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Strip-based lateral flow-type indicator displacement assay for γ-hydroxybutyric acid (GHB) detection in beverages

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ABSTRACT

The use of gamma-hydroxybutyric acid (GHB) in drug-facilitated sexual assault has increased due to its availability and high solubility in aqueous solutions and alcoholic beverages, necessitating the development of rapid methods for GHB detection. In this respect, portable testing methods for use in the field, based on lateral flow assays (LFAs) and capable of detecting trace concentrations of target analytes, are particularly attractive and hold enormous potential for the detection of illicit drugs. Using this strategy, here we report a rapid, low cost, easy-to-handle strip-based LFA for GHB analysis employing a smartphone for fluorescence readout. At molecular signalling level, the ensemble is based on a Cu^{2+} complex with a tetradentate ligand and the fluorescent dye coumarin 343, which indicate GHB through an indicator displacement assay (*IDA*) in aqueous solution. When incorporated in a LFA-based strip test this system shows a detection limit as low as 0.03 μM for GHB in MES buffer solution and is able to detect GHB at concentrations of 0.1 μM in soft drinks and alcoholic beverages in only 1 min.

1. Introduction

γ-Hydroxybutyric acid (GHB) is a naturally occurring metabolite found in many living species. In stark contrast, GHB has also been used as date rape drug and in drug-facilitated sexual assault (DFSA) as it can be easily produced in a simple one-step reaction from widely available precursors. DFSA is defined as sexual assault without the consent of the victim, who is unable to give consent due to the often-insidious administration of psychoactive substances. DFSA has gained global relevance and social impact as the number of cases has increased significantly in recent years.

GHB is colorless, odorless, and soluble in aqueous/alcoholic beverages, making it undetectable to the average person. Additionally, GHB is rapidly eliminated and its detection in urine samples is only possible within 6–12 h after ingestion [1,2]. It is reported that a 0.5 g dose of GHB produces relaxation and disinhibition, a 1 g dose produces a euphoric effect and a 2–3 g dose produces a deep sleep [3]. Assuming the dose is in a drink of 150–200 mL, the minimum concentration of GHB in a spiked beverage producing sedative effect is ca. 10 mg mL^{-1} (96 mM).

Owing to the increasing use of GHB in rape-assaults, a need has arisen to develop simple and efficient assays for its identification. Analysis of GHB can be achieved using a wide range of techniques such as GC-MS and HPLC-UV [1,4,5], however, these procedures require a laboratory environment and sophisticated instrumentation and cannot

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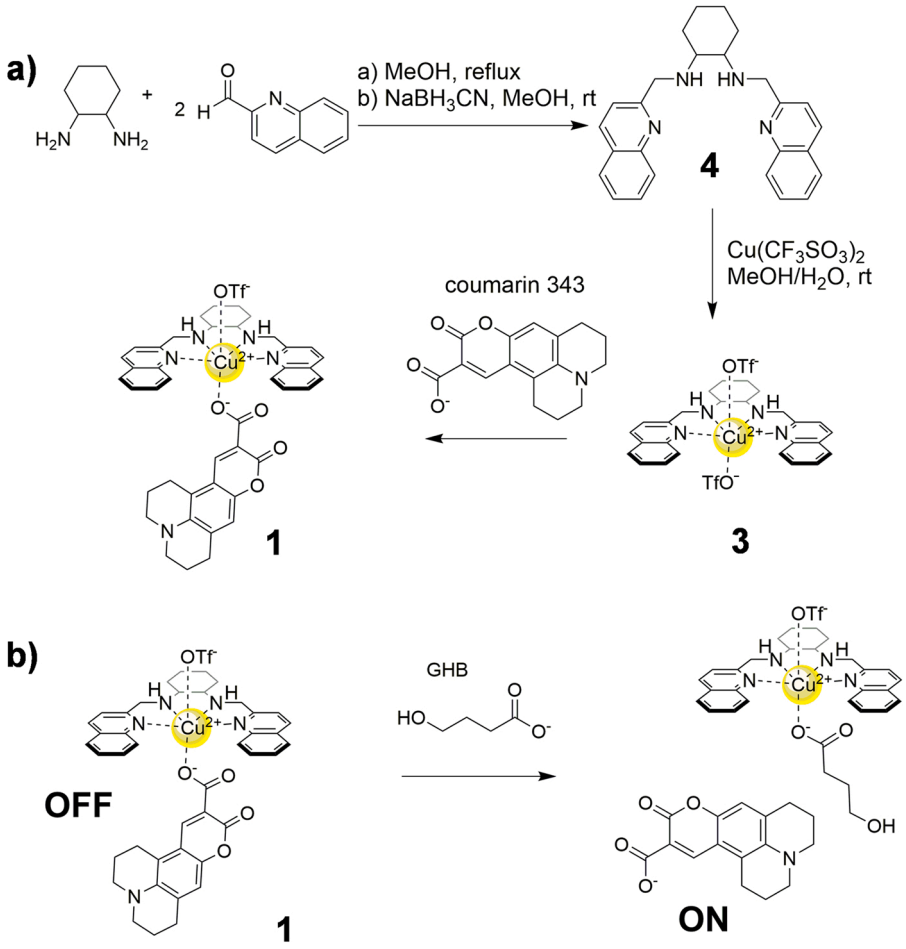


Fig. 1. a) Synthesis of tetradentate ligand **4**, Cu^{2+} complex **3** and GHB *IDA* probe **1** (formed between complex **3** and coumarin 343). b) *IDA* assay for fluorescent GHB detection using *IDA* probe **1**.

be used for *in situ* GHB detection directly at a scene of misuse. As an alternative, several potentially miniaturizable approaches based on optical sensing and the use of chromo-fluorogenic probes have recently been reported. For instance, Baumes et al. reported GHB detection in aqueous solutions using a colorimetric sensing array, based on supramolecular host–guest complexes of cucurbiturils with fluorescent dyes [6]. In addition, Chang and co-workers prepared a fluorescent BODIPY derivative which exhibited a moderate emission quenching in the presence of GHB [7]. Furthermore, some of us used non-emissive oxazole derivatives to detect GHB through color changes and marked emission enhancements in mixed aqueous solutions, allowing the detection of GHB in soft drinks and alcoholic beverages [8]. Gold nanoparticles bi-functionalized with oxazole and phenanthroline derivatives have also been reported for the colorimetric detection of GHB in water [9]. However, some of these approaches still show certain drawbacks for an effective use in GHB detection, such as the employment of organic solvents, the application of screening techniques, high detection limits in aqueous solution and difficult handling. Ultimately, methods for the simple, rapid *on-site* detection of GHB in beverages needed to prevent drug-facilitated sexual assault by enabling rapid detection and prosecution have yet to be developed.

Based on the above, we report herein an indicator displacement assay (*IDA*) for GHB detection using the probe **1**, formed by the interaction of the Cu^{2+}complex **3** and coumarin 343 (Fig. 1a). The higher affinity of Cu^{2+} for the aliphatic carboxylate group of GHB over the aromatic carboxylate group of the coumarin dye ensures the displacement of the dye. In this regard, the sensing paradigm relies on a preferential coordination of GHB by Cu^{2+} in **1** that induces displacement of coumarin 343 thus restoring its fluorescence (Fig. 1b). Fig. 1a also shows the synthesis of the tetradentate ligand **4** [10,11]. The chemical properties of the molecular complex allow to develop a system for the detection of GHB using strips and simple instrumentation. For this, *IDA* probe **1** was incorporated into a poly(ethylene glycol)-coated glass fiber (**PEG-GF**) membrane to obtain a robust and sensitive lateral flow assay that allows detection of GHB in soft drinks and alcoholic beverages in only 1 min using a smartphone for fluorescence readout. As far as we know this is the first example of a sensor based on a copper complex and a fluorophore for the detection of GHB.

2. Material and methods

2.1. Materials and reagents

2-Quinolinecarboxaldehyde, (±)-trans-1,2-diaminocyclohexane, sodium borohydride ($NaBH_4$), 2-(N-morpholino)ethanesulfonic acid (MES), tetraethylorthosilicate (TEOS), 3,4-methylenedioxypyrovalerone hydrochloride (MDPV), and scopolamine hydrobromide (SCP) were purchased from Sigma Aldrich (Madrid, Spain). Copper (II) trifluoromethanesulfonate 98% ($Cu(CF_3SO_3)_2$) was purchased from Acros Organics (Waltham, United States). 3-(methoxy(polyethyleneoxy)trimethoxy silane was purchased from CymitQuimica. The drug tested, γ-hydroxybutyric acid (GHB), morphine, cocaine, heroin, and 3,4-methylenedioxymethamphetamine (MDMA) were kindly provided by "Agencia Española de Medicamentos y Productos Sanitarios" (AEMPS). Saturated sodium chloride (Brine, NaCl sat.) and anhydrous magnesium sulfate ($MgSO_4$) were purchased from Scharlab S.L. (Barcelona, Spain).

Glass fiber and fusion 5 strips were obtained from WhatmanTM (Maidstone, United Kingdom). Filter paper was obtained from Thermofisher Scientific (Waltham, United States). All solvents were ACS reagent grade or better quality and were used without further purification. Methanol, anhydrous methanol, and anhydrous dichloromethane, were purchased from Scharlab S.L.

2.2. Synthesis of IDA probe **1**

IDA probe **1** [10] and N,N-bis(quinolin-2-ylmethylene)− 1,2-cyclohexanediamine (**4**) [12,13] were prepared following literature procedures with some changes.

2.3. Synthesis of complex **3** [12]

In a round bottom flask (50 mL), **4** (100 mg, 0.252 mmol) was dissolved in anhydrous MeOH (20 mL). An aqueous solution of $Cu^{II}(CF_3SO_3)_2$ (91.2 mg, 0.252 mmol) in milli-Q water (1.8 mL) was added and the reaction mixture was stirred for 3 h at rt. The solvent was removed under vacuum and the colorless solid obtained was washed with cold MeOH and dried to obtain complex **3** (Fig. 1a). HRMS: Calculated for $C_{26}H_{28}CuN_4$ (M^+H^+) 460.0840 *m/z*; measured 460.1561 *m/z* (M^+H^+).

2.4. Displacement kinetics in the presence of GHB

In a typical experiment, 2 mL of *IDA* probe **1** (212 μM of complex **3** in MeOH/H_2O 4:1 v/v and 0.1 μM coumarin 343 in MES buffer (50 mM), pH 6.0) was divided into two aliquots of 1 mL. Then, 50 μL of a 10 mM solution of GHB in MES buffer (50 mM of MES, pH 6) were added to one of the aliquots (final concentration of 100 μM) and, simultaneously, 50 μL of MES buffer were added to the other aliquot, constituting the blank control (blank aliquot). Both solutions were stirred at 25 °C and, after certain time intervals, the fluorescence of the displaced coumarin 343 (λ_{ex} = 444 nm, λ_{em} = 494 nm) was measured.

2.5. Concentration dependent studies with GHB

To 2 mL of a solution of *IDA* probe **1** in MeOH/H_2O 4:1 v/v, increasing volumes of a 1 mM solution of GHB in MES buffer were added. Subsequently, the solution was stirred at 25 °C for 15 s and then the fluorescence of coumarin 343 (λ_{ex} = 444 nm, λ_{em} = 494 nm) was measured.

2.6. Preparation of PEG-coated glass fiber paper (**PEG-GF**) [14]

A mixture of 24 mL toluene and 600 μL 3-(methoxy(polyethyleneoxy)trimethoxy silane was placed in a 50 mL tube containing 15 glass fiber strips of 8 × 2.5 cm. After reaction for 12 h at 25 °C with orbital stirring, the **PEG-GF** strips were washed with ethanol, dried at 37 °C for 2 h and cut into the appropriate size prior to use.

2.7. Preparation of silica- and PEG-coated glass fiber paper (**S-PEG-GF**)

A mixture of 3.4 mL Milli-Q water, 7.4 mL ethanol, 2.7 mL TEOS, 300 μL 3-(methoxy (poly-ethyleneoxy)propyl)trimethoxy silane (**PEG**) and 180 μL NH_3 (32%) was placed in a 20 mL vial containing 30 glass fiber strips of 8 × 2.5 cm. After reaction for 24 h at room temperature with orbital stirring, the **S-PEG-GF** strips were washed with ethanol and dried at 37 ºC.

2.8. Concentration dependent studies with GHB on strip

PEG-GF strips of 2.5 × 0.5 cm were prepared, and 5 μL of a solution of the *IDA* probe **1** were deposited at ca. 1 cm from one end of the strip (zone A). The strips containing *IDA* probe **1** were then dipped into 75 μL of the MES-buffered sample solutions containing different concentrations of GHB and the flow was left to develop for 1 min. Afterwards, the fluorescence emission intensity of the strips was measured by introducing the strip in a 3D-printed smartphone holder equipped with a 465 nm LED as excitation source, powered by the smartphone via a USB-OTG link, and a long-pass filter (>550 nm) for collection of the coumarin emission. The amount of dye released for each concentration was calculated according to the ratio of the fluorescence of zone B and the fluorescence of the blank.

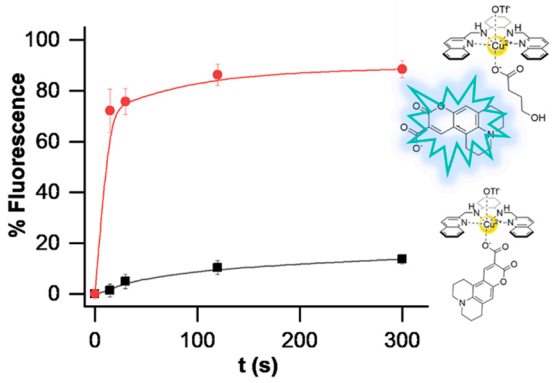


Fig. 2. Kinetics of C343 displacement from **1** in MeOH/H_2O 4:1 v/v in the absence (black line) and the presence of GHB 100 μM (red line). Error bars are expressed as 3σ for three independent experiments.

2.9. Concentration dependent studies with GHB in alcoholic beverages on strip

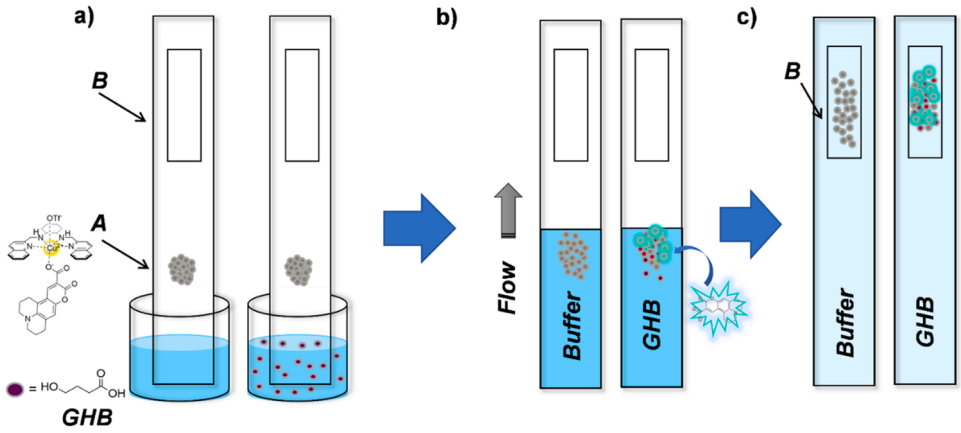
The same procedure described in the section above was carried out in a solution of gin two-fold diluted with MES buffer.

2.10. Selectivity studies in alcoholic beverages on strip

PEG-GF strips of 2.5 × 0.5 cm were prepared, and 5 μL of a solution of *IDA* probe **1** in MeOH/H_2O 4:1 v/v were deposited at ca. 1 cm from one end of the strip (zone A). The strips containing *IDA* probe **1** were then dipped into 75 μL of a sample of gin two-fold diluted in MES buffer containing 500 μM of different drugs (MDMA, morphine, heroin, cocaine, MDPV, scopolamine and GHB). Afterwards, the strip's fluorescence was measured using the smartphone setup previously described. The amount of dye released for each concentration was calculated according to the ratio of the fluorescence of zone B and the fluorescence of zone B of the blank.

3. Results and discussion

For the preparation of the sensing ensemble, the tetradentate ligand **4** was synthetized by a reductive amination of 1,2-cyclohexyl diamine with 2-quinolinecarboxaldehyde. The structure of **4** was confirmed using ^1H and ^{13}C NMR and HRMS and was in agreement with the reported values in the literature [12,13]. **4** was then reacted with Cu$(CF_3SO_3)_2$ to obtain the corresponding complex **3**, that was further reacted with coumarin 343 (C343) to obtain the final *IDA* probe **1** (Fig. 1a). The choice of **1** as a suitable *IDA* probe for the envisioned indicator displacement assay is based on the following reasons: (i) C343 displays a strong fluorescence in its neutral and deprotonated state in organic and aqueous solvents [15], (ii) coordination of anionic C343 to the paramagnetic central Cu^{2+} ion of **3** leads to an efficient quenching of the fluorescence [16], (iii) the pK_a of C343 (pK_a = 4.65) [17] is close to that of GHB (pKa = 4.72) [18], minimizing pH-dependent effects, and (iv) the fluorescence bands of neutral and deprotonated C343 are rather similar [17], qualifying the *IDA* probe for operation across the relevant



Scheme 1. Design of the lateral flow assay on strip with *IDA* probe **1** integrated into a coated **PEG-GF** membrane. a) Schematic of the integration of *IDA* probe **1** in the zone A of the lateral flow strip immersed in the GHB solution. b) No release occurs in absence of the analyte (negative test), yet dye is released in its presence (positive test). c) Coumarin 343 displaced from *IDA* probe **1** only in GHB presence is detected in zone B.

pH range.

To assess the proposed *IDA* sensing paradigm for GHB detection, the fluorescence response of **1** in organic-aqueous media (MeOH/H_2O 4:1 v/v) in the presence/absence of GHB was monitored. In a typical experiment, to a solution of **1**, GHB or MES buffer was added and the emission of C343 at 494 nm (λ_{ex} = 444 nm) was measured after certain time intervals. Kinetic emission profiles of **1** in the absence and presence of GHB are displayed in Fig. 2. In the absence of GHB, emission of C343 is highly quenched in **1**. In contrast, fluorescence is remarkably enhanced in the presence of GHB, which is ascribed to a preferential coordination of GHB's carboxylate group with the copper cation in **1**, leading to a displacement of the C343 anion. Moreover, addition of increasing amounts of GHB to a solution of the *IDA* probe **1** in MeOH/H_2O 4:1 v/v induces a progressive increase in the fluorescence of C343, in agreement with a concentration-dependent displacement of the fluorophore from **1** by GHB coordination with Cu^{2+}. From the obtained titration profile (Fig. S1) a limit of detection of 0.06 μM for GHB was determined. This detection limit is better than those previously reported in the literature [9,19–21]. This value allows detection of a common dose of GHB (1.5–2 g) in 150–200 mL (96 mM).

Hence, encouraged by the results obtained in mixed aqueous buffered solutions we immersed **1** in PEG-modified glass fiber membranes through the deposition of few microliters of a stock solution, aiming to develop a low-cost, straightforward, portable, and easy-to-handle lateral flow assay combined with a smartphone setup for fluorescence readout for *in situ* and *on-site* detection of GHB.

Within the field of point-of-care (POC) or point-of-need testing [22–24], lateral flow assays (LFAs) [25] or lateral flow test strips (LFTS) [26] are considered as excellent tools for qualitative, semi-quantitative, and even quantitative detection of various analytes, especially biomarkers [27,28], but recently also toxins [29], pesticide residues [30] and other small molecule analytes of concern [31–33]. The ASSURED criteria, mentioned by the World Health Organization (WHO), insist that all POC devices must provide features such as being (i) affordable; (ii) sensitive; (iii) specific; (iv) user friendly; (v) rapid and robust; (vi) equipment-free; and (vii) deliverable to the end-users [34,35] . In this respect, LFA technology meets these specifications and has undergone rapid technological advances for a great variety of applications. In brief, LFA tests consist of a liquid sample that runs along the porous membrane of a pad in which different (macro)molecules are placed to give finally a visual output. The sample flows by capillary forces, moving along the analyte through the various zones of the strip. The test strip we have developed consists of two different zones; zone A (in which *IDA* probe **1** is deposited) and zone B, which is an area of the strip through which the solvent front travels and in which the signal of the indicator is measured. The presence of GHB is expected to increase the concentration of free

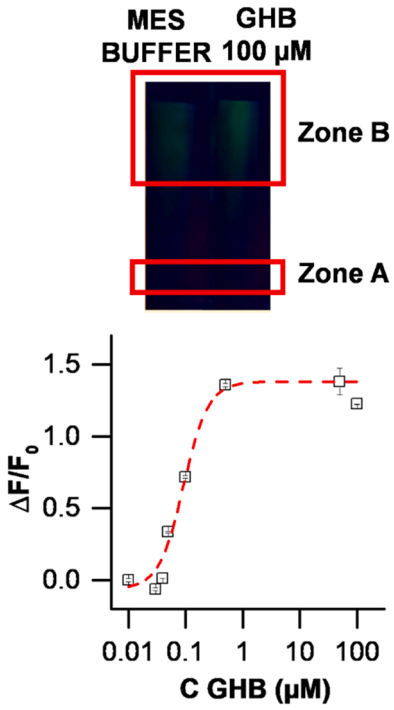


Fig. 3. Top: Smartphone readout for the determination of the fluorescence of C343 displaced from *IDA* probe **1** in the presence and absence of GHB. Bottom: Increase in fluorescence intensity of C343 displaced from *IDA* probe **1** measured in zone B in the lateral flow assay in the presence of different amounts of GHB in MES buffer (50 mM) at pH 6.0 after 1 min of dipping. Error bars are expressed as 3σ for three independent experiments.

C343 in zone B (Scheme 1). After LFA development, the amount of C343 released can be detected with a digital camera of a mobile communication device with the necessary optical accessories [36,37].

Considering this design, we prepared the strips and carried out an optimization in which different materials were examined as membranes. The materials tested were glass fiber (**GF**), filter paper (**PF**) and fusion 5 (**F5**). Moreover, these materials were further modified with a mixture of TEOS (**S**) and/or 3-(methoxy (polyethylene-oxy)propyl)trimethoxy silane (**PEG**), resulting in the materials **PEG-GF**, **PEG-PF**, **PEG-F5**, **S-PEG-GF**, **S-PEG-PF** and **S-PEG-F5**. Then, a spot of 5 μL of a methanolic aqueous solution (4:1 v/v) of **1** was deposited on the different materials in zone A and left to dry for 5 min. For blank controls, the strips were

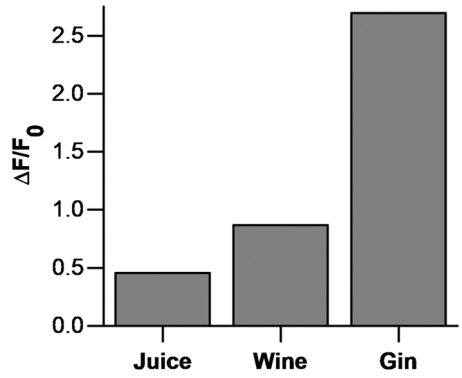


Fig. 4. Increase in fluorescence intensity registered with the smartphone/holder setup associated with C343 displaced from **1** in zone B of lateral-flow strips after 1 min of development with spiked and non-spiked soft and alcoholic beverages two-fold diluted in MES buffer. ($\Delta F/F_0 = (F_i\text{-}F_0)/F_0$).

dipped into a MES buffer solution of pH 6.0. Simultaneously, for GHB detection, a second series of strips was dipped into a MES buffered solution of GHB (1 mM). After 60 s of development, the test strips were placed in a holder and the fluorescence was measured with a smartphone camera equipped with a 3D-printed case containing an LED with an excitation wavelength of 465 nm and a long-pass filter to collect the fluorescence emission above 500 nm [14]. Images were taken with the smartphone camera under proper light conditions (ISO 400, exposure time of 0.5 s). The intensity of fluorescence of the photographs of the strips was analyzed, extracting the integrated density of fluorescence of the zone B with the software ImageJ [37]. The measured fluorescence of C343 in zone B on the different membranes employed and, in the absence, and presence of GHB is shown in Fig. S2. The highest blank/-sample difference in emission was observed with the **PEG-GF** strip, which was selected for all further assays. Accordingly, this membrane was further characterized by SEM, TGA and elemental analysis, revealing insights about the morphology and the functionalization degree with PEG (Fig. S4, S5). A content of 88 ± 0.015 μmol g^{-1} SiO_2 was obtained for the **PEG-GF** membranes. For the **PEG-GF** strip, a low fluorescence of C343 was recorded in zone B in the absence of GHB, while a remarkable enhancement in the emission was detected in the presence of GHB (Fig. 3). Based on the procedure detailed above, the response of the LFA as a function of the concentration of GHB in MES buffer was studied. For this purpose, **PEG-GF** membranes (2.5 × 0.5 cm in size) containing **1** were dipped into different solutions with increasing

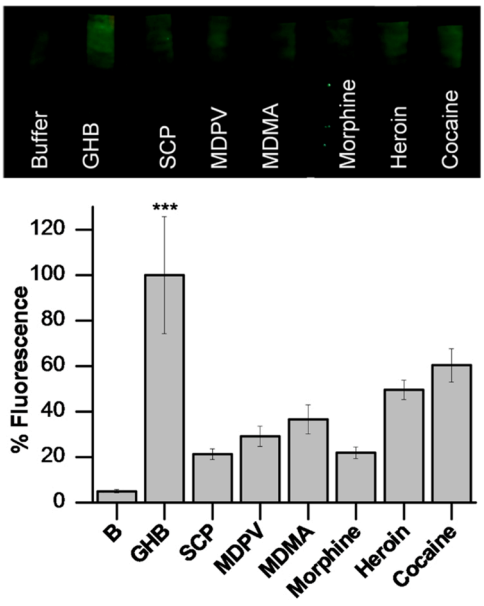


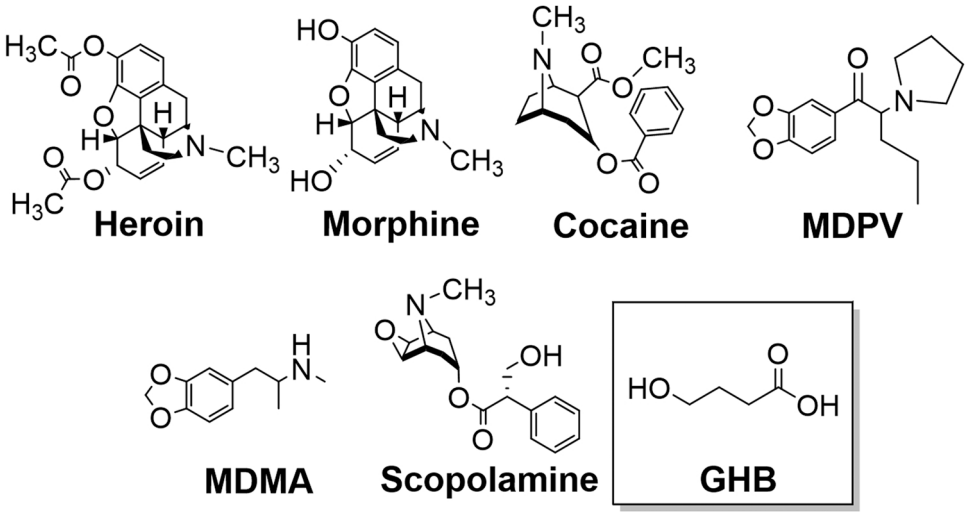
Fig. 5. Top: Collage of the photographs registered with the smartphone showing the C343 released in zone B from *IDA* probe **1**, shot under proper light excitation. For better eye visualisation, contrast of the original picture has been increased by 40%. Analysis has been performed with raw images. Bottom: Fluorescence response of **1** in the presence of the indicated drugs (500 μM) measured *via* the displacement of C343 from **1** in zone B of the test strip after dipping into diluted gin with TRIS HCl buffer (50 mM, pH 8.0) and 1 min of development. Error bars are expressed as 3σ for three independent experiments (***$p < 0.0003$).

concentrations of GHB (from 0.01 μM to 100 μM).

As can be seen in Fig. 3 when the GHB concentration increases an enhancement of the fluorescence is observed. From the titration profile an LOD of 0.03 μM for GHB was determined (Fig. 3). This limit of detection is better than those reported for other chromo-fluorogenic sensors used for GHB detection, which are in the millimolar range for aqueous or in the micromolar range for DMSO solutions.

We also tested *IDA* probe **1** immersed into the test strips for the detection of GHB in soft drinks and alcoholic beverages (such as orange juice, wine, and gin). For these experiments, we used commercial beverages diluted two-fold with MES buffer that had been either spiked or not with GHB (1 mM).

Overall, the experiments demonstrate the ability of *IDA* probe **1** to



Scheme 2. Chemical structures of the drugs used as interferents for the selectivity studies.

identify GHB in alcoholic and soft drinks by using a simple, portable, and rapid (1 min) assay without the need of trained personnel (Fig. 4). Besides, the limit of detection of GHB in gin using complex **1** was as low as 0.1 μM (Fig. S3). The limit of detection values obtained in gin is slightly higher than that measured in MES buffer solution (*vide ante*), most probably due to the presence of a higher competitive environment. To demonstrate the selectivity of the system, the fluorogenic response of *IDA* probe **1** immersed in the test strips was evaluated in the presence of other common drugs, such as cocaine, heroin, MDPV, scopolamine (SCP), MDMA, and morphine (Scheme 2). Fig. 5 shows the relative emission of C343 in zone B of the test strips, after dipping into two-fold diluted gin spiked with the selected drugs (500 μM) and 1 min LFA development.

4. Conclusions

Herein we report the development of a lateral flow assay based on the *IDA* probe **1** for the rapid and highly sensitive detection of GHB in soft drinks and alcoholic beverages with a smartphone setup for fluorescence readout. The recognition system consists of an *IDA* probe formed by coumarin 343 coordinatively bound to a Cu^{2+} complex. The response mechanism relies on a displacement of C343 from the ensemble as a consequence of the higher binding constant between GHB and *IDA* probe **1**. The probe shows a high sensitivity in MES buffer (detection limit of 0.03 μM). System design and optimization led to straightforward integration into a lateral-flow assay for GHB detection without further treatment or conditioning of the test strips while guaranteeing fast overall assay times of 1 min. Finally, we demonstrated the remarkable robustness of the probe that is able to detect GHB spiked in soft and alcoholic drinks. The lateral flow assay approach using mobile phones for fluorescence measurements offers a promising methodology for the construction of rapid test kits for practical applications such as roadside drug testing or the detection of substances in the workplace or recreational settings.

CRediT authorship contribution statement

Eva Garrido: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Guillermo Hernández-Sigüenza:** Methodology, Validation, Investigation. **Estela Climent:** Methodology, Validation, Investigation, Writing – review & editing, Supervision. **M. Dolores Marcos:** Writing – review & editing, Supervision. **Knut Rurack:** Investigation, Writing – review & editing, Visualization, Supervision. **Pablo Gaviña:** Writing – review & editing. **Margarita Parra:** Writing – review & editing. **Félix Sancenón:** Writing – review & editing, Visualization, Supervision. **Vicente Martí-Centelles:** Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Ramón Martínez-Máñez:** Conceptualization, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All data are included in the published article and its supplementary material.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.133043.

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