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Supporting Information (SI)

SELECTIVE DUALPLEX LATERAL FLOW ASSAY FOR SIMULTANEOUS SCOPOLAMINE AND "CANNIBAL DRUG" DETECTION BASED ON RECEPTOR-GATED MESOPOROUS NANOPARTICLES

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1. EXPERIMENTAL PROCEDURES

1.1. Materials and methods

General techniques: Powder X-ray diffraction (PXRD), thermogravimetric analysis (TGA), elemental analysis, transmission electron microscopy (TEM), Fourier transform infrared (FTIR), dynamic light scattering (DLS) and N_2 adsorption-desorption isotherms were employed to characterize the synthesized materials. PXRD measurements were taken on a D8 Advance diffractometer using Cu Kα radiation (Bruker, Billerica, MA, USA). Thermogravimetric analyses were carried out on a TGA/SDTA 851e balance (Mettler Toledo, Columbus, OH, USA) in an oxidizing atmosphere (air, 80 mL min^{-1}) with a heating rate program between 393-1273 °C at 10 °C min^{-1}, followed by an isothermal heating step at 1273 °C for 30 min. TEM images were taken with a 100 kV CM10 microscope (Philips, Amsterdam, NL). FTIR measurements were taken on a Tensor 27 (Bruker, Billerica, MA, USA). DLS experiments were performed using a ZetaSizer Nano ZS (Malvern). N_2 adsorption-desorption isotherms were recorded with a Tristar II Plus automated analyser (Micromeritics, Norcross, GA, USA). The samples were degassed at 90 °C or 120 °C under vacuum overnight. Specific surface areas were calculated from the adsorption data within the low-pressure range using the Brunauer-Emmett-Teller (BET) model. Pore size was determined following the Barrett-Joyner-Halenda (BJH) method. Fluorescence spectroscopy measurements were taken on a Fluoromax4 from HORIBA Scientific. A ColorQube8580 (Xerox) wax printer was employed for the generating channel features on the test strips. For the

smartphone-based approach, a 3D-box was printed with black PLA using an Ultimaker 3 printer. LEDs and optical filters were purchased from Thorlabs. Photographs were taken with a Samsung Galaxy S7 and values retrieved from images via the integrated density with the software ImageJ, i.e., the product of mean grey value $\bar{G}$, $\bar{G}$ = (red + green + blue)/3, and the selected area (in square pixels).

Solvents: All solvents were ACS reagent grade or better quality and were used without any further purification. Ethanol and 1,4-dioxane were purchased from Scharlab S.L.

Chemical reagents: Tetraethyl orthosilicate (TEOS), *n*-cetyltrimethylammonium bromide (CTABr), sodium hydroxide, (3-aminopropyl) triethoxysilane, succinic anhydride, benzoic anhydride, zinc chloride ($ZnCl_2$), carbamyl-β-methylcholine chloride (bethanechol chloride), rhodamine B, tris(hydroxymethyl)aminomethane (TRIS), 3,4-methylenedioxypyrovalerone hydrochloride (MDPV) and scopolamine hydrobromide (SCP) were purchased from Sigma Aldrich (Madrid, Spain). Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) and trifluoroacetic acid (TFA) was purchased from Acros Organics. Recombinant human muscarinic acetylcholine receptor M_2-AChR protein was purchased from Abcam PLC. Glass fibre strips (C grade) were obtained from Whatman™ and the adsorbent pad was obtained from Ahlstrom. Other drugs tested, i.e., morphine, cocaine, heroin and MDMA were kindly provided by "Agencia Española de Medicamentos y Productos Sanitarios" (AEMPS).

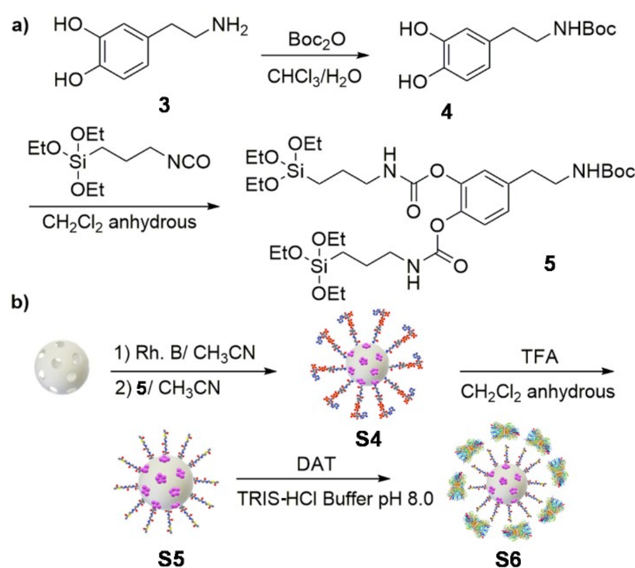
1.2. Synthesis of nanomaterial for MDPV detection.

Synthesis of 4-(2-((tert-butoxycarbonyl)amino)ethyl)-1,2-phenylene bis((3-(triethoxysilyl)propyl)carbamate) (5): Precursor **5** was prepared using a procedure reported by us previously.[1]

Synthesis of S4:[1] In a typical synthesis, a mixture of calcined mesoporous silica nanoparticles (100 mg) and rhodamine B (38.3 mg, 0.08 mmol) were suspended in anhydrous CH_3CN (5 mL) and purged with Argon. The suspension was stirred at room temperature for 24 h to load the mesopores. Then, compound **5** (191.5 mg, 0.26 mmol) was added, and the final suspension was stirred at room temperature for 5.5 h. Finally, the resulting pink solid was filtered and then dried under vacuum overnight, giving rise to **S4** (195 mg).

Synthesis of S5:[1] Solid **S4** (10 mg) was suspended in anhydrous CH_2Cl_2 (2 mL) and purged with Argon. The suspension was then cooled to 0 °C, and TFA (0.1 mL, 1.31 mmol) was added for *N*-Boc deprotection. The mixture was stirred at room temperature for 10 min, and the solid was isolated by centrifugation, washed with CH_2Cl_2 (2 × 8 mL), and dried overnight at 37 °C.

Synthesis of S6:[1] Solid **S5** (1 mg) was suspended in Tris-HCl buffer (300 µL, 20 mM of Tris-HCl and $MgCl_2 \cdot 6H_2O$, pH 8.0), and then the recombinant human transporter dopamine protein (150 µL, 0.017 µg/µL) was added. The mixture was stirred in a thermo-shaker at 4 °C overnight. Then, the suspension was centrifuged at 12,000 rpm for 5 min. **S6** was then washed with Tris-HCl buffer (300 µL) to eliminate the non-encapsulated dye and the unattached protein.



Scheme S1. a) Synthetic route followed for the preparation of the precursor **5**. b) Synthetic route for the synthesis of solid **S6** in the presence of dopamine active transporter (DAT).

Synthesis of MCM-41 (Mobile Composition of Matter No. 41) mesoporous nanoparticles:[2] NaOH (2.00 mol L^{-1}, 3.5 mL) was added to a solution of *n*-cetyltrimethylammonium bromide (CTABr; 1.00 g, 2.74 mmol) in deionized H_2O (480 mL) at 40 °C. The solution temperature was adjusted to 80 °C and then tetraethyl orthosilicate (TEOS; 5.00 mL, 2.57×10^{-2} mol) was added dropwise. The mixture was stirred for 2 h to give a white precipitate. The solid was isolated by centrifugation and washed with deionized H_2O, and then dried at 70 °C for 12 h to give MCM-41. To remove the template phase, MCM-41 was calcined at 550 °C for 5 h in an oxidizing atmosphere.

Synthesis of 4-oxo-4-((3-(triethoxysilyl)propyl)amino)butanoic acid (2):[3] Succinic anhydride (**1**, 1.5 g, 15 mmol) was dissolved in 1,4-dioxane (10 mL) before (3-aminopropyl) triethoxysilane (3.3 g, 15 mmol) in 1,4-dioxane (10 mL), was added dropwise. The mixture was heated at 80 °C for 30 min. Afterwards, the solvent was removed under reduced pressure to obtain product **2** as a pale-yellow oil (4.82 g, 15 mmol).

Synthesis of S1: In a typical synthesis, a mixture of calcined MCM-41 (100 mg) and rhodamine B (38.32 mg, 0.8 mmol) were suspended in ethanol (3.5 mL). The suspension was stirred at room temperature for 24 h to load the MCM-41 pores. Then, compound **2** (1.52 g, 4.73 mmol) was added and the final suspension was stirred at room temperature for 5.5 h. Finally, the resulting pink solid was filtered and dried under vacuum overnight, giving rise to **S1** (164 mg).

Synthesis of S2:[4] **S1** (164 mg) was suspended in ethanol (40 mL). Benzoic anhydride (1.07 g, 4.73 mmol) and $ZnCl_2$ (26.86 mg, 4.73 mmol) were successively added to the solution. The mixture was stirred at room temperature for 10 min before bethanechol chloride (930 mg, 4.73 mmol) was added. The reaction was left at room temperature for 2.5 h. Afterwards, the solid was isolated by centrifugation (12,000 rpm for 5 min). The final solid was dried overnight at 37 °C. The proper functionalization of solid **S2** was assured by FTIR measurements, elemental and thermogravimetric analysis (Figure S3-S4).

Synthesis of S3: Solid **S2** (1 mg) was suspended in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl buffer; 300 µL, 20 mM of Tris-HCl and $MgCl_2 \cdot 6H_2O$, pH 8.0) before the recombinant human muscarinic acetylcholine receptor protein, M_2-AChR, (71.43 µL, 0.07 µg/µL) was added. The mixture was stirred in a thermo-shaker at 4 °C overnight. After that, the suspension was centrifuged at 12,000 rpm for 5 min. **S3** was then washed with Tris-HCl buffer to eliminate non-encapsulated dye and unattached protein. The proper recognition between the receptor and the bethanechol derivative was estimated by means of a BCA protein assay.

Release experiments of solid S2 in the presence of SCP: To check the crucial role played by the muscarinic acetylcholine receptor M_2-AChR, absent in **S2** but present in **S3**, 1 mg of solid **S2** was suspended in 800 µL of Tris-HCl buffer and this volume was divided into two aliquots of 400 µL. Both samples were centrifuged for 5 min at 12,000 rpm and the fluorescence (λ_{exc} = 565 nm, λ_{em} = 572 nm) of the supernatant (150 µL) was measured to obtain the initial point. This volume was returned to the corresponding aliquot. After that, 54 µL of a 20 mM solution of SCP in Tris-HCl buffer were added to one of the aliquots (final concentration of 2.7 mM), and 54 µL of Tris-HCl buffer were added to the second aliquot, constituting the blank control (termed 'blank aliquot' in the following). Both suspensions were stirred at 25 °C for a certain time interval before 150 µL of supernatant was taken from both suspensions, treated, and measured as above. This procedure was repeated until it allowed to construct the release kinetics of the uncapped material in both, absence and presence of the analyte (Figure S5).

Release experiments of solid S3 in the presence of SCP: The same procedure as described for **S2** was used for studying the release kinetics of the capped solid **S3** (Figure 2a in the manuscript).

Concentration-dependence studies of S3 with SCP in Tris-HCL buffer: 1 mg of **S3** was suspended in 2.4 mL of Tris-HCl buffer and then divided into twelve aliquots of 200 µL. All samples were centrifuged at 12,000 rpm for 5 min and the fluorescence (λ_{exc} = 565 nm, λ_{em} = 572 nm) of the supernatant (150 µL) was measured to obtain the initial point. This volume was returned to the corresponding aliquot. Meanwhile, several solutions of SCP in Tris-HCl buffer within a concentration range of 0.052 to 70 mM were prepared. Then, 9 µL of each SCP solution were added to every aliquot, yielding final concentrations of 2.24 to 3150 µM. Simultaneously, 9 µL of Tris-HCl buffer were added to the blank aliquot. All suspensions were stirred at 25 ^{o}C for 10 min. After that, all aliquots were centrifuged, and the fluorescence of the rhodamine B released in the supernatant was measured (Figure 2b in the manuscript).

Determination of limit of detection (LOD): Limits of detection (LODs), which describe the smallest concentration of analyte that can be reliably detected, were derived calculating first the limits of blank (LOBs; describing the smallest signal that can be observed with the detection method employed) and then the corresponding minimum concentration to be detected taking into account the measurement uncertainties of the low concentration samples as follows:[5]

$LOB = \text{mean blank} + 1.645(\sigma_{blank})$

$LOD = LOB + 1.645(\sigma_{\text{low concentration sample}})$

Selectivity studies with S3: To test the selectivity of **S3** towards SCP, 1 mg of solid **S3** was suspended in 1.4 mL of Tris-HCl Buffer and this volume was separated into seven aliquots of 200 µL. Beforehand, several solutions of different drugs (MDMA, morphine, heroin, cocaine, MDPV, and SCP) at a concentration of 8 mM were prepared in Tris-HCl buffer. Sample handling and measurement were then carried out as described for the SCP titrations above, only that a single concentration of every drug was probed (345 µM) (Figure 3 in the manuscript).

Release experiments of solid S3 in the presence of SCP in saliva: 1 mg of **S3** was suspended in 400 µL of 30 % extracted saliva and this volume was divided into two aliquots of 200 µL. Both samples were centrifuged for 5 min at 12,000 rpm and the fluorescence (λ_{exc} = 565 nm, λ_{em} = 572 nm) of the supernatant (150 µL) was measured to obtain the initial point. This volume was returned to the corresponding aliquot. After that, 54 µL of a 20 mM solution of SCP in Tris-HCl buffer were added to one aliquot (final concentration of 2.7 mM), and simultaneously, 54 µL of

Tris-HCl buffer were added to the blank aliquot. Both suspensions were stirred at 25 ^oC and release kinetics were recorded as detailed above (Figure S6).

Concentration-dependence studies of S3 with SCP in saliva: 1 mg of **S3** was suspended in 2.4 mL of 30% extracted saliva and divided into twelve aliquots of 200 µL each one. The experimental procedure followed was the same as that described for Tris-HCl buffer (Figure S7).

Preparation of wax-patterned glass fibre paper (WAX-GF). First, a wax pattern of approximately 13 x 13 cm presenting wax free channels of 3 mm diameter was printed on an aluminium foil using a wax printer. In a second step, the wax pattern was transferred to glass fibre (GF) strips by lamination. Finally, the strips were cured at 110 °C for 30 min. This step melts the wax and creates hydrophobic barriers across the thickness of the paper. The as-prepared **WAX-GF** were cut in individual 0.5 x 4 cm strips and were stored at room temperature until the next step.

Concentration-dependence studies of S3-WAX-GF strips with SCP in Tris-HCl buffer: WAX-GF strips of 0.5 x 2.5 cm containing an adsorbent pad of 0.5 x 1 cm at the top of the strip were prepared, 2 µL of a suspension (1 mg/mL) of **S3** were deposited at ca. 1 cm from the bottom of the strip (zone A), yielding **S3-WAX-GF**. **S3-WAX-GF** strips were dipped into 75 µL of a Tris-HCl solution at pH 8.0 containing different concentrations of SCP from 8.6 µM to 9.99 mM in a microwell plate for 10 min. Afterwards, the strips were dried for 2 min at room temperature and the fluorescence of the released dye was measured with a smartphone setup using an LED emitting at 522 nm as excitation source powered by the smartphone via a USB-OTG link and filtered by a short-pass filter (532 nm) while collecting the emission through a long-pass filter (550 nm) after inserting the developed strip in a 3D-printed, customized holder previously described.[6] The amount of dye released for each concentration was calculated according to the fluorescence intensity of zone B using the program ImageJ (Figure S8).[7]

Concentration-dependence studies of S3-WAX-GF strips with SCP in saliva: WAX-GF strips of 0.5 × 2.5 cm with an absorbent pad were prepared, and 2 µL of a suspension (1 mg/mL) of solid **S3** in 30% extracted saliva were deposited at ca. 1 cm from the bottom of the strip (zone A). The experimental procedure for LFA development followed the one described above (Figure 4a in the manuscript).

Selectivity studies with S3-WAX-GF strips: S3-WAX-GF strips of 0.5 × 2.5 cm were prepared as in the previous experiment and dipped into 75 µL of diluted saliva (diluted to 30%) containing 200 µM of different drugs (MDMA, morphine, heroin, cocaine, MDPV and scopolamine) in a microwell plate for 10 min before drying the strips for 2 min at room temperature. Afterwards,

the strips' fluorescence was measured using the smartphone setup described above. The amount of dye released for each concentration was calculated from the images as above (Figure S9).

Concentration-dependence studies with S6-WAX-GF strips with MDPV in saliva: WAX-GF strips of 0.5 x 2.5 cm with an absorbent pad were prepared in a similar way to **S3-WAX-GF**. Then 2 µL of a suspension (1 mg/mL) of **S6** in 30% extracted saliva were deposited at ca. 1 cm from the bottom of the strip (zone A). Thereafter, the materials containing the nanosensor **S6** (**S6-WAX-GF**) were dipped into 75 µL of a 30% extracted saliva containing different concentrations of MDPV from 2.1 µM to 9.99 mM in a microwell plate for 10 min. After drying for 2 min at room temperature, the amount of released dye was measured with the smartphone setup and analysed with ImageJ (Figure 4b in the manuscript).

Selectivity studies with S6-WAX-GF strips: These experiments were carried out in analogy to those with **S3-WAX-GF** using the drugs MDMA, morphine, heroin, cocaine, MDPV and scopolamine (Figure S10).

Dualplex lateral flow assay for SCP and MDPV detection in saliva: On a wax-printed dual-channel strip,[8] 2 µL of a 1mg/mL suspension of solids **S3** and **S6** were deposited separately into the respective channels of the strip (**S6/S3-WAX-GF**). **S6/S3-WAX-GF** were immersed in a microplate with extracted saliva solutions (300 µL) containing different concentrations of both SCP and MDPV for 10 min. Then, the strips were left to dry for 5 min at room temperature, and the fluorescence of rhodamine released from each strip was measured and analysed as before (Figure 5 in the manuscript).

2. CHARACTERIZATION OF THE PREPARED MATERIALS

The MCM-41 scaffold and mesoporous solids **S1**, **S2** and **S3** were characterized following standard techniques, including transmission electron microscopy (TEM), powder X-ray diffraction (PXRD), N_2 adsorption/desorption analysis, dynamic light scattering (DLS) and FTIR. On the other hand, content of recombinant human muscarinic acetylcholine receptor M_2-AChR protein in solid **S3** was determined from BCA protein assay[9] whereas bethanechol derivative and rhodamine B on the nanoparticles were determined from thermogravimetric and elemental analysis.

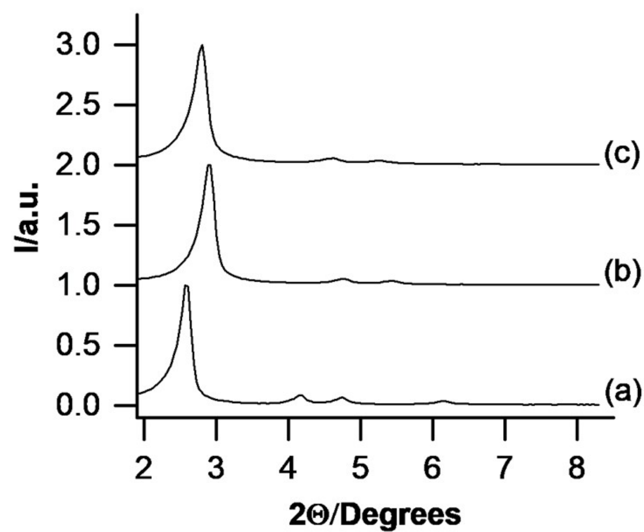


Figure S1. Powder X-ray diffraction (PXRD) patterns of solids (a) as-synthesized MCM-41, (b) calcined MSNs and (c) solid **S2**. Diffractograms are y-shifted for clarity.

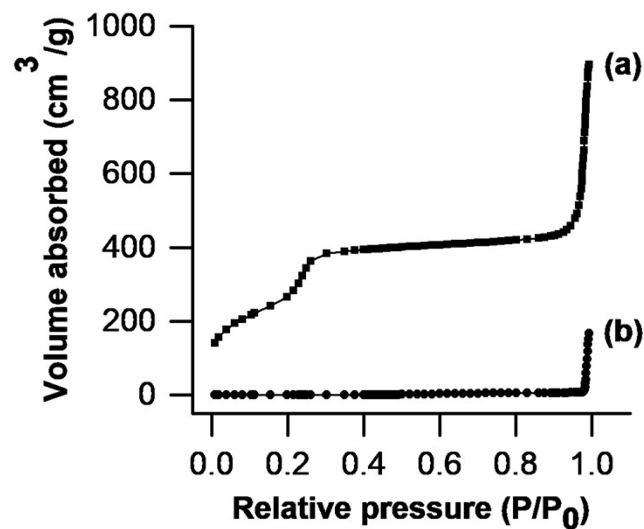


Figure S2. N_2 adsorption-desorption isotherms for (a) calcined MCM-41 nanoparticles and (b) **S2** material.

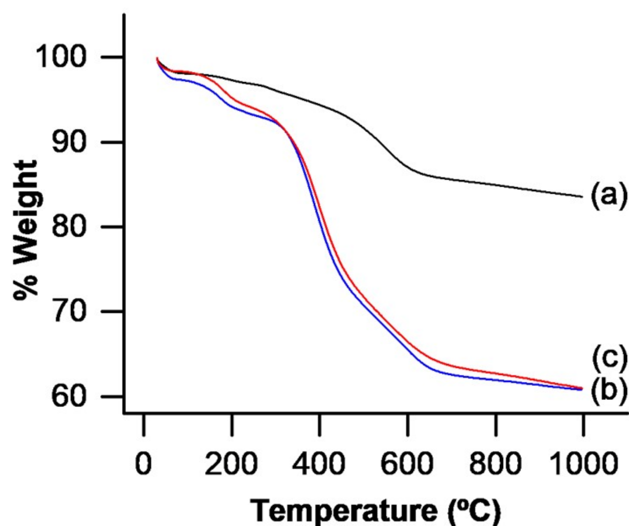


Figure S3. Thermogravimetric analysis for (a) calcined MCM-41 nanoparticles loaded with rhodamine B, (b) **S1** material and (c) **S2** material.

FTIR spectrum of functionalized solid **S1** shows typical absorption bands at ca. 1100, 1500 and 3400 cm^{-1} related to the bond stretching vibrations of Si-O-Si, C=O and of O-H and N-H groups, respectively. Moreover, the spectrum showed the stretching vibrations of C=O groups at 1711 cm^{-1}, which is present in FTIR spectrum of solid **S2** whereas the O-H band is reduced because of the reaction between **2** and bethanechol. Finally, solid **S3** displays a broad band of N-H, C=O and O-H groups at ca. 1629 and 3319 cm^{-1} ascribed to the vibration of peptide bonds and terminal NH_2 and COOH groups from M_2-AChR.

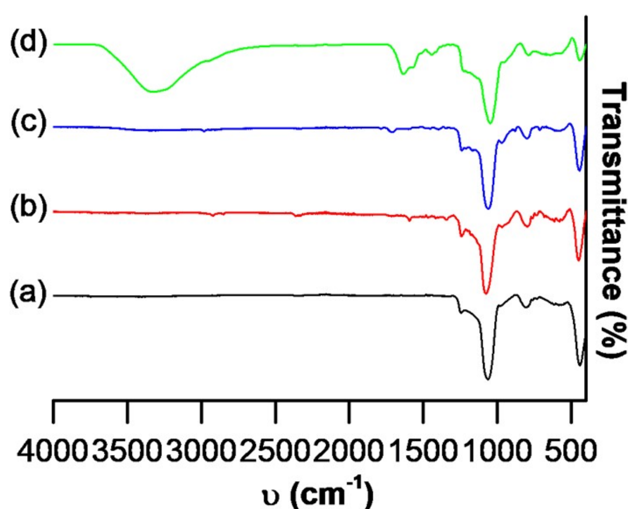


Figure S4. FTIR spectrum of (a) calcined MCM-41 nanoparticles, (b) **S1** nanoparticles, (c) **S2** solid and (d) **S3**. Spectra are y-shifted for clarity.

3. RESULTS AND DISCUSSION

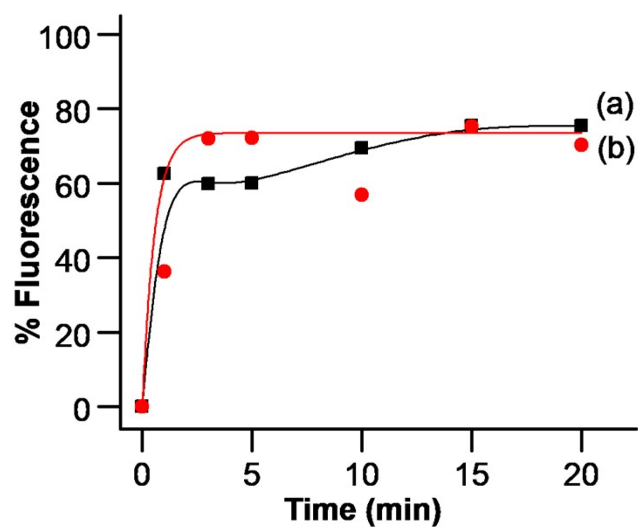


Figure S5. Release profiles of rhodamine B from Tris-HCl buffer suspensions of solid **S2** at pH 8.0 (a) in absence and (b) in the presence of SCP (2.7 mM).

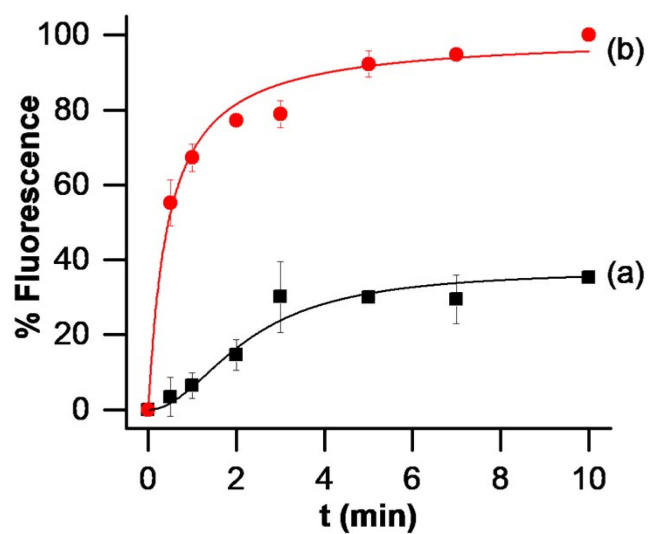


Figure S6. Release profiles of rhodamine B from 30% extracted saliva suspensions of solid **S3** (a) in the absence and (b) in the presence of SCP (2.7 mM). Error bars are expressed as 3σ.

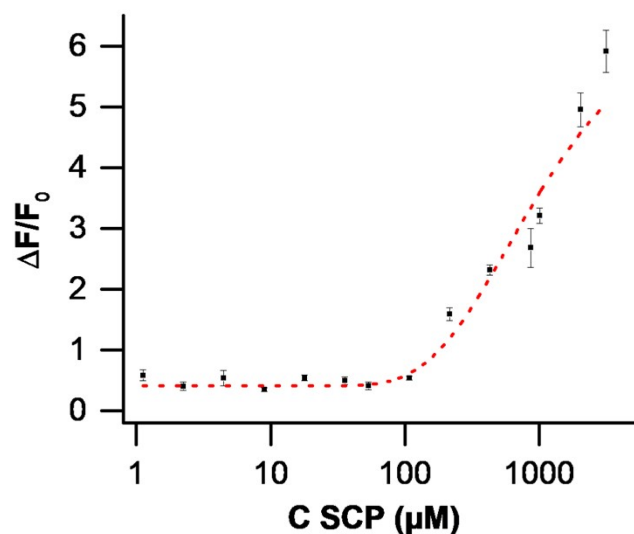


Figure S7. Release of rhodamine B from solid **S3** in the presence of different amounts of SCP in 30% extracted saliva after 10 min of addition. Error bars are expressed as 3σ.

3.1. Lateral flow tests with fluorescence read-out protocol

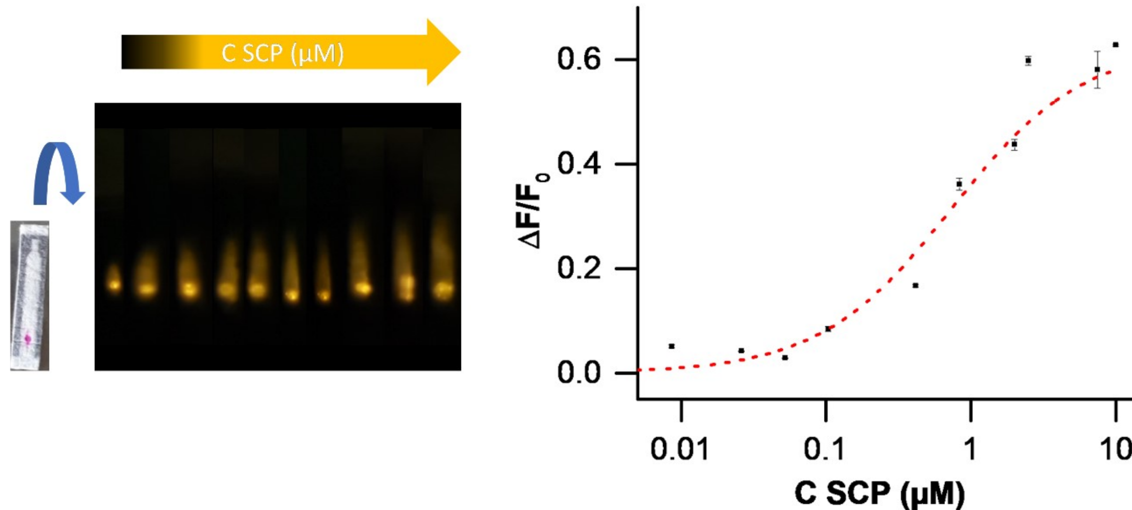


Figure S8. Left: Collage of the photographs registered with the smartphone showing the rhodamine B released in zone B from solid **S3**, shot under proper light excitation. Right: Release of rhodamine B from solid **S3** in the presence of different amounts of SCP in TRIS-HCl buffer at pH 8.0 after 10 min of dipping. Error bars are expressed as 3σ for three independent experiments.

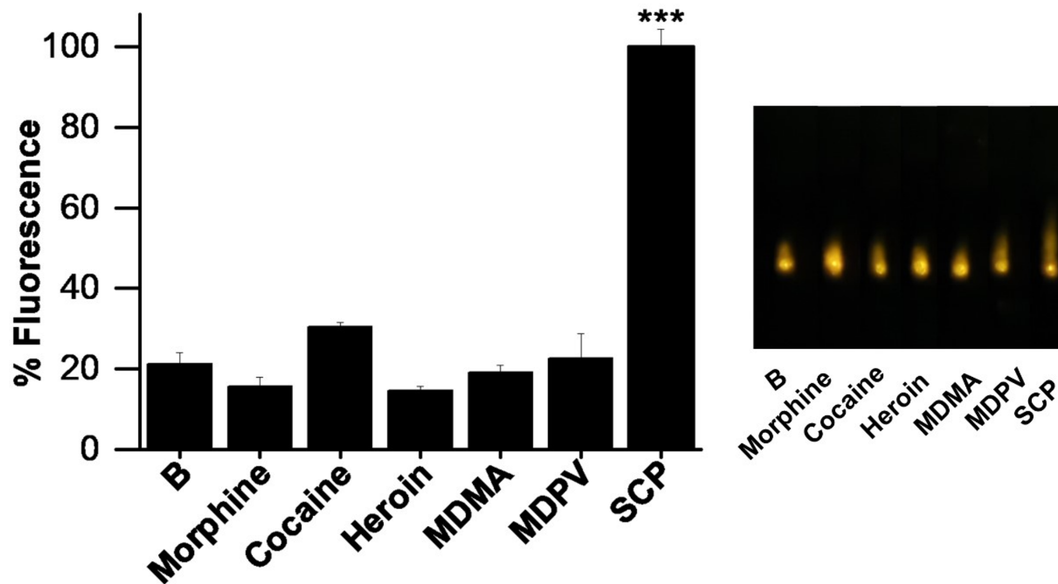


Figure S9. Effect of the indicated drugs (200 µM) on the relative rhodamine B release in **S3-WAX-GF** strips in 30% extracted saliva 10 min after dipping. Error bars are expressed as 3σ for three independent experiments.

3.2. Lateral flow assay for the detection of illicit drugs MDPV

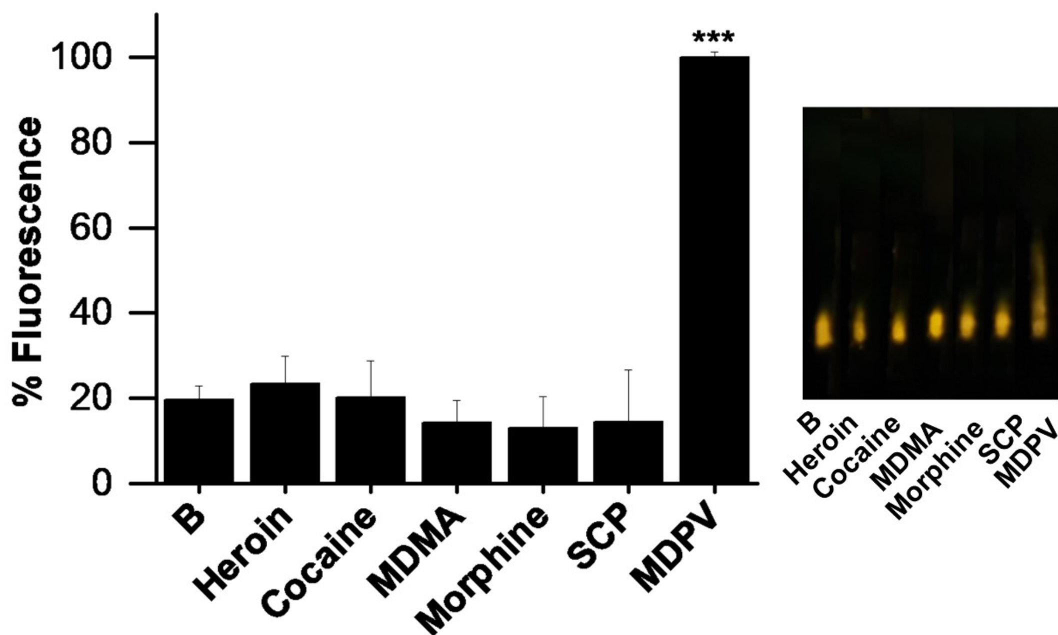


Figure S10. Effect of the indicated drugs (200 µM) on the relative rhodamine B release in **S6-WAX-GF** strips from solid **S6** in 30% extracted saliva 10 min after dipping. Error bars are expressed as 3σ for three independent experiments.

3.3. Duplex lateral flow assay for the detection of illicit drugs MDPV and SCP

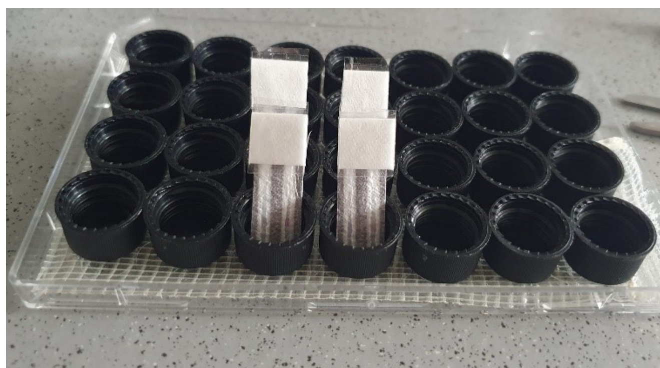


Figure S11. Elution process illustration of the dual-channel strip with **S6/S3-WAX-GF** membranes.

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