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ORIGINAL PAPER

Biosensor for on-line fluorescent detection of trifluoroperazine based on genetically modified calmodulin

Martin González-Andrade · Elena Benito-Peña · Rachel Mata · Maria C. Moreno-Bondi

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Abstract This paper describes the development of a novel on-line biosensor based on a fluorescently labeled human calmodulin (CaM), *h*CaM M124C-*mBBr*, immobilized on controlled-pore glass (CPG), for the analysis of trifluoroperazine (TFP); a phenothiazine drug in human urine samples. The device was automated by packing *h*CaM M124C-*mBBr*-CPG in a continuous-flow microcell connected to a monitoring system, composed of a bifurcated optical fiber coupled to a spectrofluorometer. Operating parameters of the on-line biosensor (flow rate, sample injection volume, and carrier solution and buffer pH) were studied and optimized. Under the optimal conditions, the biosensor provides a detection and a quantification limit of 0.24 and 0.52 μg mL^{-1}, respectively, and a dynamic range from 0.52 to 61.05 μg mL^{-1} TFP (n=5, correlation coefficient 0.998). The response time (t_{100}) was shorter than 42 s (recovery time <4.5 min) and reproducibility and repeatability of the TFP measurements, within the linear response range, were lower than 1.4 and 2.7%, respectively. The device was successfully applied to the analysis of TFP in spiked human urine samples with recoveries ranging between 97 and 101% and with RSDs lower than 5.9%.

Keywords On-line biosensor · Fluorescence · CaM-CPG · Trifluoroperazine · Biomimetic element

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M. González-Andrade · E. Benito-Peña · M. C. Moreno-Bondi (✉)
Departamento de Química Analítica, Facultad de Química, Universidad Complutense,
28040 Madrid, Spain
e-mail: mcmbondi@quim.ucm.es

M. González-Andrade
Instituto Nacional de Medicina Genómica (INMEGEN), Secretaría de Salud,
Mexico City, Federal District 14610, Mexico

R. Mata
Facultad de Química, Universidad Nacional Autónoma de México,
Mexico City, Federal District 04510, Mexico

Introduction

Biosensors and biochips are tools widely used over the last few decades and have rapidly evolved in different fields of science and technology such as pharmaceutical, biology, biotechnology, environmental, bioprocessing, and clinical medicine [1, 2]. Scientific and technological advances in this field are driven by the need to replace existing diagnostic tools with low cost, environmentally friendly, simple, and fast monitoring techniques [3–5]. In this context, the availability of biomimetic recognition elements for sensor development has attracted extraordinary interest in recent years, in the search of highly sensitive and selective analytical devices for chemical analyses [6].

The discovery of new chemical entities of pharmacological interest has been a priority for pharmaceutical companies as well as for researchers around the world. This research requires the availability of high throughput analytical methods, including biosensors, for pharmacological screening since samples to be tested are typically extracted from natural products, generated by combinatorial chemistry, or produced by biotechnology (e.g., natural products, synthetic, or biotechnological compounds).

CaM is a 148-amino acid protein devoid of enzymatic activity that acts as the primary transducer of calcium mediated signals in eukaryotes that has proven to be a promising recognition element for biosensor development. It is ubiquitous

and 100% conserved among vertebrates. CaM is highly acidic, with an isolelectric point (p*I*) of approximately 4.6 [7] and binds to a diverse array of Ca^{2+}-dependent intracellular signaling targets, playing a key role in several cellular physiological processes and, consequently, in a variety of functions, both in normal and dysfunctional cells, including phatologies such as AIDS, Alzheimer's disease, or some cancers, among others [8, 9].

We have reported the preparation of two CaM fluorescently labeled proteins, namely *h*CaM M124C-*mBBr* and *h*CaM L39C-*mBBr*/V91C-*mBBr*, which probe to be useful for the detection of CaM inhibitors [10, 11]. The first mutant, *h*CaM M124C-*mBBr*, was engineered by rational design replacing Met124 by cysteine using site-directed mutagenesis. This residue was further modified by chemically attaching the fluorophore monobromobimane (*mBBr*) preserving the structural and functional properties of the native protein. CaM inhibitors, such as drugs or pesticides, specifically bind to this region of the protein promoting a conformational change that results in the fluorescence quenching of the reporter dye [8, 9, 12, 13].

Trifluoroperazine (TFP) is a phenothiazine derivative, acting as a CaM antagonist, which has been widely used as antipsychotic, antiparkinsonian, and antihistaminic drug. The analysis of TFP, as well as other phenothiazine drugs, is important for quality assurance in pharmaceutical preparations as well as for obtaining optimum therapeutic concentrations in body fluids to minimize the risk of toxicity. Several analytical methods have been optimized for the analysis of this pharmaceutical in clinical samples based on gas chromatography with flame photometric or mass spectrometry (MS) detection [14, 15], or high-performance liquid chromatography (HPLC) coupled to UV or MS [16–18]. The application of these methods requires, in most cases, a previous solid phase extraction step for sample clean up and preconcentration and the detection limits range from ng to μg mL^{-1} in urine samples. Recently, a DNA-modified electrode has been applied to the analysis of phenothiazine drugs based on electrogenerated chemiluminiscent detection [19].

This paper describes the development of an on-line optical biosensor for the analysis of TFP based on the covalent immobilization of *h*CaM M124C-*mBBr* in controlled pore glass beads (controlled-pore glass, CPG), previously functionalized with 3-aminopropyltriethoxysilane (APTES), using glutaraldehyde as cross-linking agent [20] (Fig. 1). The beads were packed in a flow through microcell connected to a continuous flow device with a monitoring system composed of a bifurcated fiber optic coupled to a spectrofluorometer. Several parameters affecting biosensor performance have been optimized, such as sample flow rate, sample volume, as well as nature, concentration, and pH of the carrier buffer. The biosensor has been applied to the analysis of TFP in human urine samples and validated by comparison with a chromatographic method.

Materials and methods

Reagents

The *h*CaM M124C-*mBBr* was purified and chemically modified with *mBBr* as previously described [10]. *mBBr* was purchased from Toronto Chemical Research (Toronto, Canada). APTES and CPG (mean diameter 41 μm, mean pore diameter 200–240 Å, specific surface area 300 m^2/g) were purchased from Fluka (Buchs, Switzerland). Glutaraldehyde, benzamide (BZM), and trifluoperazine (TFP) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Acetonitrile (ACNHPLC-grade) and methanol (MeOH; HPLC-grade) were provided by SDS (Peypin, France), and trifluoroacetic acid (TFA; HPLC-grade, 99%) was from Fluka (Buchs, Switzerland). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). All solutions prepared for HPLC were passed through a 0.45-μm nylon filter before use. Other reagents, including buffers (HAc/Ac, 2-(*N*-morpholino) ethanesulfonic acid buffer (MES) and tris (hydroxymethyl)aminomethane hydrochloride buffer (Tris–

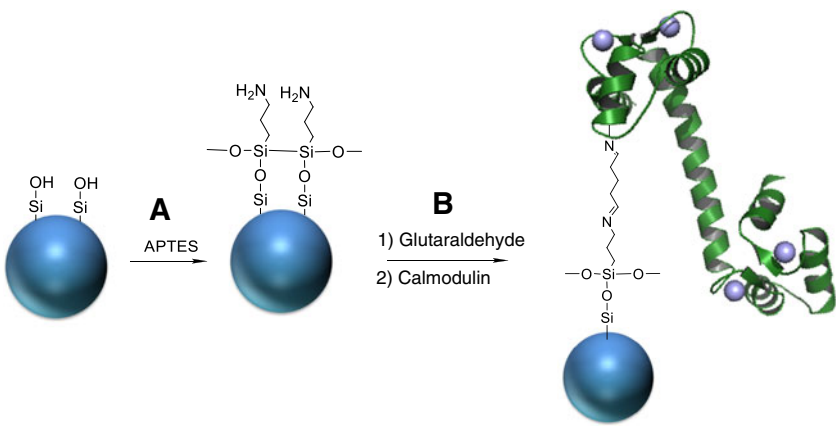

Fig. 1 Covalent binding of *CaM M124C-mBBr* onto CPG: (A) CPG surface functionalization with APTES; (B) Derivatization with glutaraldehyde and covalent binding of the mutant to CPG

HCl)), were of analytical grade reagents and were purchased from Sigma-Aldrich (St. Louis, MO).

Immobilization of *h*CaM M124C-*mBBr* on CPG

The immobilization of *h*CaM M124C-*mBBr* in CPG was performed in three steps: (a) washing of CPG, (b) glass functionalization with APTES, and (c) covalent binding of the protein onto CPG. Initially, CPG was washed with 2 M NaOH and 10% *v*/*v* H_2O_2 (2:1, *v*/*v*). The mixture was heated at 70 °C for 30 min and washed repeatedly (five times) with distilled water. Thereafter, CPG was placed in a mixture of H_2O, 2 M HCl, and 10 % *v*/*v* H_2O_2 (5:1:1) and heated at 70 °C for 30 min; the resulting material was rinsed thoroughly with distilled water and dried overnight at 90 °C. For amination, 1 g of CPG was suspended in a 10% aqueous solution of APTES, pH 3–4, at 75 °C for 1.5 h. The resulting beads were rinsed with deionized water and dried overnight at 95 °C. The process was repeated twice to assure complete activation of the CPG. For covalent binding of the protein to CPG, glutaraldehyde (47.6 μL of 42 mM), APTES-activated CPG (300 μL, sedimented volume), and *h*CaM M124C-*mBBr* (400 μL of a 1.25 mM protein solution) were mixed in 2 mL of 100 mM phosphate buffer (pH 7.4) and stirred for 4–5 h at room temperature. A molar ratio glutaraldehyde to protein of 1:10 was used to avoid intra-protein cross-linking and CPG aggregation [21]. Then, 15 mg of sodium cyanoborohydride was added to reduce the imine groups resulting from the reaction between the aldehyde groups in the protein and the amine residues of the APTES-activated sorbent; the reaction was maintained at 4 °C for 15 h. The particles were then washed thoroughly with deionized water and 1 mL of a 0.1 M ethanolamine solution was added to block free remaining aldehyde groups. After incubation at RT for 2 h, the modified CPG particles were washed once with distilled water and suspended and stored in 300 μL of PBS buffer at 4 °C, protected from light.

Determination of the amount of protein bound to CPG

The amount of protein was determined with the bicinchoninic acid method (BCA assay) [22] and it was calculated as the difference between the concentration of protein in solution before and after immobilization.

Measuring system

The experimental set-up used for the assay is shown schematically in Fig. 2. The system is fully automated and consists of a syringe dispenser module (VersaPump 6) equipped with a 2.5-mL syringe, connected to six-way distribution valve (Kloehn Ltd, Las Vegas, NV) for program-assisted uptake and delivery of the buffer and TFP samples into the flow-through system. The whole set-up is controlled with the Winpump software (V6 Firmeware version 5) provided by Kloehn Ltd. The output of the pump is connected to a glass flow-through microreactor, internal dimensions 9.3×2.0×0.25 mm, consisting of a flow-through chamber of 5 μL and an array of posts (spacing 22 μm), at the end, to retain the packing material, developed at the Institute of Microtechnology (Neuchatel, Switzerland) [23]. The microreactor was packed with the *h*CaM M124C-*mBBr* immobilized in CPG as described previously [23]. An optical fiber (Horiba Jobin Yvon, Longjumeau Cedex, France) was used to illuminate the *h*CaM M124C-*mBBr*-CPG pad and to guide the emitted light back to spectrofluorometer. Fluorescence intensity measurements (λ_{ex}=381 nm; λ_{em}=466 nm) were carried out in a Fluoromax 2 (Horiba Jobin Yvon, Longjumeau Cedex, France) fitted with the optical fiber adaptor, and the instrumental parameters and data processing were controlled with the original software (Datamax). Measurements were performed according to the following procedure. Initially, the carrier buffer, HAc/Ac (50 mM, pH 5.1), was pumped through the flow system, flow rate 1.2 mL min^{-1}, in order to obtain a baseline signal. Calibration solutions (5 mL) containing increasing concentrations of TFP in the range of 0.52–61.05 μg mL^{-1} (seven points, each point corresponding to the average of five replicates) in HAc/Ac 50 mM, pH 5.1, were then injected into the system (flow rate 1.2 mL min^{-1}) monitoring the decrease in the fluorescence intensity that was proportional to TFP concentration. Before a new measurement, the microrreator was washed with 5.4 mL of carrier buffer at a flow rate of 1.2 mL min^{-1}.

Chromatographic analysis was carried out with an HP-1100 HPLC from Agilent Technologies (Palo Alto, CA, USA) equipped with a quaternary pump, an online degasser, an autosampler, an automatic injector, a column thermostat, and a diode array detector (DAD). Data analysis was performed using HP Chemstation software.

Samples analysis and validation

Urine samples were collected from a healthy individual, not medicated with TFP for more than 6 months, and analyzed using the standard addition method. To that aim, portions of 2 mL, previously enriched with known amounts of TFP (final concentration 5.08 and 10.17 μg mL^{-1}), were spiked with increasing concentrations of the drug (five concentration levels in the range of 0–56.27 μg mL^{-1}) and made up to a final volume of 3 mL with HAc/Ac pH 5.1 (final buffer concentration 50 mM). At least three replicate samples were analyzed to validate the results.

Urine samples were also analyzed by liquid chromatography. Chromatographic analysis was performed on a LUNA C_{18} (150×4.6 mm, 5 μm) HPLC column protected by an RP18 guard column (4.0×3.0 mm, 5 μm), both of Phenomenex (Torrance, CA). A gradient program was used

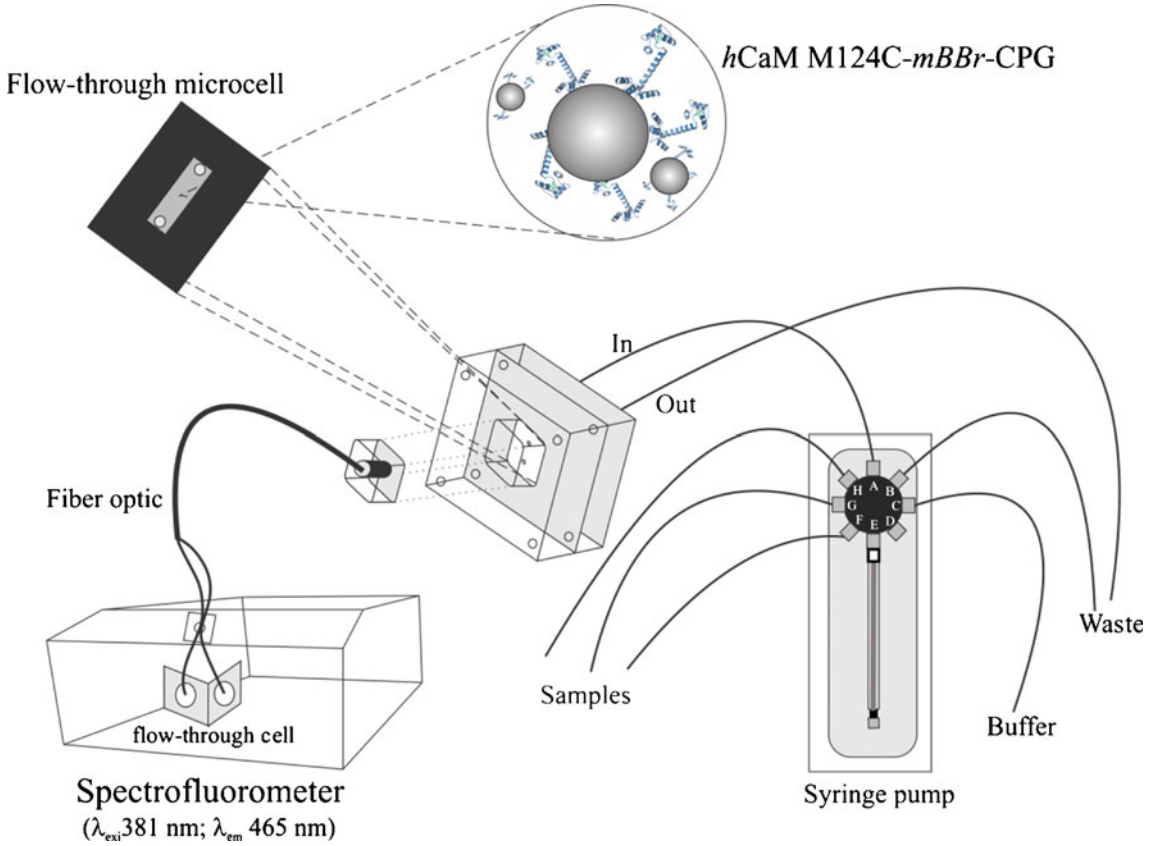

Fig. 2 Schematic diagram of the automated flow injection set-up used for the measurements with the fluorescent *h*CaM M124C-*mBBr*-CPG based sensor

with a mobile phase comprising solvent A (Milli-Q water with 0.1% TFA), solvent B (ACN with 0.1% TFA), and solvent C (MeOH) as follows: 8% B and 10% C (12 min), 8–26.5% B and 10% C (7.5 min), and 26.5–65% B and 10% C (9.5 min). Analyses were performed at a flow rate of 1.5 mL min^{-1}, and the column temperature was kept at 25 °C. The injection volume was 4 μL. Quantification was performed using external calibration peak area measurements. Linear calibration graphs were obtained in the 0–20.54 μg mL^{-1} range for TFP (R^2>0.999), and the absorbance signal was recorded at 250 nm. All the analyses were carried out in triplicate.

Results and discussion

Characterization and immobilization efficiency of *h*CaM M124C-*mBBr*-CPG

As reported previously [10, 11], the response of the biosensor *h*CaM M124C-*mBBr*-CPG is based on the fluorescence quenching of *mBBr* covalently bound to the cysteine residue (Cys) at position 124 of the mutant protein. According to the X-ray analyses of cocrystallized Ca^{2+}-CaM-TFP complex, 14 side chains, including this residue, participate in the binding site. Upon drug binding, there is a conformational change and the fluorescent emission of *mBBr* is quenched in a concentration-dependent way [10, 11].

Protein immobilization for biosensor preparation is an important factor that directly affects the sensitivity and long-term stability of the on-line devices. Regardless the immobilization procedure, or the solid support, the protein should maintain its functionality as well as their properties for optimum biosensor performance. Some factors affecting the stability of immobilized *h*CaM M124C-*mBBr*-CPG include: (a) pore size of the nanostructured support, (b) the number of reactive sites per protein for covalent binding with the cross-linking agent, and (c) the relationship between the amount of cross-linking agent and amount of immobilized protein, which is critical for optimal functionality of this system.

In the present work, the protein *h*CaM M124C-*mBBr* has been immobilized on CPG, using glutaraldehyde as cross-linking agent, following the procedure described in the "Experimental Section". This approach has been broadly applied for protein immobilization in optical fiber sensors [24] as it requires mild reaction conditions and provides high operational stability with low cost. The immobilization efficiency, evaluated as the difference between the initial

amount of protein and protein present in all the washing fractions collected during the immobilization procedure, was always higher that 85%. Figure 3 shows the fluorescence spectra of a (1 μM) solution of *h*CaM M124C-*mBBr* and of *h*CaM M124C-*mBBr*-CPG in HAc/Ac buffer (50 mM, pH 5.1). The fluorescence maximum of the immobilized mutant shows a slight hypsochromic shift (6 nm) with respect to that of *h*CaM M124C-*mBBr*. Fluorescence microscope images of both CPG and *h*CaM M124C-*mBBr*-CPG confirmed that the fluorescence emission of *h*CaM M124C-*mBBr*-CPG was due exclusively to the immobilized mutant and not to the CPG particles.

On-line *h*CaM M124C-*mBBr*-CPG biosensor optimization and analytical characterization

Several parameters affecting the performance of the on-line biosensor have been evaluated: sample flow rate, sample volume, the nature of the carrier solution as well as buffer concentration, and pH. The optimized conditions for the analysis of TFP are summarized in Table 1.

Figure S1A, in the Electronic Supplementary Material, shows the effect of the flow rate on the response of the device to a standard solution of TFP (2.0 μg mL^{-1}). As expected, higher responses were obtained with decreasing flow rates that can be attributed to the increased contact time between the analyte and the immobilized protein. However, analysis times were also longer. Thus, an optimum flow rate of 1.2 mL min^{-1} was selected for further experiments as a compromise between sample throughput and biosensor sensitivity.

The effect of the sample volume injected on the analytical response was tested over the 400–600 μL range for a 2.0 μg mL^{-1} TFP standard solution; as shown in Fig. S1B, in the Electronic Supplementary Material, biosensor response did not improve significantly for sample volumes higher than 500 μL that was selected for further experiments.

Table 1 Optimized measuring conditions with the *h*CaM M124C-*mBBr*-CPG on-line biosensor

Parameter	Evaluated range	Optimized value
Flow rate (mL min^{-1})	0.5, 0.8, 1.0, 1.2, 1.4, 1.6, and 1.8	1.2
Buffer solution		
Nature		
	0.05 M Tris–HCl, pH 7.0	HAc/Ac
	0.05 M MES, pH 6.0	
	0.05 M HAc/Ac, pH 5.1	
Concentration (mM)[a]		
	5, 10, 50, and 100	50
pH[a]		
	3.7, 4.0, 4.5, and 5.1	5.1
Sample volume (μL)	400, 500, 550 and 600	500

[a] Concentration and pH were optimized for buffer HAc/Ac

The effect of buffer composition and pH on the response of the biosensor was examined taking into account the physicochemical properties of both *h*CaM M124C-*mBBr*-CPG and the analyte tested (TFP). CaM is stable over a wide pH range (2–12) [26] and TFP solubility is highest at pH 6. Therefore, different buffer solutions, HAc/Ac (50 mM, pH 5.1), MES (50 mM, pH 6.0), and Tris–HCl (50 mM, pH 7.0) were tested for assay optimization using the on-line system (Table 1).

The highest response was obtained with HAc/Ac buffer (50 mM, pH 5.1). The use of MES buffer (50 mM, pH 6.0), which increases TFP solubility, or TRIS buffer, did not improve biosensor sensitivity. No effect on the signal was observed when HAc/Ac buffer concentration was increased in the range from 5 to 100 mM. Finally, a 50-mM HAc/Ac buffer solution, pH 5.1, was selected for the assay.

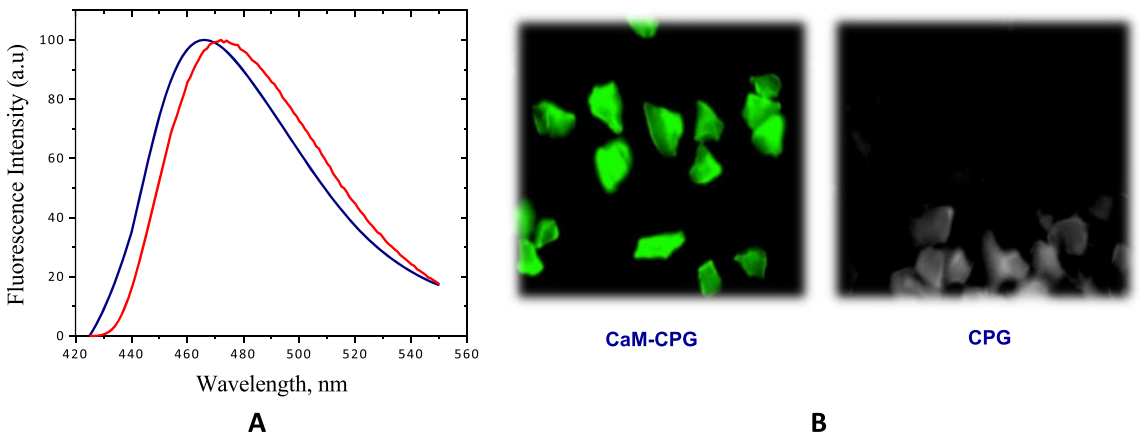

Fig. 3 (**A**) Normalized emission spectra of *h*CaM M124C-*mBBr*-CPG (*blue*) and *h*CaM M124C-*mBBr* (1 μM) in 50 mM HAc/Ac, pH 5.1 (*red*) and (**B**) fluorescence microscopy images of *h*CaM M124C-*mBBr*-CPG (*left*) and CPG (*right*); λ_{ex}=350 nm, λ_{em}=400 nm

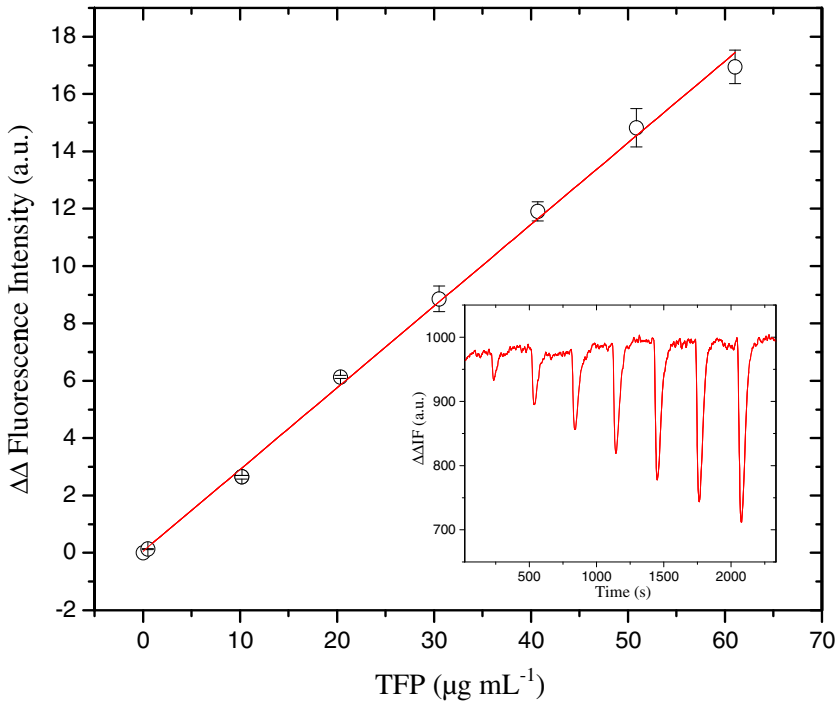

Fig. 4 Calibration plot obtained with the biosensor for TFP standard solutions in 50 mM HAc/Ac pH 5.1 (n=3). *Inset* dose–response curve corresponding to the analysis of TFP standard solutions in the calibration range measured with the on-line biosensor

Figure 4 (inset) shows a dose–response curve measured with the automated on-line biosensor in the optimized conditions. Biosensor response was fully reversed by flowing the buffer solution though the sensing phase due to the nature of the non-covalent interactions between TFP and the biomimetic recognition element (*h*CaM M124C-*mBBr*).

The calibration data were fitted to a linear regression curve (uncertainty values calculated at a 95% confidence limit):ΔIF (a.u.)=(0.02±0.18)+(0.285±0.005)[TFP] (R^2= 0.998, n=5 replicates/standard) where ΔIF represents the variation of the fluorescence intensity corresponding to different TFP standard solutions, in μg mL^{-1}. No signal change was measured in the presence of benzamidine that was used as negative control.

The detection limit, calculated as the concentration of TFP which produced an analytical signal that is three times the standard deviation of the blank (IUPAC criterion), was 0.24 μg mL^{-1} and the quantification limit ($10s_b$) was 0.52 μg mL^{-1}. Lower detection limits have been reported previously using chromatographic techniques (between 0.017 and 0.30 μg mL^{-1}; [14–19]) however, these methods required a previous clean up and preconcentration step while the biosensor allows direct analysis of the diluted sample. Therefore, analysis times are reduced considerably (t_{100}=43 s and recuperation time <4.5 min) and this device could be applied for the on-line screening of a large number of samples in pharmaceutical or clinical analysis.

Intra-day reproducibility was determined by analyzing three replicates of calibration curves on the same day, and inter-day reproducibility was evaluated by the analysis of three replicates of calibration curves on three different days during the study period. The intra-day and the inter-day coefficient of variation were lower than 1.4% and 2.7%, respectively, indicating that the method is precise. Finally, to evaluate biosensor stability, the same sensing layer was calibrated every 15 days over a period of 6 months. The device was stored at 4 °C, protected from light, in HAc/Ac buffer between measurements. No significant differences (confidence level, 95%) have been found in the calibration curves measured during that period, demonstrating the excellent performance and stability of the on-line biosensor based on the engineered CaM mutant.

Table 2 Mean recoveries (%) and relative standard deviations (RSD, %) for TFP in human urine samples spiked with the drug a two concentration levels (n=3) using the on-line biosensor and HPLC with diode array detection

Spiking level (μg/mL)	*h*CaM M124C-*mBBr*-CPG biosensor			HPLC-DAD		
	Found (μg/mL)	Recovery (%)	RSD (%)	Found (μg/mL)	Recovery (%)	RSD (%)
5.08	5.12	101	5.9	5.25	102	0.95
10.17	9.84	97	4.9	10.02	98	0.60

Analysis of TFP in spiked human urine samples

For validation purposes, the optimized biosensor was applied to the analysis of free-analyte human urine samples spiked with TFP. Calibration curves were prepared by spiking buffer and urine samples with increasing concentrations of TFP potassium salt, at seven concentration levels, in the range of 0.63–40.70 μg mL^{-1}. A slight matrix effect was observed for the analyses of urine samples, therefore the standard addition method was applied and the results are summarized in Table 2. The recoveries were close to 100% demonstrating the validity of the biosensor for the proposed analysis.

The samples were also analyzed by HPLC-DAD as alternative technique. The results were statically compared with those of the biosensor by applying the *F* test for precision and the *t* test for accuracy. The calculated *F* values exceeded the tabulated values, $F=38.51$ at the 95% confidence level for 2 degrees of freedom, for the highest TFP assayed concentration in urine. However, the calculated *t* values, using the modified *t* test for methods with different standard deviations [25], did not exceed the tabulated values at the 95% confidence level, therefore both methods do not differ significantly with respect to accuracy.

Conclusions

An automated on-line biosensor based on an engineered CaM protein, *h*CaM M124C-*mBBr*, immobilized on CPG has been optimized for the analysis of TFP, an anti-CaM ligand, in human urine samples. The response of the device is based on the fluorescence quenching of the labeled mutant upon ligand binding. The developed biosensor is low cost because the components are not expensive, the mutant is not discarded after every assay, and the biosensor shows satisfactory long-term stability of more than 6 months, demonstrating the high stability of the immobilized engineered protein. Biosensor automation allows the analysis of about 12 samples per hour, a great advantage over existing routine analytical methods for the determination of CaM inhibitors. The on-line method has been applied to the analysis of TFP in human urine samples, without further sample clean up or extraction, and the results were fully comparable to those obtained by HPLC-DAD as alternative technique. The optimized device demonstrates the potential of engineered proteins for the development of biomimetic sensors for the analysis of pharmaceuticals in biological fluids.

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