Multiplexed Detection of Analytes on Single Test Strips with Antibody-Gated Indicator-Releasing Mesoporous Nanoparticles

Estela Climent, Mustafa Biyikal, Delia Gröninger, Michael G. Weller, Ramón Martínez-Máñez, and Knut Rurack*

anie_202009000_sm_misellaneous_information.pdf
Table of Contents

TABLE OF CONTENTS .......................................................................................................................................................... 2
EXPERIMENTAL PROCEDURES ............................................................................................................................................. 3
1 GENERAL METHODS ........................................................................................................................................................ 3
2 SYNTHESIS OF HAPTENS AND HAPten DERIVATIVES ....................................................................................................... 3
3 POLYCLONAL ANTIBODIES FOR TATP, TNT AND PETN...................................................................................................... 4
4 SYNTHESIS AND CHEMICAL CHARACTERIZATION OF MATERIALS ..................................................................................... 5
4.1 SYNTHESIS OF MCM-41 MESOPOROUS SILICA NANOPIRICLE SUPPORT (MCM) ......................................................... 5
4.2 SYNTHESIS OF MI1H1 ..................................................................................................................................................... 5
4.3 SYNTHESIS OF MI2H2 ..................................................................................................................................................... 5
4.4 SYNTHESIS OF MI3H3 ..................................................................................................................................................... 5
4.5 SYNTHESIS OF MI1H2 AND MI1H3 ................................................................................................................................... 5
4.6 SYNTHESIS OF ANTIBODY-CAPPED MATERIALS MINHNAN .............................................................................................. 5
5 CHARACTERIZATION OF MATERIALS ................................................................................................................................ 5
RESULTS AND DISCUSSION ................................................................................................................................................. 9
6 CONTROL EXPERIMENTS WITH MI1H2A2........................................................................................................................... 9
7 RAW SPECTRA OF RESPONSE TO ANALYTES ..................................................................................................................... 9
8 DETECTION OF EXPLOSIVES IN TURBID MEDIA: MILK AS AN EXAMPLE ............................................................................ 9
9 CROSS-REACTIVITIES ...................................................................................................................................................... 10
10 LATERAL FLOW TESTS WITH FLUORESCENCE READ-OUT PROTOCOL ............................................................................ 11
11 MULTIPLEXING DETECTION OF EXPLOSIVES TATP, TNT AND PETN.................................................................................. 12
11.1 SMARTPHONE SETUP AS READOUT DEVICE WITH A SINGLE STRIP ............................................................................ 12
11.2 FLUORESCENCE READER AS READOUT DEVICE WITH A SINGLE STRIP ....................................................................... 12
11.3 SMARTPHONE SETUP AS READOUT DEVICE WITH PATTERNED STRIPS ..................................................................... 13
12 ADDITIONAL NOTES ON ANALYTICAL PERFORMANCE ................................................................................................. 15
12.1 LIMITS OF DETECTION ............................................................................................................................................... 15
12.2 REPEATABILITY ......................................................................................................................................................... 16
12.3 STABILITY .................................................................................................................................................................. 16
12.4 CUT-OFF VALUES ....................................................................................................................................................... 16
12.5 PREVIOUSLY REPORTED IMMUNOASSAYS FOR EXPLOSIVES DETECTION .................................................................. 16
REFERENCES ..................................................................................................................................................................... 18
AUTHOR CONTRIBUTIONS................................................................................................................................................ 18
SUPPORTING INFORMATION

**Experimental Procedures**

1 General methods

Chemicals and solvents were purchased from Sigma-Aldrich, ACBR, Merck and J.T. Baker. Buffers were prepared with ultrapure reagent water, which was obtained by running demineralized water (by ion exchange) through a Milli-Q® ultrapure water purification system (Millipore Synthesis A10). Phosphate-buffered saline (PBS 10X: 70 mmol dm⁻³ Na₂HPO₄, 10 mmol dm⁻³ NaH₂PO₄, 145 mmol dm⁻³ NaCl, pH 7.6), PBS containing 0.005% BSA (w/v), 0.05% Tween-20 (v/v) and 200 ppm of the corresponding dye were used for capping processes. Controlled release experiments were performed using a solution of PBS 10X containing 0.05% Tween-20 (v/v) and 2.5% EtOH. The common explosives PETN, TNT, RDX and HMX were obtained from BAM’s Testing and Evaluation of Explosives and Pyrotechnics Division, and nitroglycerin (containing ca. 25% water) were purchased from Sigma Aldrich.

Absorption and fluorescence spectroscopy, elemental analysis, thermogravimetric analysis (TGA), transmission electron microscopy (TEM), N₂ adsorption-desorption, dynamic light scattering (DLS), mass spectrometry and NMR techniques were employed to characterize the synthesized materials and test their behaviour towards the corresponding analytes. UV-vis spectra were measured with a Specord 210plus from Analytik Jena. Fluorescence measurements were carried out with a Fluoromax4 from HORIBA Scientific. Thermogravimetric analyses were conducted on a STA7200 (Hitachi High-Tech Analytical Science) thermobalance, using in a first step a nitrogen atmosphere (80 mL min⁻¹) with a heating program consisting of a ramp of 10 °C min⁻¹ from 25 °C to 600 °C and in a second step an oxidizing atmosphere (air, 80 mL min⁻¹) from 600 °C until 1000 °C with a heating program consisting of a ramp of 10 °C min⁻¹. Elemental analyses were carried out with a Euro EA-Elementaranalysator. TEM images were obtained with a Tecnai G 2 20 Twin Transmission Electron Microscope, FEI Company. N₂ adsorption-desorption isotherms were recorded with a Micromeritics ASAP2010 automated sorption analyzer. The samples were degassed for 3h at 200 °C in a vacuum. The specific surface areas were calculated from the adsorption data in the low-pressure range using the Brunauer-Emmett-Teller (BET) method. Parentheses were determined following the Barrett-Joyner-Halenda (BJH) method. DLS studies were conducted using a Malvern Zetasizer Nano ZS. Mass spectra were measured with a CT Premier XE-TOF mass spectrometer. ¹H and ¹³C NMR spectra were acquired with Bruker AV-500 and AV-600 spectrometers, using residual protomated solvents as internal standards [¹H: δ(CDCl₃) = 7.24 ppm and ¹³C: δ(CDCl₃) = 77.23 ppm]. Fluorescence measurements on strips were recorded with an ESE-Quant FL and a universal strip holder from Qiagen. 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 G, G = (red + green + blue)/3, and the selected area a (in square pixels).

2 Synthesis of haptenes and hapten derivatives

TATP, TNT and PETN hapten derivatives H₁, H₂ and H₃ employed to prepare the corresponding sensing materials were prepared through an amidation reaction of the corresponding haptenes I, II or III with 3-aminopropyltriethoxysilane (APTES) using a modification of the active ester method (see Scheme S1).

Haptens I, II and III were prepared following previously described procedures. For the hapten derivatives, three different solutions, each containing N-hydroxysuccinimide (NHS, 18.89 mg, 0.16 mmol) and N,N-dicyclohexylcarbodiimide (DCC, 33.86 mg, 0.16 mmol) dissolved in anhydrous THF (0.75 mL), were prepared. Then, the individual solutions were added to a solution of hapten I, II or III (0.14 mmol) in anhydrous THF (0.25 mL). The mixtures were stirred at room temperature for 5 h, and the white solids formed (dicyclohexylurea) were removed from each fraction by centrifugation. In a second step, three portions of APTES (33.53 µL, 0.14 mmol) were added to each solution and the reaction mixtures were stirred for 24 h at room temperature. The remaining dicyclohexylurea of the different reactions was removed by centrifugation and the solvent was evaporated from each fraction under reduced pressure to give yellow sticky oils H₁, H₂ and H₃ (0.12 mmol, 0.13 mmol and 0.12 mmol; yields 85.7 %, 92.8% and 85.7%), respectively.

TATP-based H₁: [¹H NMR (CDCl₃): δ (ppm) = 0.63 (t, 2 H, -CH₂-Si-), 0.96 (q, 6H, CH₂CH₂C-)] 1.22 (t, 9 H, SiO-CH₂-CH₂) 1.22 (m, 2H, -NH-CH₂-CH₂-Si), 1.32 (s, 9H, CH₃C-) 1.58 (m, 8H, CH₂-CH₂-CH₂-CH₂-CH₂CO-NH-), 1.84 (m, 4 H, CH₂CH₂C-), 2.16 (m, 2 H, -CH₂-CH₂-CH₂-CH₂-CH₂CO-NH-), 2.34 (t, 2H, -NH-CH₂-CH₂-Si), 3.81 (s, 6H, CH₂-CH₂-O-Si). [¹³C NMR (CDCl₃): 7.7, 18.3, 18.6, 21.3, 22.8, 23.7, 24.9, 25.3, 25.6, 29.4, 33.8, 36.8, 41.8, 58.5, 107.4, 109.3, 173.1. Exact mass (M+H) 554.336; found 554.326.]

TNT-based H₂: [¹H NMR (CDCl₃): δ (ppm) = 0.65 (t, 2 H, -CH₂-Si-(O-CH₂-CH₂)₃), 1.22 (t, 9 H, -CH₂-Si-(O-CH₂-CH₂)₃), 1.62 (m, 6H, -CH₂-CH₂-CH₂-CH₂CO=NH-CH₂-CH₂), 2.50 (t, 2H, -NH-CH₂-CH₂CO=NH-), 2.61 (m, 2H, -CH₂-CH₂-CH₂-CH₂CO=NH-), 3.13 (m, 4H, Si-(O-CH₂-CH₂C-)), 3.82 (q, 6H, Si-(O-CH₂-CH₂C-), 7.57 (d, 1H, CH₃(NO₂)=), 8.35 (d, 1H, CH₃(NO₂)=), 8.73 (s, 1H, CH₃(NO₂)=). [¹³C NMR (CDCl₃): 7.7, 18.3, 22.8, 25.4, 25.6, 28.3, 32.8, 36.4, 41.8, 58.4, 120.2, 124.2, 126.8, 133.2, 144.5, 169.1. Exact mass (M+H) 486.2193; found 486.2278.]

PETN-based H₃: [¹H-NMR (CDCl₃) δ (ppm) = 0.63 (t, 2 H, -CH₂-Si-(O-CH₂-CH₂)₃), 1.22 (t, 9 H, -CH₂-Si-(O-CH₂-CH₂)₃), 1.30 (m, 2H, -CO-O-CH₂-CH₂-CH₂-CH₂-CH₂CO-NH-), 1.60 (m, 4H, -CO-O-CH₂-CH₂-CH₂-CH₂-CH₂CO-NH-), 1.90 (m, 2H, -NH-CH₂-CH₂-Si), 2.16 (t, 2H, -CO-O-CH₂-CH₂-CH₂-CH₂-CH₂CO-NH-), 3.24 (t, 2H, -NH-CH₂-CH₂-Si), 3.81 (q, 6H, Si-(O-CH₂-CH₂C-), 4.09 (t, 2H, -CH₂CO-NH-), 7.57 (d, 1H, -CO-O-CH₂-CH₂-CH₂-CH₂-CH₂CO-NH-), 8.35 (d, 1H, -CH₃(NO₂)=), 8.73 (s, 1H, -CH₃(NO₂)=).]
-CH₂-OCO-O), 4.21 (s, 2H, -CO-O=CH₂-CH₂-CH₂-CH₂-CO-NH-), 4.68 (s, 6H, NO₂- CH₂-C=). ¹³C NMR (CDCl₃): 7.7, 18.3, 22.8, 25.4, 25.6, 28.3, 36.4, 41.8, 49.1, 58.4, 64.5, 67.1, 69.5, 154.2, 172.4. Exact mass (M+H) 633.2209; found 633.2294.

Scheme S1. Synthesis of TATP, TNT and PETN hapten derivatives H₁, H₂ and H₃ from haptens I, II and III.

Figure S1. Absorbance (solid), fluorescence excitation (dash-dotted) and emission spectra (dotted lines) of the dyes F27, SRB and RB3 in PBS (80 mM) containing 2.5% MeOH.

3 Polyclonal antibodies for TATP, TNT and PETN

The polyclonal antibodies against TATP, TNT and PETN were obtained as described previously by us. For immunizing purposes, the corresponding haptens were covalently attached, through their carboxylic acid moieties to the lysine residues of BSA, using a modification of the active-ester method. The conjugates were employed to immunize six 9 to 12 weeks old (2–2.5 kg) rabbits with subcutaneous injections (two rabbits per analyte), and 8–12 different sera were obtained from each rabbit. These sera were used to evaluate the titer and affinity maturation of the hapten-specific antibodies via ELISA. Based on these results, the sera of boosts 11, 8 and 5 for TATP, TNT and PETN were selected as the best suited for the preparation of the antibody-capped hybrid materials.
4 Synthesis and chemical characterization of materials

4.1 Synthesis of MCM-41 mesoporous silica nanoparticle support (MCM)

Mesoporous MCM-41 nanoparticles were synthesized as reported previously.[3] n-Cetyltrimethylammonium bromide (CTABr, 1.00 g, 2.74 mmol) was first dissolved in 480 mL of deionized water. Then, 3.5 mL of 2 M NaOH in deionized water were added to the CTABr solution, followed by heating the solution to 80 ºC. Tetraethylorthosilicate (TEOS) (5.00 mL, 2.57×10⁻² mol) was then added dropwise to the surfactant solution. The mixture was stirred for 2 h at 80°C to give a white precipitate. Finally, the solid product was centrifuged, washed with deionized water and ethanol, and dried at 60ºC (MCM -41 as-synthesized). Removal of the template was achieved by calcination for 8 h at 550°C, yielding the calcinated porous material M.

4.2 Synthesis of MI1H1

MCM-41 mesoporous nanoparticles M (100 mg) and the dye 2,7-dichlorofluorescein sodium salt (F27 or I1; 33.0mg, 0.8 mmol g⁻¹ solid) were suspended in EtOH (5 mL). The suspension was stirred for 24 h at rt to achieve maximum loading of the dyes into the pores of the MCM-41 scaffold. Afterwards, 25 µmol of H1 were dissolved in 40 µL THF and the final mixture was stirred for 6 h at room temperature. Finally, the solid MI1H1 was centrifuged (5 min at 9500 rpm), washed with EtOH (1 mL) and isolated by centrifugation, before drying for 12 h at 35 ºC in a vacuum.

4.3 Synthesis of MI2H2

ML2H2 was prepared following the same procedure as for MI1H1, i.e., M (100 mg) and the dye tris(bipyridine)ruthenium(II) chloride (RB3 or I2; 59 mg, 0.8 mmol g⁻¹ solid) were suspended in acetonitrile (10 mL), stirring the suspension for 24 h at rt. Afterwards, 12.5 µmol of H2 were dissolved in 40 µL THF and the final mixture was stirred for 6 h at room temperature. Finally, the solid MI2H2 was centrifuged (5 min at 9500 rpm), washed with acetonitrile (1 mL) and isolated by centrifugation, before drying for 12 h at 35 ºC in a vacuum.

4.4 Synthesis of MI3H3

ML3H3 was prepared following the same procedure as for MI1H1, i.e., M (100 mg) and the dye sulforhodamine B sodium salt (SRB or I3; 46.5 mg, 0.8 mmol g⁻¹ solid) were suspended in acetonitrile (20 mL), stirring the suspension for 24 h at rt. Afterwards, 12.5 µmol of H3 were dissolved in 40 µL THF and the final mixture was stirred for 6 h at room temperature. Finally, the solid MI3H3 was centrifuged (5 min at 9500 rpm), washed with acetonitrile (1 mL) and isolated by centrifugation, before drying for 12 h at 35 ºC in a vacuum.

4.5 Synthesis of MI1H2 and MI1H3

Materials MI1H2 and MI1H3, both containing F27 = I1 as an indicator in the pores yet also the other two hapten derivatives H2 and H3 grafted to the surface, were analogously prepared by suspending M (100 mg) and I1 (33.0mg, 0.8 mmol g⁻¹ solid) in EtOH (5 mL). After 24 h stirring at rt, the suspension was divided into two fractions, and 25 µmol of H2 and H3, dissolved in 15 µL THF, were added to each fraction. The final mixtures were stirred for 6 h at room temperature. Finally, the solids MI1H2 and MI1H3 were centrifuged (5 min at 9500 rpm), washed with EtOH (1 mL) and isolated by centrifugation before drying for 12 h at 35 ºC in a vacuum.

4.6 Synthesis of antibody-capped materials MInHnAn

M11H1A1, M12H2A2, M13H3A3, M12H2A2 and M13H3A3 were prepared using a modified procedure reported previously.[2d] 1 mg of the corresponding materials MI1H1, MI2H2, MI3H3, MI1H2 and MI1H3 were suspended in 0.5 mL of a solution of phosphate-buffered saline (PBS) at pH 7.4 containing 200 ppm of the respective dye, the respective antibody in PBS (4.5 µL A1-3 mg S1-3-1), 0.005% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween-20 and stirred for 20 h at room temperature. Afterwards, the capped materials MI1H1A1, MI1H2A2, MI1H3A3, MI1H2A2 and MI1H3A3 were isolated by centrifugation and washed several times with PBS, before drying in a vacuum as reported above. All the capped materials were stored in a dry state at 4°C.

5 Characterization of materials

All solids prepared were characterized using standard procedures. The presence of the mesoporous structure was confirmed with nitrogen adsorption-desorption isotherms and transmission electron microscopy (TEM) analysis, whereas the respective contents of
antibody, hapten and dye in and on the materials were estimated from elemental analysis, thermogravimetry and absorbance measurements. TEM images revealed that the as-prepared MCM-41 nanoparticles are obtained as spherical particles with diameters ranging of 70–110 nm. The micrographs also show the hexagonal mesoporosity and the typical channels of the MCM-41 matrix, visualized as alternating black and white stripes (Figures S2a, S2b). Figure S2b also shows a TEM image of the capped material MI1H1A1. Here, it is possible to observe an increase in the disorder of the mesochannels, ascribed to the increase of organic matter (antibodies and other proteins) on the surface.

Nitrogen adsorption-desorption isotherms of the calcinated MCM-41 nanoparticles are shown in Figure S3. A behaviour typical for mesoporous solids can be recognized, consisting of an adsorption step at intermediate P/P₀ values (0.25–0.35). This curve corresponds to a type-IV isotherm, in which the observed step is characteristic for nitrogen condensation inside the mesopores. No hysteresis loop is observed in this interval which, together with the narrow BJH pore distribution, is indicative of uniform cylindrical mesopores. Employing the BJH model on the adsorption branch of the isotherm, the pore diameter can be determined to 2.15 nm and the pore volume to 0.85 cm³ g⁻¹. Furthermore, the application of the BET model resulted in a value of 999.3 m² g⁻¹ for the total specific surface area. A second adsorption step at a higher relative pressure (P/P₀>0.9) is also observed, which corresponds to the filling of the large voids between the particles (pore volume of 0.55 cm³ g⁻¹ calculated according to the BJH model) and can, therefore, be ascribed to textural porosity. N₂ adsorption-desorption isotherms of the functionalized materials could not be obtained because of the large amount of material that would have been to be prepared. However, significantly reduced pore volumes and specific surface areas would be expected already for dye-loaded materials MI1, MI2 and MI3.

Figure S2. TEM micrographs of a) MCM-41 nanoparticles and b) capped material MI1H1A1, showing the typical hexagonal porosity of the MCM-41 mesoporous matrix and the change of appearance on the surface on the MCM-41 support after the various steps of functionalization.
Figure S3. a) N₂ adsorption-desorption isotherm for calcined MCM-41 nanoparticles M. b) Pore size distribution of M. Inset shows a zoom out into the 50–200 nm region of the pore size distribution, ascribed to the textural porosity.

The size of the particles was also determined by dynamic light scattering (DLS). All measurements were performed in triplicate on previously sonicated and highly diluted Milli-Q water dispersions (100 µg mL⁻¹) at pH 6. The results of the measurements of M, MI₂H₂, and MI₂H₂A₂ are shown in Figure S4 as representative examples, revealing mean hydrodynamic diameters of 122 ± 25, 110 ± 15 and 205 ± 35 nm for M, MI₂H₂ and MI₂H₂A₂, respectively. The differences in the particle diameters between M and MI₂H₂ are minor, but the increase upon antibody attachment is significant, which is because, along with the antibodies, stabilizing BSA is loosely attached as well during the incubation step used for capping.⁴

Figure S4. Hydrodynamic diameter measured by DLS for M, MI₂H₂ and MI₂H₂A₂.

The contents of hapten derivatives H₁, H₂ and H₃ as well as dyes F27, RB3 and SRB in the prepared solids were determined by elemental analysis and thermogravimetric studies. Furthermore, the amounts of loaded dye in the final systems after antibody capping were determined indirectly from the difference between the amount measured for MIₙHₙ and the amount washed out into the aqueous washing fractions after the capping process.
Table S1. Contents (in µmol g⁻¹ solid) of the hapten derivatives H₁, H₂ and H₃ and the dyes F27, RB3 and SRB in the prepared solids.

| Solid | TATP hapten | TNT hapten | PETN hapten | F27 (I₁) | RB3 (I₂) | SRB (I₃) |
|-------|-------------|------------|-------------|----------|----------|----------|
| MI₁H₁ | 111.8       | –          | –           | 213.4    | –        | –        |
| MI₁H₂ | –           | 65.7       | –           | –        | 310.0    | –        |
| MI₁H₃ | –           | –          | 33.5        | –        | –        | 232.5    |
| MI₂H₁ | 111.8       | –          | –           | 190.4    | –        | –        |
| MI₂H₂ | –           | 65.7       | –           | –        | 124.8    | –        |
| MI₂H₃ | –           | –          | 33.5        | –        | –        | 140.9    |
| MI₃H₁ | –           | 98.1       | –           | 172.2    | –        | –        |
| MI₃H₂ | –           | 98.1       | –           | 143.0    | –        | –        |
| MI₃H₃ | –           | –          | 85,1        | 156.5    | –        | –        |
| MI₂H₃ | –           | –          | 85,1        | 127.3    | –        | –        |
Results and Discussion

6 Control experiments with MI1H2A2

Figure S5. Increase in fluorescence vs time for F27 (λ<sub>exc</sub> = 490 nm; λ<sub>em</sub> = 525 nm) released from MI1H2A2 in PBS-Tween 20 containing 2.5% MeOH (pH 7.4), in the presence (circles) and the absence (stars) of 10.5 ppm of TNT. The lines are included only as a guide to the eye for better illustration.

7 Raw spectra of response to analytes

Figure S6. Increase in fluorescence of the three fluorescent dyes a) F27 (λ<sub>exc</sub> = 490 nm), b) RB3 (λ<sub>exc</sub> = 460 nm) and c) SRB (λ<sub>exc</sub> = 560 nm) vs concentration of the corresponding analyte for MI1H1A1–MI3H3A3 in PBS-Tween 20 containing 2.5% MeOH (pH 7.4) after 5 min of reaction.

8 Detection of explosives in turbid media: milk as an example

Rapid tests for point-of-need measurements have to be applicable in real samples without the possibility of extensive clean-up. To assess the general suitability of the title materials, the suspensions assays were thus also performed in milk containing 3.5% fat. Milk was chosen because it is a complicated liquid sample that (i) is turbid so that it cannot usually be used directly in spectroscopic analysis, (ii) it is a liquid food stuff, food stuff being one of the sample types frequently encountered in explosives-related security applications and (iii) it contains a high load of a variety of matrix components, in so far also being related to soil extracts which would be typical sample types in environmental analysis. As can be seen in Figure S7, the detection of TATP, TNT and PETN using materials MI1H1A1, MI2H2A2 and MI3H3A3 could also be successfully performed and results similar to those in buffered media were found.
Figure S7. Increase in fluorescence of, from left to right, F27 (λ<sub>exc</sub> = 490 nm; λ<sub>em</sub> = 525 nm), RB3 (λ<sub>exc</sub> = 464 nm; λ<sub>em</sub> = 626 nm) and SRB (λ<sub>exc</sub> = 564 nm; λ<sub>em</sub> = 588 nm) released from MI1H1A1 (left), MI2H2A2 (middle) and MI3H3A3 (right) in the presence of increasing concentrations of TATP (left), TNT (middle) and PETN (right) in full-fat milk (3.5 % fat, black) and PBS-Tween 20 containing 2.5% MeOH (pH 7.4, red) after 5 min of reaction. The lines exemplify four-parametric logistic fits.

Following a similar approach, the crosstalk within the three systems was also tested using a cocktail of all three materials. The response in milk was again found to be comparable to that in buffered media, Figure S8.

Figure S8. Increase in fluorescence of F27 = I₁ (λ<sub>exc</sub> = 490 nm; λ<sub>em</sub> = 525 nm), RB3 = I₂ (λ<sub>exc</sub> = 464 nm; λ<sub>em</sub> = 626 nm) and SRB = I₃ (λ<sub>exc</sub> = 564 nm; λ<sub>em</sub> = 588 nm), released from MI1H1A1–MI3H3A3 in the presence of increasing concentrations of TATP (left), TNT (middle) and PETN (right) in full-fat milk (3.5 % fat) after 5 min of reaction.

9 Cross-reactivities

Figure S9. Relative dye release of the three sensory materials MI₁H₁A₁, MI₂H₂A₂ and MI₃H₃A₃ in the presence of the target analytes, the primary competitors of the tri-plexing assay and other common explosives such as picric acid (PA), octogen (HMX), nitroguanidine (NG) and hexogen (RDX).
Nitrocellulose strips of 0.5 × 4 cm were prepared, and 1 µL of a suspension (1 mg mL⁻¹) of the corresponding materials $M_iH_iA_n$ was deposited at ca. 1 cm from one end of the strip (zone A). The strips containing materials $M_iH_1A_1$, $M_2H_2A_2$ or $M_3H_3A_3$ were then dipped into the PBS-buffered sample solutions containing different concentrations of the respective analytes TATP, TNT and PETN, and the flow was left to develop for 3 min. Afterwards, the strips were dried at room temperature and the fluorescence was measured using a flow assay reader at 520 nm ($\lambda_{exc} = 470$ nm) for $M_iH_iA_1$, 625 nm ($\lambda_{exc} = 470$ nm) for $M_2H_2A_2$ and 625 nm ($\lambda_{exc} = 560$ nm) for $M_3H_3A_3$. 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 entire strip. As an example, Figure S10 shows the SRB release from $M_3H_3A_3$ in the absence and in the presence of PETN. It is evident that a significant signal in zone B is only registered in the presence of PETN, whereas virtually no fluorescence is observed in zone B in the absence of analyte. The dependence of dye release on the concentration of PETN was found to be similar as in solution. LODs of 0.5 ± 0.2, 0.6 ± 0.2 and 0.4 ± 0.2 ppb were thus determined for the detection of TATP, TNT and PETN, respectively.

For the multiplexing detection of the explosives using a mixture of the materials $M_1H_1A_1$, $M_2H_2A_2$ and $M_3H_3A_3$, first, the chromatographic separation efficiency of the dyes F27, RB3 and SRB had to be assessed for different strip materials. Three different membranes were thus tested, Immunopore RP from Whatman and HF120 and HF135 from Millipore. For that purpose, 1 µL of a mixture of 1 ppm solutions of SRB, F27 and RB3 were deposited at one end of the strip, the strips were dipped into a microwell plate and the flow was left to develop, before taking several pictures with a digital camera under visible and UV light ($\lambda_{exc} = 356$ nm) and recording the fluorescence with fluorescence reader. The results are shown in Figure S11, revealing the most efficient separation of the dyes used here was achieved with HF135 from Millipore.
11 Multiplexing detection of explosives TATP, TNT and PETN

11.1 Smartphone setup as readout device with a single strip

The release of the different dyes was measured with a smartphone setup using two LEDs of 500 nm and 515 nm as excitation sources 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. The corresponding dye release of the strips containing a mixture of materials MI1H1A1, MI2H2A2 and MI3H3A3 is depicted in Figure S12.

11.2 Fluorescence reader as readout device with a single strip

The release of the different dyes was also measured with a fluorescence reader, which contained different excitation wavelength/detection filter settings. F27 was recorded at 520 nm (λ<sub>exc</sub> = 470 nm), RB3 at 625 nm (λ<sub>exc</sub> = 470 nm) and SRB at 625 nm (λ<sub>exc</sub> = 560 nm). Corresponding dye release of the strips containing a mixture of materials MI1H1A1, MI2H2A2 and MI3H3A3 are depicted in Figure S13.
11.3 Smartphone setup as readout device with patterned strips

Wax-patterning strips with three separate channels branching out from a single sample reservoir and containing the sensing hybrids materials were prepared. 30 µl of a solution containing different concentrations of buffer employed, TATP, TNT or PETN were deposited on the sample deposition zone and then fluorescence intensity of dye released on the corresponding B zones was evaluated taking pictures of the strips with a smartphone. Figure S14 shows differences in fluorescence intensity of dye released as a function of the concentration of different explosives used.

Figure S13. a–c) Release of F27, RB3 and SRB from materials MI1H1A1, MI2H2A2 and MI3H3A3 deposited on nitrocellulose strips as a function of the concentration of the corresponding explosives TATP, TNT and PETN added, evaluated using a fluorescence reader. d) F27, RB3 and SRB release from materials MI1H1A1, MI2H2A2 and MI3H3A3 deposited on nitrocellulose strips in the presence of 600 ppb of different explosives. The lines exemplify four-parametric logistic fits.
Figure S14. Collage of the photographs corresponding to the plots in Figure 5, shot under proper light excitation and showing the release of F27 (I) from materials MI1H1A1, MI1H2A2, and MI1H3A3 in the presence of various amounts of TATP, TNT or PETN.

Following a similar procedure as described, several strips were exposed to different solutions of a mixture of 3 explosives tested (Figure S15, experiment 2) or a mixture of only two explosives (Figure S15, experiments 3-6), with the aim to evaluate the possible interference on the sensing materials. Strips exposed only to buffered media were also evaluated as reference (Figure S15, experiment 1). As can be seen in Figure 15, fluorescence intensity on the respective zones Bi were increased only when the corresponding explosive was present on the buffered media, showing no fluorescence enhancement on the channel corresponding to the explosive absent.
**12 Additional notes on analytical performance**

12.1 **Limits of detection**

For the experiments in suspension, the fluorescence emission of the supernatants of the suspensions of the materials in the presence of different concentrations of explosives were recorded, and the values of the fluorescence enhancement observed during the titrations were plotted vs. analyte concentration. These curves were fitted to a four-parameter logistic fitting function (equation S1).[^6]

\[
\frac{I}{I_0} = \frac{F_1 - F_2}{\left(1 + \frac{x}{x_0}\right)^p} + F_2
\]

(S1)

Here, \(F_1\) and \(F_2\) correspond to the minimum and maximum enhancement of fluorescence observed, and \(p\) corresponds to the slope of the sigmoidal curve. Limits of detection (LODs), which describe the smallest concentration of analyte that can be reliably detected, were derived calculating in a first instance the Limits of Blank (LOBs; describing the smallest concentration of analyte that can be observed) and calculating the corresponding concentration of the signal as follows:\[^6\]

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

\[
\text{LOD} = \text{LOB} + 1.645(\sigma_{\text{low concentration sample}})
\]
Generally, three repeat experiments were carried out for both the kinetics and the concentration-dependent measurements. Measurement uncertainties were derived as detailed in ref. [7].

12.2 Repeatability

Regarding the strip-to-strip repeatability, within the same batch, repeat errors of 4-6% were found between replicates, the main problem being the slightly different flow behavior on the nitrocellulose membranes, having a certain impact on the area of the dye in the detection zone.

12.3 Stability

When kept under normal laboratory conditions (in dry state at 4°C under air atmosphere) in a conventional refrigerator, the materials are stable for at least 3 months. After that period, the amount of dye that could be maximally released decreased, and a certain blank release was also noticed. When the material is kept on the strips (in dry state at 4°C under air atmosphere), this combination can be stored up to 2 months in a refrigerator. Afterwards, flow on the strips is becoming slower, possibly due to aggravated wetting. However, we tentatively assume that the stability of the material, as well as the final assay strip, can be improvable when the strips would be packaged under CA (controlled atmosphere) conditions.

12.4 Cut-off values

Simple YES/NO responses are the preferred format in which results of rapid on-site tests are commonly delivered to the user, because decision making in the application scenarios usually has to be fast. Commercial hand-held sensors such as the Fido X3, which can detect various classes of explosives (https://www.flir.com/products/fido-x3/), are thus also providing only concise YES/NO information to the use. The evaluation of the analytical results is usually done on the basis of a threshold or cut-off value, in the case of the Fido X3 for instance also amounting to <5%.

12.5 Previously reported immunoassays for explosives detection

Table S2. Representative selection of immunoassays for explosives detection published in the literature.

| No. | Analyte | Type of IA | Format | Detection | LOD /ppb | Range | Time | Multiplexing and/or hand-held | Ref. |
|-----|---------|------------|--------|-----------|----------|-------|------|--------------------------------|-----|
| 1   | TNT     | competitive| ELISA  | colorimetric | 2       | 2–30,000 | hours | no                       | [8]  |
| 2   | TNT     | competitive| ELISA  | colorimetric | 0.06    | 0.06–2  | 2 h   | no                       | [9]  |
| 3   | TNT     | competitive, indirect | ELISA | CL       | 0.2      | 0.2–50  | hours | no                        | [10] |
| 4   | TNT     | competitive| ELISA  | colorimetric | 0.0006  | 0.005–150 | hours | no                       | [26] |
| 5   | RDX     | competitive| ELISA  | colorimetric | 0.3     | 0.3–6   | hours | no                       | [11] |
| 6   | TATP    | competitive| ELISA  | colorimetric | 65      | 65–10,000 | hours | no                       | [12] |
| 7   | TATP    | competitive| ELISA  | colorimetric | 0.01    | 30–500  | hours | no                       | [26] |
| 8   | PETN    | competitive| ELISA  | colorimetric | 0.5     | 1–1,000 | hours | no                       | [26] |
| 9   | TNT     | displacement| reader-based | fluorometric | 0.5    | 0.5–100 | 30 min | no                       | [13] |
| 10  | TNT     | displacement| FIA    | fluorometric | 2.5     | 20–1,200 | few min. | no                       | [14] |
## Supporting Information

| No. | Analyte          | Type of IA | Format $^{[a]}$ | Detection $^{[b]}$ | LOD /ppb | Range          | Time    | Multiplexing and/or hand-held | Ref. |
|-----|------------------|------------|-----------------|--------------------|----------|----------------|--------|------------------------------|------|
| 11  | RDX              | displacement | FIA             | fluorometric       | 15       | 18–300         | few min.| no                           | [15] |
| 12  | TNT or RDX       | displacement | FIA             | fluorometric       | <1       | 1–600          | few min.| no                           | [16] |
| 13  | TNT              | displacement, reversed | FIA             | fluorometric       | 2.5      | 20–10,000      | 10 min | no                           | [17] |
| 14  | TNT              | displacement, reversed | microcapillary-based IA | fluorometric | 0.25 | 1–1000 | 5 min | no                           | [18] |
| 15  | TNT              | displacement | microfluidic chip-based IA | fluorometric | 1–10 | 3 orders of magnitude | 1.5–2 h | no                           | [19] |
| 16  | TNT              | competitive  | electrophoretic chip-based IA | fluorometric | 1 | 1–300 | 50 min | no                           | [20] |
| 17  | TNT              | competitive  | electrophoretic chip-based IA | fluorometric | 1 | 1–300 | 50 min | no                           | [21] |
| 18  | TNT              | competitive, indirect | gold chip-based IA | SPR                | 0.09 | 0.09–1000 | 25 min | no                           | [22] |
| 19  | TNT              | competitive, indirect | gold chip-based IA | SPR                | 0.002 | 0.008–30 | 5 min | no                           | [23] |
| 20  | TNT              | competitive, indirect | gold chip-based IA | SPR                | 0.008 | 0.008–30 | 5 min | no                           | [24] |
| 21  | TNT              | competitive, indirect | gold chip-based IA | SPR                | 0.15 | 0.5–7 | 10 min | no                           | [25] |
| 22  | TNT              | competitive, indirect | gold chip-based IA | SPR                | 0.01 | 0.01–100 | few min.| no                           | [26] |
| 23  | TNT              | displacement  | gold chip-based IA | SPR                | 0.08 | 10 s mins | no | [27] |
| 24  | TNT              | displacement  | gold chip-based IA | SPR                | 0.4  | 1–100 | 10 s mins | no | [28] |
| 25  | TNT              | displacement  | QCM-based IA     | frequency shift    | 0.1   | 0.1–10,000   | 10 min | no                           | [29] |
| 26  | TNT              | competitive  | LF-IA            | colorimetric       | 1000  | not reported | 5 min | hand-held                    | [10] |
| 27  | TNT              | displacement  | bead-based cytometer IA | fluorometric | 0.1 | 1–10,000 | 30 min | potentially multiplexable | [20] |
| 28  | TNT              | competitive  | bead-based cytometer IA | fluorometric | 1 | 1–1000 | 40 min | potentially multiplexable | [21] |
| 29  | TNT              | competitive  | bead-based cytometer IA | fluorometric | 1 | 100–10,000 | 40 min | potentially multiplexable | [22] |
| 30  | TATP and PETN    | displacement  | gAID             | fluorometric       | ca. 0.5 | from 1–15 to 5–300 | 5 min | both this work               |      |

$^{[a]}$ ELISA = enzyme linked immunosorbent assay, FIA = flow immunoassay, IA = immunoassay, QCM = quartz crystal microbalance, LF-IA = lateral flow immunoassay. $^{[b]}$ CL = chemiluminescence, SPR = surface plasmon resonance.
E.C. and K.R. conceived the experiments. E.C. with M.B. and D.G. performed the experiments. M.G.W. curated the immunoassay data. E.C. and K.R. with M.G.W. and R.M.M. prepared the manuscript. All authors discussed the results and commented on the manuscript.

Author Contributions

E.C. and K.R. with R.M.M. conceived the experiments. E.C. with M.B. and D.G. performed the experiments. M.G.W. curated the immunoassay data. E.C. and K.R. with M.G.W. and R.M.M. prepared the manuscript. All authors discussed the results and commented on the manuscript.

References

[1] J. G. Tatak, M. M. Knapp, C. Ressler, Bioconjugate Chem. 1991, 2, 124-132.
[2] a) M. A. Walter, U. Panne, M. G. Weller, Biosensors 2011, 1, 93-106; b) S. Ramin, M. G. Weller, J. Mol. Recognit. 2012, 25, 89-97; c) A. Hesse, M. Biyikal, K. Rurack, M. G. Weller, J. Mol. Recognit. 2016, 29, 88-94; d) E. Climent, D. Groninger, M. A. Walter, R. Martinez-Manez, M. G. Weller, F. Sancenón, A. Amorós, K. Rurack, Chem. Eur. J. 2013, 19, 4117-4122.
[3] E. Climent, L. Mondragon, R. Martinez-Manez, F. Sancenón, M. Dolores Marcos, J. Ramon Murguia, P. Amoros, K. Rurack, E. Perez-Paya, Angew. Chem. Int. Ed. 2013, 52, 8935-8942.
[4] E. Costa, E. Climent, K. Gawlitza, W. Wan, M. G. Weller, K. Rurack, J. Mater. Chem. B 2020, 8, 4950-4961.
[5] Y. Xiang, J. Donley, E. Setelskaia, S. Shingare, J. Kamerud, B. Gorovits, AAPS J. 2018, 20, Art. No. 45.
[6] D. A. Armbruster, T. Pry, Clin. Biochem. Rev. 2008, 29 Suppl 1, S49-S52.
[7] E. Costa, E. Climent, S. Ast, M. G. Weller, J. Canning, K. Rurack, Analyst 2020, 145, 3490-3494.
[8] P. Julicher, E. Muskenbrock, R. Renneberg, K. Cammann, Anal. Chim. Acta 1995, 315, 279-287.
[9] A. Zeck, M. G. Weller, R. Niessner, Fresenius J. Anal. Chem. 1999, 364, 113-120.
[10] S. Giroti, S. A. Eremin, A. Montoya, M. J. Moreno, P. Caputo, M. D’Elia, L. Ripani, F. S. Romolo, E. Maiolini, Anal. Bioanal. Chem. 2010, 396, 687-695.
[11] D. O. Ulaeto, A. P. Hutchinson, S. Nicklin, Monoclonal Antibodies Immunodiagn. Immunother. 2015, 34, 225-227.
[12] M. A. Walter, D. Pfeifer, W. Kraus, F. Emmerling, R. J. Schneider, U. Panne, M. G. Weller, Langmuir 2010, 26, 15418-15423.
[13] E. R. Goldman, G. P. Anderson, N. Lebedev, B. M. Lingerfelt, P. T. Winter, C. H. Patterson, J. M. Mauro, Anal. Bioanal. Chem. 2003, 375, 471-475.
[14] J. P. Whelan, A. W. Kusterbeck, G. A. Wemhoff, R. Bredehorst, F. S. Ligler, Anal. Chem. 1993, 65, 3561-3565.
[15] J. C. Barf, L. L. Judd, A. W. Kusterbeck, Sens. Actuators B 1997, 39, 411-418.
[16] S. Y. Rabbany, W. J. Lane, W. A. Marganski, A. W. Kusterbeck, F. S. Ligler, J. Immunol. Methods 2000, 246, 69-77.
[17] T. M. Green, P. T. Charles, G. P. Anderson, Anal. Biochem. 2002, 310, 36-41.
[18] P. T. Charles, J. G. Rangasamy, G. P. Anderson, T. C. Romanoski, A. W. Kusterbeck, Anal. Chim. Acta 2004, 525, 199-204.
[19] P. T. Charles, A. A. Adams, P. B. Howell, S. A. Trammell, J. R. Deschamps, A. W. Kusterbeck, Sensors 2010, 10, 876-889.
[20] A. Bromberg, R. A. Mathies, Anal. Chem. 2003, 75, 1188-1195.
[21] A. Bromberg, R. A. Mathies, Electrophoresis 2004, 25, 1895-1900.
[22] D. R. Shankaran, K. V. Gobi, T. Sakai, K. Matsumoto, K. Toko, N. Miura, Biosens. Bioelectron. 2005, 20, 1750-1756.
[23] D. R. Shankaran, T. Kawaguchi, S. J. Kim, K. Matsumoto, K. Toko, N. Miura, Anal. Bioanal. Chem. 2006, 386, 1313-1320.
[24] T. Kawaguchi, D. R. Shankaran, S. J. V. Gobi, K. Matsumoto, K. Toko, N. Miura, Talanta 2007, 72, 554-560.
[25] P. Singh, T. Onodera, Y. Mizuta, K. Matsumoto, N. Miura, K. Toko, Sens. Mater. 2007, 19, 261-273.
[26] T. Kawaguchi, D. R. Shankaran, S. J. Kim, K. Matsumoto, K. Toko, N. Miura, Sens. Actuators B 2008, 133, 467-472.
[27] Y. Mizuta, T. Onodera, P. Singh, K. Matsumoto, N. Miura, K. Toko, Biosens. Bioelectron. 2008, 24, 191-197.
[28] T. Onodera, Y. Mizuta, K. Horikawa, P. Singh, K. Matsumoto, N. Miura, K. Toko, Sens. Mater. 2011, 23, 39-52.
[29] J. Wang, M. Almakhaita, S. L. Biswal, L. Segatori, J. Biosens. Bioelectron. 2012, 3, 1-7.
[30] G. P. Anderson, S. C. Moreira, P. T. Charles, I. L. Medintz, E. R. Goldman, M. Zeinali, C. R. Taitt, Anal. Chem. 2006, 78, 2279-2285.
[31] G. P. Anderson, J. D. Lamar, P. T. Charles, Environ. Sci. Technol. 2007, 41, 2888-2893.
[32] G. P. Anderson, E. R. Goldman, J. Immunol. Methods 2008, 339, 47-54.