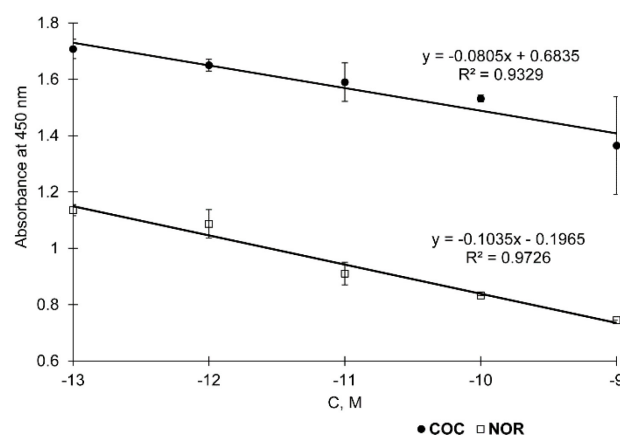


Fig. 4 Competitive pseudo-ELISA assay for detection of COC and NOR using nanoMIPs in PBS solution.

It was found that a limit of detection of BZE was in one order of magnitude lower than in case with COC and NOR suggesting about higher affinity of nanoMIPs towards BZE in comparison with other tested drugs, which could be explained by the fact that BZE was used as a template. There was also no response from nanoNIPs which suggested about specificity of nanoMIPs towards cocaine and its metabolites BZE and NOR.

Following the success of testing in model solutions the measurements of COC were also repeated in the biological samples. A sample of human blood serum was spiked with COC and tested using optimised ELISA assay based on nanoMIPs polymerised in water. In this case, only a very slight response for cocaine concentration range from 10^{-12} to 10^{-9} M was observed (Supplementary Information section, Fig. S6). This could be explained by the complexity of the human serum matrix containing lots of proteins and other interfering compounds. In an attempt to increase the affinity towards COC required for its detection in the complex biological matrices, a batch of

nanoMIPs was made in organic solvent. A similar approach was reported during imprinting and recognition of melamine.³⁴ To reduce the non-specific binding and improve nanoMIPs adhesion to microplate, nanoMIPs were post-synthetically grafted with a shell of PEG950. PEGylated MIPs were tested in real samples in assay with COC and NOR. A high affinity towards COC and NOR was observed. The assay for COC demonstrated a linear response in a concentration range between 10^{-13} and 10^{-9} M and for NOR- from 10^{-12} to 10^{-7} M (Fig. 5).

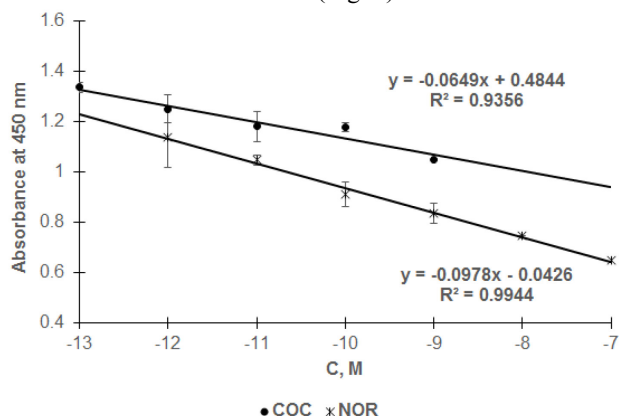


Fig. 5 Competitive pseudo-ELISA assay for detection of COC and NOR using nanoMIPs in blood serum.

The cross-reactivity of nanoMIPs in blood serum was tested using caffeine and paracetamol, compounds which could potentially be present in human blood during testing for COC. It was observed that cocaine-specific nanoMIPs did not show any response for caffeine and paracetamol. At the same time a high selectivity for COC and analogues demonstrated by nanoMIPs was not even affected by interfering compounds in the blood serum.

The results presented here show that detection of COC and their metabolites could be made in microtiter plate format using nanoMIPs instead of antibodies, offering shorter preparation time, lower price and higher stability of the synthetic receptors over their natural counterparts. Additionally, much lower limits of detection were achieved using nanoMIPs (4.2×10^{-12} M) (Table 2) when compared to commercial ELISA that offered a detection limit of 1 ng mL^{-1} of cocaine that corresponds to about 3.3×10^{-9} M.³⁵ We believe that such superior performance of nanoMIPs in pseudo-ELISA format may open new opportunities not only for the analysis of cocaine and its analogues in blood serum but, potentially, for development of antibody-free test systems for any other compounds of interest.

Table 2 Limit of detection of pseudo-ELISA for COC, BZE and NOR

Drug	Polymerisation method	Media	LOD, M
COC	Chemical, water	PBS	4.24×10^{-12}
	UV, DMF, PEG	Blood serum	3.91×10^{-13}
BZE	Chemical, water	PBS	5.16×10^{-13}

NOR	UV, DMF, PEG	B serum	3.49×10^{-12}
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Conclusions

Solid-phase imprinting protocol allowed us to develop nanoMIPs with high specificity and affinity for cocaine and metabolites superior to nanoMIPs made in solution.³⁶⁻³⁸ Synthesised nanoMIPs were used in a 'pseudo'-ELISA for measurement of drug content in human blood serum. Optimisation of the blocking solution was performed using the Box-Behnken design. The developed assay allowed to determinate cocaine in range of 10^{-13} to 10^{-9} M, LOD - 4.24×10^{-12} M and norcocaine with the range of 10^{-12} to 10^{-7} M, LOD - 3.49×10^{-12} M. The results showed that nanoMIPs have a promising application in the measurement of cocaine and its metabolites. Therefore, we believe that nanoMIPs or "plastic antibodies" could potentially be a superior alternative to natural antibodies for the analysis of any drug or analyte of interest.^{24,31}

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