Supporting Information

An aptamer-gated silica mesoporous material for thrombin detection

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Chemicals

The chemicals tetraethylorthosilicate (TEOS), n-cetyltrimethylammonium bromide (CTABr), sodium hydroxide (NaOH), rhodamine B, 3-aminopropyltriethoxysilane and human serum – from human male AB plasma (sterile-filtered) were provided by Aldrich. Human α-thrombin was provided by Haematologic Technologies Inc. Analytical-grade solvents were from Scharlab (Barcelona, Spain). All reactives were used as received.

General Techniques

XRD, TG analysis, elemental analysis, TEM microscopy, N_2 adsorption-desorption and UV-visible spectroscopy techniques were used to characterize the prepared materials. X-ray measurements were performed on a Brücher AXS D8 Advance diffractometer using Cu-Kα radiation. Thermo-gravimetric analysis were carried out on a TGA/SDTA 851e Mettler Toledo equipment, using an oxidant atmosphere (Air, 80 mL/min) with a heating program consisting on a heating ramp of 10 °C per minute from 393 K to 1273 K and an isothermal heating step at this temperature during 30 minutes. TEM images were taken with a JEOL TEM-1010 Electron microscope working at 100 kV. N_2 adsorption-desorption isotherms were recorded on a Micromeritics ASAP2010 automated sorption analyser. The samples were degassed at 120ºC in

vacuum overnight. The specific surfaces areas were calculated from the adsorption data in the low pressures range using the BET model. Pore size was determined following the BJH method. UV-visible spectroscopy was carried out with a Jasco V-630 Spectrometer. Fluorescence spectroscopy was carried out on a Jasco FP-8300 Spectrometer and UV-visible spectroscopy was carried out with a Jasco V-630 Spectrometer.

Buffer Solutions

Phosphate buffered saline 1x (PBS 1x) consisting in 137 mM NaCl, 1.47 mM KH_2PO_4, 7.85 mM Na_2HPO_4, 2.68 mM KCl (pH:7.5) was used for controlled release experiences.

Synthesis of the silica mesoporous nanoparticles support (SMPS)

The MCM-41 mesoporous nanoparticles were synthesized by the following procedure: n-cetyltrimethylammoniumbromide (CTABr, 1.00 g, 2.74 mmol) was first dissolved in 480 mL of deionized water. Then a 3.5 mL of NaOH 2.00 M in deionized water was added to the CTABr solution, followed by adjusting the solution temperature to 80ºC. TEOS (5 mL, 2.57. 10^{-2} mol) was then added dropwise to the surfactant solution. The mixture was allowed stirred for 2 h to give a white precipitate. Finally the solid product was centrifuged, washed with deionized water and dried at 60ºC (MCM-41 as-synthesized). To prepare the final porous material (MCM-41), the as-synthesized solid was calcinated at 550 ºC using oxidant atmosphere for 5 h in order to remove the template phase.

Synthesis of S1

The amino-functionalised solid **S1** was prepared following literature procedures.[1] 500 mg of calcinated MCM-41 and 33.2 mg (0.10 mmol) of dye rhodamine B were suspended in 40 mL of acetonitrile inside a round-bottom flask connected to a Dean-Stark in an inert atmosphere. The suspension was refluxed (110 ºC) in azeotropic distillation, collecting 10 mL in the trap in order to remove the adsorbed water. Then, the mixture was stirred during 24 hours at 36ºC with the aim of achieving maximum loading in the pores of the MCM-41 scaffolding. Afterward an

[1] E. Climent, R. Martínez-Máñez, F. Sancenón, M. D. Marcos, J. Soto, A. Maquieira, P. Amorós, *Angew. Chem. Int. Ed.* **2010**, *49*, 7281.

excess of 3-aminopropyltriethoxysilane (APTS, 0.936 mL, 4.0 mmol) was added, and the suspension was stirred for 5.5 h. Finally, the pink solid (**S1**) was filtered off and dried at 70 ºC for 12h.

Synthesis of aptamers

The aptamers TBA d(5'-GGT TGG TGT GGT TGG-3') and TBA-flu d(5'-GGT TGG TGT GGT TGG-3'-fluorescein) were synthesized in a 1 µmol scale using a 3400 Applied Biosystems DNA synthesizer following standard protocols. Ammonia deprotection was performed overnight at 55 ºC. The resulting products were desalted by Sephadex G-25 (NAP-10, GE Healthcare) and used without further purification. Controlled pore glass (CPG) functionalised with fluorescein (3'-(6-FAM)-CPG, Link Technologies) was used for the introduction of fluorescein at the 3'-end of TBA.

Synthesis and optimization of solid S1-TBA

In order to estimate the proper amount of TBA for the preparation of **S1-TBA**, capping and release experiments in presence of different amounts of TBA were carried out. First of all, portions of 50 µL of a suspension of 1 mg **S1**/1 mL PBS were added to a solution containing the aptamer **TBA-flu** d(5'-GGT TGG TGT GGT TGG-3'-fluorescein) in concentrations of (0.02, 0.04, 0.06 and 0.08 µmol of **TBA-flu** g solid^{-1}) in Milli-Q water, and each suspension was stirred for 30 min at 37ºC, respectively. Afterwards, the absorbance of **TBA-flu** in the solution after the capping process was measured, in order to estimate the content of **TBA-flu** retained into solid. Results are shown in Table S1.

Table S1. Content of TBA added for the synthesis of **S1-TBA** and TBA retained during the process in mmol g^{-1} S1.

TBA added (mmol g^{-1} S1)	**TBA retained (mmol g^{-1} S1)**	**% retained (mmol g^{-1} S1)**
0.02	0.0123	61.55
0.04	0.0164	44.25
0.06	0.0134	22.44
0.08	0.0027	3.43

Some release studies were carried out. The isolated solids were divided in two parts. To one part, a solution of 250 µL PBS 1x containing human α-thrombin in concentrations of: 0.04, 0.06 and 0.08 µmol of thrombin g $solid^{-1}$ were added and to the other part 250 µL PBS 1x were added. The suspensions were stirred for one hour; during this period two aliquots of the suspensions were taken. Finally, after centrifugation of all the aliquots the fluorescence of rhodamine B emission at 572 nm (λex = 555nm) delivered to the solution was measured. It was found that all the concentrations were able to induce an effective capping of the pores, but in terms of dye release the concentration of the TBA at 0.04 mmol g $solid^{-1}$, showed more dye delivery than the others two TBA concentrations (0.06 and 0.08 mmol g $solid^{-1}$). Results are collected in Figure S1.

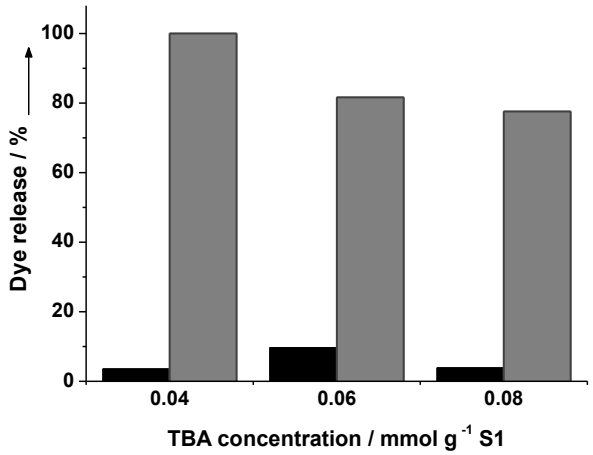

Figure S1. Optimization of the concentration of TBA to reach an effective capping of the pores. The black bars represent the blanks (absence of thrombin) and the grey bars represent the samples in presence of thrombin 0.04, 0.06 and 0.08 µmol of α-thrombin g solid^{-1}, respectively. All the measurements were carried out in PBS 1x (pH 7.5).

With the aim to obtain solid **S1-TBA**, 50 µL of a suspension of 1 mg **S1** in 1 mL PBS were suspended in 20 µl of a solution containing the aptamer TBA d(5'-GGT TGG TGT GGT TGG-3') in a concentration of 28.6 µM in Milli-Q water, and the suspension was stirred at 37ºC for 30 min. The final **S1-TBA** solid was isolated by centrifugation and washed with 100 µL of PBS buffer (pH 7.5) in order to remove the residual dye and the free aptamer TBA.

Materials Characterisation

Solid **S1** was characterised using standard procedures. Figure S2 shows powder X-ray patterns of the nanoparticulated MCM-41 as synthesized support, calcinated MCM-41 and the **S1** functionalised material. The PXRD of siliceous nanoparticulated MCM-41 as-synthesized (curve a) shows four low-angle reflections typical of a hexagonal array that can be indexed as (100), (110), (200), and (210) Bragg peaks. A significant displacement of the (100) peak in the XRD powder of the nanoparticulated MCM-41 calcinated sample is clearly appreciated in the curve b, corresponding to an approximate cell contraction of 6.6 Å. This displacement and the broadening of the (110) and (200) peaks are related to further condensation of silanol groups during the

calcination step. Finally, curve c corresponds to the **S1** XRD pattern. In this case, a loss of the (110) and (200) reflections is observed, most likely related to a loss of contrast due to the filling of the pore voids with the rhodamine B dye. Nevertheless, the value and intensity of the (100) peak in this pattern strongly evidences that the loading process with the dye and the further functionalisation with APTS have not damaged the mesoporous 3D MCM-41 scaffolding. The presence in the final functionalised solids of the mesoporous structure is also confirmed from the TEM analysis, in which the typical channels of the MCM-41 matrix are visualized as alternate black and white stripes (see Figure S2 for MCM-41 calcinated and solid **S1**). The figure also shows that the prepared MCM-41 and solid **S1** are obtained as spherical particles with diameters ranging from 100 to 200 nm.

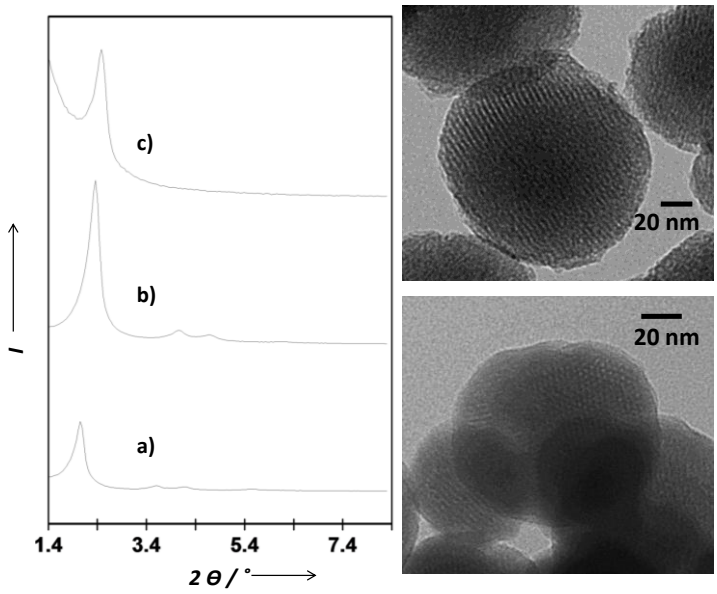

Figure S2. Left: powder X-ray patterns of the solids (a) MCM-41 as synthesized (b) calcinated MCM-41 and (c) solid **S1** containing the dye rhodamine B and functionalized with 3-aminopropyltriethoxysilane. Right: TEM images of (a) calcinated MCM-41 sample and (b) solid **S1** showing the typical hexagonal porosity of the MCM-41 mesoporous matrix.

The N_2 adsorption-desorption isotherms of the nanoparticulated MCM-41 calcinated material shows two sharp adsorption steps. The isotherm shows a first step at intermediate P/P_0 value (0.1-0.3) typical of these solids (see Figure S3, curve a). This step can be related to the nitrogen condensation inside the mesopores by capillarity. The absence of a hysteresis loop in this interval and the narrow BJH pore distribution suggests the existence of uniform cylindrical mesopores with pore volume of 0.84 $cm^3\ g^{-1}$ calculated by using the BJH model on the adsorption branch of the isotherm. The application of the BET model resulted in a value for the total specific surface of 1066.8 $m^2\ g^{-1}$. From the XRD, porosimetry and TEM studies, the a_0 cell parameter (43.16 Å), the pore diameter (2.57 nm) and a value for the wall thickness (1.74 nm) were calculated. In addition to this adsorption step associated to the micelle generated mesopores, a second feature appears in the isotherm at a high relative pressure ($P/P_0 > 0.8$). This adsorption correspond to the filling of the large voids among the particles and present a volume of 0.47 $cm^3\ g^{-1}$ (calculated by using the BJH model) and then must be considered as a textural-like porosity. In this case, the curves show a characteristic H1 hysteresis loop and a wide pore size distribution.

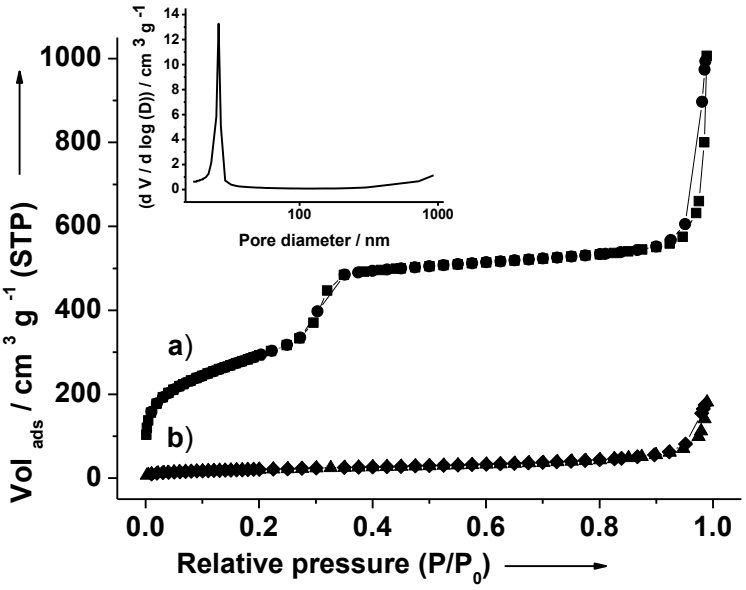

Figure S3. Nitrogen adsorption-desorption isotherms for (a) MCM-41 mesoporous material and (b) **S1** material. Inset: Pore size distribution of MCM-41 mesoporous material.

The N_2 adsorption-desorption isotherm of **S1** is typical of mesoporous systems with filled mesopores (see Figure S3, curve b), and a significant decrease in the N_2 volume adsorbed and surface area (0.06 $cm^3\ g^{-1}$ and 77.1 $m^2\ g^{-1}$ respectively) is observed. The most relevant feature is the absence of a sharp step at low-medium relative pressure ($0.1 < P/P_0 < 0.4$). In fact, this solid shows flat curves when compared (at the same scale) to those of the MCM-41 parent material, this indicates a significant pore blocking and the subsequent absence of appreciable mesoporosity. Additionally, a certain textural porosity is preserved. BET specific surface values, pore volumes, and pore sizes calculated from the N_2 adsorption-desorption isotherms for MCM-41 and **S1** are listed in Table S2.

Table S2. BET specific surface values, pore volumes and pore sizes calculated from the N_2 adsorption-desorption isotherms for selected materials.

	S_{BET} ($m^2\ g^{-1}$)	Pore Volume [a] ($cm^3\ g^{-1}$)	Pore size [a] (nm)
MCM-41	1066.8	0.84	2.57
S1	77.1	0.06	-

[a] Volume (V) and diameter (D) of mesopore.

The content of APTS and rhodamine B in the prepared solids **S1** and **S1-TBA** were determined by elemental analysis, thermogravimetric and delivery studies. Values of content are detailed in Table S3. The thermal analysis of the **S1** shows typical behaviour in functionalised mesoporous materials; i.e. a first weight loss between 25 and 150ºC related to the solvent evolution, a second step, between 150 and 800 ºC due to the combustion of the organics and a final loss in the 800 - 1000 ºC range related to the condensation of the silanol groups. In addition, from elemental analysis of C, H, N, S it is possible to determine the amount 3-aminopropyltriethoxysilane and rhodamine B contained in the materials calculated in millimole per gram of **S1** (mmol g^{-1} S1) using equation 1:

$$\alpha_A = \frac{\Delta W_i\% \times 1000}{\Delta W_{SiO_2}\% \times nM_i} \quad \left(\frac{mmol}{g\ S1}\right) \quad (1)$$

where $\Delta Wi\%$ (i = C, N, S) are the weight percentages of carbon, nitrogen or sulphur, M_i is the corresponding atomic weight and n is the number of the corresponding atom type in one molecule. $\Delta W_{SiO2}\%$ is the inorganic SiO_2 content in weight percentage. In addition, the content of dye in solid **S1-TBA** was determined from the dye content in **S1** after interaction with TBA aptamer, and determining the amount of dye delivered when washing the solids. Also, the amount of dye delivered from **S1-TBA** in the presence of thrombin was determined by using values of intensity of the fluorescence bearing in mind that, at low concentrations, relation between concentration and intensity are linear. Values of content are detailed in Table S3.

Table S3. Content of APTS and rhodamine B in the prepared solids **S1** and **S1-TBA** in mmol g^{-1} SiO_2.

Solid	**α_{APTS} (mmol g^{-1} S1)**	**$\alpha_{Rhodamine\ B}$ (mmol g^{-1} S1)**	**α_{TBA} (mmol g^{-1} S1)**
S1	1.65	0.092	-
S1-TBA	1.65	0.057	0.0164

Taking into account the different amounts of APTS and aptamer TBA in solid **S1-TBA** it is possible to estimate the distribution of aptamer onto surface. Using these values, we can estimate that **S1-TBA** material contains 9.87 x 1018 oligonucleotide molecules/g solid. Additionally, considering surface value of S1 calculated from N_2 adsorption measurements (77.1 m^2 g^{-1}) and the aptamer content calculated previously, the average surface coverage on solid S1-TBA by aptamer molecules is 0.36 molecules nm^{-2}. This aptamer surface coverage resulted in an average distance between aptamer molecules of about 2.8 nm. On the other hand, and bearing in mind again a typical value of external surface of S1 (77.1 m^2 g^{-1}) and the content of APTS (in mmol g^{-1} SiO_2) calculated with elemental analysis and thermogravimetric studies, the average surface coverage on solid **S1-TBA** by APTS molecules is 12.8 molecules nm^{-2}. Finally, using the aptamer and APTS contents calculated previously a ratio of 100 APTS molecules aptamer $molecule^{-1}$ was estimated

In vitro characterisation of S1-TBA in simulated human blood plasma

With the aim to study the possible application of **S1-TBA** for real sample analysis, we tested **S1-TBA** in simulated human blood plasma. Thus, the response of **S1-TBA** in presence of several amounts of α-thrombin in simulated human blood plasma[2] was studied. In the absence of α-thrombin, solid **S1-TBA** showed negligible dye release, whereas an enhancement in the emission intensity at 572 nm was observed upon addition of increasing quantities of α-thrombin. In this case, the maximum delivery of the dye was observed at an α-thrombin concentration of 1700 nM, showing a limit of detection of 2 nM. Figure S4 shows the fluorescence emission spectra of rhodamine B released from **S1-TBA** in absence and in presence of different amounts of α-thrombin.

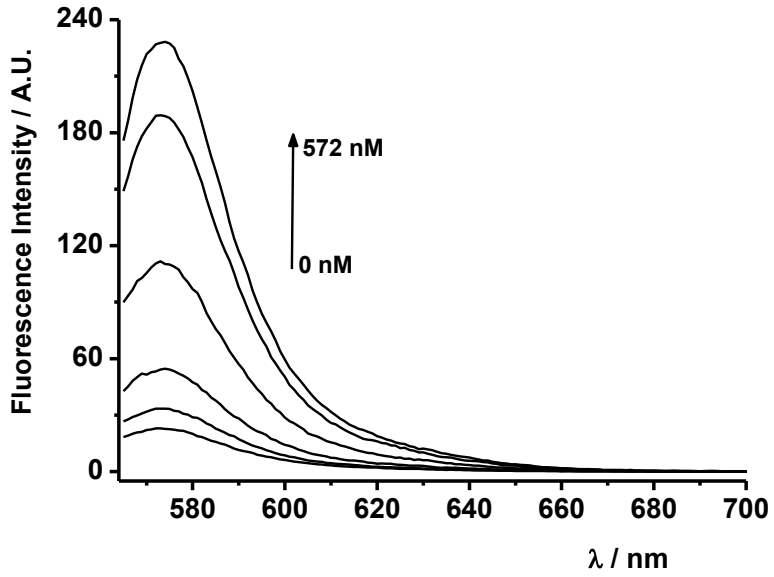

Figure S4. Fluorescence emission spectra of the rhodamine B delivered from **S1-TBA** in the presence of different concentrations of α-thrombin in simulated human blood plasma (pH 7.25). From top to bottom: 572, 381, 267, 114, 19 and 0 nM. (λ_{exc} = 555 nm).

[2] M. R. C. Marques; R. Loebenberg; M. Almukainzi. Simulated Biological Fluids with Possible Application in Dissolution Testing. Dissolution Technologies 2011, 18, Issue 3, 15-28.

In vitro characterisation of S1-TBA in human serum

The delivery experiments of **S1-TBA** in human blood plasma showed faster response than the response observed in simulated human blood plasma (related with stronger ionic strength), showing a tolerance of the system using 10% of human serum. Calibration curve for thrombin containing 10 % of human serum was obtained (Figure S5), showing the maximum delivery of the dye at 15 min employing 98.82 nM of thrombin.

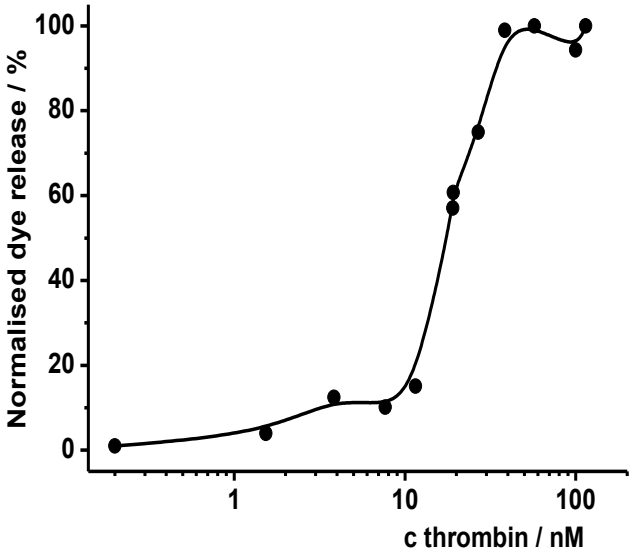

Figure S5. Release of rhodamine B from solid **S1-TBA** as a function of the concentration of human α thrombin in PBS containing 10% of human serum – from human male AB plasma.

Determination of thrombin in human serum

With the aim to verify the feasibility of the developed method, we prospectively used solid **S1-TBA** for the determination of thrombin in human serum. For this purpose, in a typical experiment, three portions of 1 mL of human serum sample were spiked with 75, 150 and 300 nM of thrombin, respectively. Samples were diluted with PBS for a final amount of 10%, and finally each sample was doped with different amounts of human thrombin in order to obtain a calibration curve by standard addition. Then, 40 µL of each doped sample was carefully added to 100 µl of **S1-TBA** suspension (12 µg in 140 µl) in order to prepare a calibration curve. After 15 minutes, the suspensions were centrifuged (3 min at 7000 rpm) and the emission intensity of

rhodamine B (λ_{ex} = 555 nm) at 572 nm was measured in the solution. These fluorescence intensities at 572 nm plotted vs. human thrombin concentration yielded three calibration curves (data not shown). The content of α-thrombin spiked in human serum was calculated through the difference between the y-intercept of the calibration curve and the residual emission of a blank solution divided by the slope of the calibration curve. The obtained results are shown in Table S4. As can be seen, remarkable recoveries in the range of 91-120% of thrombin were achieved.

Table S4. Determination of thrombin in 10% human serum samples.

Thrombin added (nM)	**Thrombin found (nM)**	**Recovery**
75	68.35 ± 5.56	91
150	147.32 ± 4.08	98
300	361.22 ± 60.47	120