Implementation of oligonucleotide-gated supports for the electrochemical detection of Ochratoxin A

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Abstract. We report herein the design a hybrid material for the recognition of Ochratoxin A (OTA) by using an aptamer gated-material able to release electroactive species, which can be measured using differential pulse voltammetry. Mesoporous silica nanoparticles (MSN) loaded with Methylene Blue and capped with an OTA aptamer were prepared and characterized. A highly selective and sensitive response due to the displacement of the capping aptamer in the presence of OTA and subsequent MB release was found in aqueous media. Finally, the possible use of the gated material for the detection of OTA in a real matrix was also explored.

Keywords: molecular gates, mesoporous materials, ochratoxin A, sensing, differential pulse voltammetry

# Introduction

Hybrid materials generated from the combination of molecular and supramolecular entities with inorganic supports have represented a remarkable improvement in the development of systems with advanced functionalities.([[1]](#endnote-1)) Among different scaffolds, MCM-41-type mesoporous silica materials are of great interest. This kind of support possesses nontoxic nature, high surface area and empty channels (mesopores) with large specific pore volume organized in a honeycomb-like structure.([[2]](#endnote-2)) In addition, its surface can be easily functionalized due to the presence of silanol groups. This last characteristic confers a valuable feature to the material. The chemical attachment of organic molecules to the surface results in hybrid organic-inorganic materials.([[3]](#endnote-3)) Among them, gated mesoporous materials have emerged with great force in the last years. These kind of materials, take advantage of the porous structure to accommodate molecules or other cargoes in the pore voids. Also, it allows anchoring molecular and supramolecular functional moieties to cap the pores, which are able to respond to a predefined stimulus and uncap the pores allowing the release of a previously entrapped cargo.([[4]](#endnote-4),[[5]](#endnote-5),[[6]](#endnote-6)) Gated materials have found key application as systems to release cargoes in a finely controlled way in the presence of physical (i.e. temperature, light, magnetic fields),([[7]](#endnote-7),[[8]](#endnote-8),[[9]](#endnote-9),[[10]](#endnote-10)) chemical (i.e. pH, redox changes, presence of small molecules)([[11]](#endnote-11),[[12]](#endnote-12),[[13]](#endnote-13)) or biochemical (i.e. enzymes, antigens, oligonucleotides)([[14]](#endnote-14),[[15]](#endnote-15),[[16]](#endnote-16),[[17]](#endnote-17)) stimuli. In particular, therapeutic applications where the cargo is preferentially released in a target cell/organ is one of the most common utility for such materials.([[18]](#endnote-18),[[19]](#endnote-19)) However, a growing new application for such gated materials is related with the recognition of selected chemical species. In this case, the gating mechanism situated in the pore outlets is only triggered by the presence of a certain analyte.([[20]](#endnote-20)) The recognition of the target species triggers the release of cargo molecules which act as a signaling agent. This unique feature foresees a promising advance in the field of the recognition of target species. At present it can be found in the literature disseminated examples for the detection of cationic,([[21]](#endnote-21)) anionic ([[22]](#endnote-22)) and neutral ([[23]](#endnote-23)) species with special emphasis in the development of systems able to recognize biologically related targets.([[24]](#endnote-24))

To allow controlled release features, gated materials have used the rupture/formation of covalent bonds,([[25]](#endnote-25), [[26]](#endnote-26)) electrostatic ([[27]](#endnote-27)) or supramolecular interactions,([[28]](#endnote-28), [[29]](#endnote-29)) or changes in the physical properties of molecules or macromolecules.([[30]](#endnote-30)) In this filed, an appealing approach is the use of aptamer-molecule interactions. Aptamers are a rising class of ligands composed by oligonucleotides or small peptides selected from a library by an easy and effective method.([[31]](#endnote-31)) This kind of ligands is non-toxic and it can be obtained through a fast and low-cost production process with minimal batch-to-batch variability.([[32]](#endnote-32)) Exploring the combination of mesoporous materials with aptamers to obtain capped materials Ozalp and coworkers developed the first apta-gate for the recognition of ATP.([[33]](#endnote-33)) In this example, the authors used an amino-terminated oligonucleotide sequence containing an ATP aptamer. The hairpin-like structure was grafted into the pore outlets of MSNs loaded with fluorescein. In their system, the hairpin structure of the oligonucleotide was able to block the pores of the material. The presence of ATP which selectively binds with the aptamer, induced a conformational change from a duplex to a less bulky single stranded DNA close to the surface which triggered the release of entrapped fluorescein. After this first example, other apta-gate designs were developed. Among them, examples where the aptamer is displaced from the material surface when the recognition takes place are especially appealing due to their simplicity.([[34]](#endnote-34)) In the reported examples, electrostatic or supramolecular interactions based on amine-phosphate interaction or hydrogen bonds between DNA strands are responsible to cap the pores. Following this approach several apta-gated systems able to recognize ATP,([[35]](#endnote-35)) Hg2+,([[36]](#endnote-36)) K+,([[37]](#endnote-37)) Mg2+,([[38]](#endnote-38)) Pb2+,([[39]](#endnote-39)) adenosine,([[40]](#endnote-40)) *BRCAI* gene mutations,([[41]](#endnote-41)) thrombin,([[42]](#endnote-42)) PSA,([[43]](#endnote-43)) cocaine,([[44]](#endnote-44)) arsenic,([[45]](#endnote-45)) or Bisphenol A ([[46]](#endnote-46)) has been developed.

Finally, most of the examples described in the literature take advantage of fluorogenic or chromogenic species as cargo which can be easily monitored by spectroscopic methods. Examples of gated materials able to detect selected species using alternative methodologies to monitor cargo release are still scarce. In particular, systems using electrochemical methods such as differential pulse voltammetry are certainly limited.([[47]](#endnote-47),[[48]](#endnote-48))

In this work we centered the attention in the possible recognition of Ochratoxin A (OTA). OTA is a mycotoxin produced by various species of *Aspergillus* and *Penicillium* which can contaminate preferentially cereals (wheat, corn, barely) and wines.([[49]](#endnote-49)) This mycotoxin is a known nephrotoxic, hepatotoxic, neurotoxic, immunotoxic, and also teratogenic agent which can produce, for example, lymphoid and gastro-intestinal tract lesions, myelotoxicity, and intestinal fragility as well as inhibition of lymphocytes T and B proliferation and IL2 production. Moreover, OTA has been classified as possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC) indicating the need of control of this molecule in food stuff. In line with this, European Commission has established regulatory limits to the presence of OTA for raw cereal grains (5 μg/kg), dried fruits (10 μg/kg), roasted coffee (5 μg/ kg), grape juice and wines (2 μg/kg).([[50]](#endnote-50)) Due to its harmful properties, several OTA detection methods have been developed.([[51]](#endnote-51)) Particularly, HPLC, mass spectroscopy and antibody based techniques are the most common procedures.([[52]](#endnote-52)) However, in general all these methods require qualified staff, finely prepared protocols and expensive equipment.

As a part of our interest in the development of gated materials for signaling applications, we report herein the design of an OTA recognition system based on aptamer-gated materials for an easy, quick and simple OTA selective response by the release of an electroactive species able to be measured using differential pulse voltammetry. In this work, mesoporous silica nanoparticles (MSN) loaded with a suitable electroactive guest molecule (Methylene Blue) and capped with an OTA aptamer was prepared and characterized. The ability of the prepared material to recognize OTA in aqueous solutions was studied. Also, the response as a function of the OTA concentration and potential selectivity was assessed. Finally, the possible application in a real matrix was also explored.

# Results and discussion

*Design and synthesis*

In this work, we specifically selected mesoporous silica nanoparticles (MSN) as inorganic scaffold and a suitable electroactive guest molecule (Methylene Blue) for monitoring purposes. The outer surface of MSNs was functionalized with isocyanate moieties able to form a urea bond with a linking oligonucleotide. Finally, the addition of OTA blocked the pores due to the hybridization of the linking oligonucleotide and the aptamer as depicted in Scheme 1.



Scheme 1. Performance of the gated solid **S3** able to detect OTA by DPV.

As described in the Experimental Section (*vide infra*), MSNs of ca. 100 nm in diameter were prepared following well-known procedures using TEOS as a hydrolytic inorganic precursor and hexadecyltrimethylammonium bromide (CTABr) as a porogen species. The solid was then calcined at 550°C to obtain mesoporous nanoparticles, which were loaded with the electroactive species MB and externally functionalized with (3-isocyanatopropyl)triethoxysilane to obtain solid **S1**. In a second step, the linking oligonucleotide **O1** (NH2-(CH2)6-5’-AAA AAA CCC CCC-3’) was anchored to the surface via the formation of a urea bond between isocyanate moieties and the amino terminal group of **O1**. This isolated solid was called **S2**. Finally, the OTA-selective aptamer **O2** (5’- TTTTG GGG GGG CAT CTG ATC GGG TGT GGG TGG CGT AAA GGG GGG GGT TTT-3’) ([[53]](#endnote-53)) was hybridized with the anchored oligonucleotide **O1** to give the final capped solid **S3** where MB release is anticipated to be inhibited by the presence of the aptamer onto the pore entrances. The opening protocol will be expected to occur by an effective aptamer displacement in the presence of OTA.

*Materials characterization*

The prepared materials were fully characterized by powder X-ray diffraction, transmission electron microscopy (TEM), nitrogen adsorption-desorption isotherms, and thermogravimetry. Powder X-ray diﬀraction (PXRD) patterns of as-synthesized MSNs, calcined MSNs, and solids **S1** and **S2** are shown in **Figure 1**. The obtained PXRD pattern of as-synthesized MSN (a) shows four low-angle reflections which correspond to (100), (110), (200), and (210) planes of an hexagonal array of pores, as in the MSN. Calcined MSN nanoparticles PXRD pattern shows significant shift and broadening of (100), (110), (200) planes with respect to as synthesized MSN which clearly indicates further condensation of silanol groups and subsequent cell contraction during the calcination step. Finally, the typical reduction in contrast between pores due to the presence of loaded dye and surface functionalization can be appreciated in PXRD of solids **S1** and **S2**. Nonetheless, the absence of changes in the (100) plane in the PXRD patterns indicates that the process of pore loading and additional functionalization did not modify the mesoporous structure of the mesoporous support of **S1** and **S2** to a large extent.



Figure 1. PXRD pattern of solids (a) MSN as synthesized, (b) calcined MSN, (c) **S1** and (d) **S2**.

Additionally, the mesoporous structure of the prepared solids was also conﬁrmed by transmission electron microscopy (TEM) analysis. **Figure 2** shows representative TEM images for calcined MSN and solids **S1**, **S2** and **S3**. The TEM pictogram of MSN shows spherical particles with the typical MCM-41-like hexagonal arrangement of the mesopores. This same visualization was found for solids **S1** and **S2**. Finally, TEM images of solid **S3** indicated the presence of a dense organic layer around the nanoparticles in accordance with the presence of the capping aptamer **O2**.



Figure 2. TEM images of (a) calcined MSNs and solids (b) **S1** (c) **S2** and (d) **S3**.

Also, N2 adsorption–desorption isotherms of calcined MSN and solid **S1** were registered. A typical curve for MCM-41-like mesoporous solids was obtained. As usually, a sharp adsorption step was recorded at intermediate P/P0 values (0.25–0.4) indicating the nitrogen condensation inside the mesopores by capillarity. The absence of a hysteresis loop in this interval suggested the presence of uniform cylindrical mesopores. From the obtained data a specific total surface of 1014.9 m2g-1 and a narrow pore distribution centered at 2.52 nm was calculated by using the Brunauer, Emmett and Teller (BET)([[54]](#endnote-54)) and Barrett–Joyner–Halenda (BJH)([[55]](#endnote-55)) models, respectively. In addition, a second feature can also be observed at a high relative pressure (P/P0>0.85), which can be ascribed to the typical textural properties of MSNs.

Finally, TGA of solids **S1**, **S2** and **S3** showed the typical behavior of functionalized mesoporous materials. In the thermograms, a first weight loss between 25 and 150 ̊C related to solvent escape can be appreciated. Secondly a sharp second step between 150 to 800 ̊ C due to the combustion of organic matter can be identified. Finally, a weight loss between 800 to 1000 ̊C was related to condensation of silanol group. From TGA data, the organic content for solids **S1**, **S2** and **S3** can be calculated as 0.135, 0.012 and 0.014 g/gSiO2, respectively. Changes in the organic content, especially the reduction from **S1** to **S2** and **S3** was due to partial MB leaching from the pores before capping the pores with **O2**.

*Study of performance of S3.*

Monitorization of electrochemical signals is a considerably easy way to obtain rapid, robust and low cost measures and offer an upgrade with respect to other methods. Among electroactive species, the redox indicator MB has been widely used in electroanalytical methods. In the present work, MSN were loaded with the electroactive species MB. The performance of solid **S3** was confirmed by release experiments. In particular, 200 µg of **S3** were suspended in 1000 µL of TRIS buffer and the suspension was divided into two fractions. The first fraction was diluted with 500 µL of Milli-Q water, whereas the second one was treated with 500 µL of an aqueous solution containing Ochratoxin A (1 mM). In both cases, the suspensions were stirred for 60 minutes at 25 ̊ C. At certain time the amount of MB released was registered by differential pulse voltammetry (DPV) directly by immersion of the electrodes in the **S3** suspension. As can be observed in Figure 3, in the absence of OTA no significant peak was obtained in the voltagramm. In contrast, in the presence of OTA, a current peak at 0.3755µA was registered.



Figure 3. DPV signal obtained from solid **S3** in (a) the absence and (b) in the presence of OTA 0.5 mM.

The delivery profile kinetics of MB in the presence and absence of Ochratoxin A from the capped MSN is shown in Figure 4. In the absence of OTA the system remains practically capped whereas in the presence of OTA 0.5 mM the signaling molecule MB was quickly released to the aqueous solution.



Figure 4. MB kinetics release profile from solid **S3** in (a) the absence and (b) in the presence of OTA.

In a step forward, it was explored the possible direct correlation between the increase in the registered current and the concentration of target analyte when solid **S3** was used to recognize OTA. Following a similar procedure to that described above, delivery of MB from **S3** as a function of different concentration of OTA was studied. Results are illustrated in Figure 5. The experiment showed that the delivered amount of cargo is proportional to the OTA concentration, displaying a typical competitive assay response curve in agreement with an uncapping protocol indicated in Scheme 1. The maximum MB delivery was registered when using an OTA concentration of 50 nM. Finally, a limit of detection of 0.003 nM of Ochratoxin A was estimated, which is in line with other OTA detection systems based on aptamers.([[56]](#endnote-56))



Figure 5. Registered current from aqueous solutions of solid **S3** as a function of concentration of OTA in Tris (pH=7.5) after 20 min of reaction.

In addition, to verify the selectivity of the method, similar experiments with solid **S3** were performed in the presence of 0.1 mM of selected mycotoxins such as Fumonisin B1 and Aflatoxin B1. The response of solid **S3** for each case is depicted in Figure 6. As it can be appreciated in the figure, solid **S3** is highly selective to the presence of OTA, whereas other relevant mycotoxins induced a rather poor cargo delivery. This opens the way to the possible use of **S3** material for OTA detection in highly competitive media. The obtained results clearly confirmed that the opening protocol occurs by a highly effective aptamer displacement in the presence of OTA.



Figure 6. Registered current variations from aqueous solutions of solid **S3** in the presence of a Tris solution, OTA, Fumonisin B1 (FUMB1) and Aflatoxin B1 (AFLAB1) after 20 min of reaction.

Finally, we explored the possibility to detect OTA in wheat samples. In a typical experiment, 0.05 g of wheat was doped with 10 µL of an aqueous solution of OTA (500 nM). OTA was then extracted from sample with MeOH:H2O 6:4. Then, after centrifugation, the liquid phase was evaporated and finally the residue was dissolved in 1 mL of MilliQ water. OTA content was then obtained using solid **S3** and an addition standard method. Aliquots of 100 μL were spiked with different known amounts of a standard OTA solution (0, 10, 15, 20, 30, 50, 75 nM). In a following step 100 μg of solid **S3** were added to each fraction and filled to a final volume of 1000 μL with hybridization buffer. After 30 min, the DPV signal of MB released from solid **S3** in the different aliquots was measured. From the registered measurements, an OTA recovery of 76.35 % was obtained, which confirmed the practical application of the developed sensing material.

# Conclusions

We reported herein aptamer-capped mesoporous silica nanoparticles for the electrochemical detection of OTA. The system consists of MSNs loaded with the electroactive species MB and capped with an OTA aptamer. A highly selective and sensitive response due to the displacement of the capping aptamer and subsequent MB release in the presence of OTA was found in aqueous media and in real samples. The development of systems similar the described herein could open new perspectives in the development of new rapid sensing structures with exquisite sensibility and selectivity.

# Experimental section

*Chemicals*

Tetraethyl orthosilicate (TEOS), n-cetyl trimethyl ammonium bromide (CTABr), sodium hydroxide (NaOH), Methylene Blue, (3-isocyanatopropyl)triethoxysilane, and Ochratoxin A, Fumonisin B1 and Aflatoxin B1 were purchased from Sigma-Aldrich Química. Oligonucleotides **O1** (NH2-(CH2)6-5’-AAA AAA CCC CCC-3’) and **O2** (5’- TTTTG GGG GGG CAT CTG ATC GGG TGT GGG TGG CGT AAA GGG GGG GGT TTT-3’) were purchased from Isogen-Lifesciences. All products were used as received without further purification. Tris buffer (pH 7.5) was prepared by taking 1.21 g of Tris (hydroxymethyl) aminomethane and 3.81 g of MgCl2·6H2O in 500 mL of milliQ water. pH of the solution was adjusted using HCl.

*General techniques*

Powder X-ray difraction (PXRD), thermogravimetric analysis (TGA), elemental analysis, transmission electron microscopy (TEM), N2 adsorption – desorption isotherms, and differential pulse voltammetry techniques were employed to characterize the prepared materials. PXRD measurements were performed on a Philips D8 Advance diffractometer using Cu Kα radiation. Thermogravimetric 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. N2 adsorption-desorption isotherms were recorded on a Micromeritics TriStar II sorption analyser. The samples were degassed at 120 °C under vacuum overnight. The specific surfaces areas were calculated from the adsorption data in the low pressures range using the BET model. The pore size was determined by the BJH method.

*Synthesis of mesoporous silica nanoparticles*

In this study, we selected spherical nanometric mesoporous silica nanoparticles (MSNs) from the MCM-41 family as support. MCM-41-type mesoporous nanoparticles were synthesized by dissolving 1.00 g of n-cetyltrimethylammoniumbromide (CTABr) in 480 mL of deionized water. Then 3.5 mL of NaOH (2.00 M) in deionized water was added to the above CTABr solution, followed by adjusting the solution temperature to 80 ºC. Tetraethyl orthosilicate (5 mL) was then added drop wise to the surfactant solution with observation of slight formation of white precipitate. Then, the mixture was allowed to stir for 2 h to give a white precipitate. Finally, the formed solid was centrifuged and washed with deionized water. As-synthesized MSNs were dried at 60 ºC and calcined at 550 ºC using oxygen atmosphere for 5 h in order to remove the template phase and get the porous MCM-41-type nanoparticles.

*Synthesis of solid S1*

Solid **S1** was prepared by suspending 100 mg of synthesized MSNs and 2.55 mg (0.08 mmol) of Methylene Blue dye in 13 mL of acetonitrile in a round-bottom flask and in an inert atmosphere. The suspension was heated at 130 ºC and 10 mL of the solvent was collected using a Dean-Stark apparatus to remove the moisture in the reaction medium. Then, the mixture was allowed to stir for 24 hours at 36 ºC with the aim of achieving maximum dye loading in the pores of the MSNs scaffolding. After this time, an excess of (3-isocyanatopropyl)triethoxysilane was added directly to the mixture (3.684 mL, 5.0 mmol) and the suspension was stirred for 5.5 h. Finally, the obtained blue solid was filtered off and dried at 70 ºC for 12 h.

*Synthesis of solid S2*

1 mg of synthesized **S1** was suspended in 700 µL of anhydrous acetonitrile containing MB 0.085 mg/mL. Then 100 µL of an aqueous solution of oligonucleotide **O1** (100 µM) and 2 µL of triethylamine were added and allowed to stir for 3 h at room temperature. Above suspension was centrifuged and washed several times with milliQ water and dried overnight under inert atmosphere.

*Synthesis of the capped solid S3*

1 mg of solid **S2** was suspended in 1000 µL of Tris buffer. After sonication, the suspension was divided in 5 fractions of 200 µL. Each 200 µL fraction was treated with 5 µL of an aqueous solution of the OTA aptamer **O2** (100 µM) and the volume reaction was adjusted until 600 µL with Tris buffer. After 2 h, the suspension was centrifuged and washed several times with Tris and dried overnight under inert atmosphere.

*Release experiments*

To perform release experiments. An amount of solid S3 was suspended in 1000 µL of TRIS buffer and the suspension was divided into two fractions. The first fraction was diluted with 500 µL of Milli-Q water, whereas the second one was treated with 500 µL of an aqueous solution containing Ochratoxin A (1 mM). Relesea experiments were conducted always under mild stirring at 25 ̊ C.

*DPV measurements*

MB in the collected samples were measured by using differential pulse voltammetry (DPV) analysis in Autolab 302N impedance analyzer (Ecochemie, The Netherlands). The signal was measured in the range between -0.5 and -0.25 V using an applied potential of 50 mV/s. A current maximum at 0.3755 µA was monitored in the presence of MB which was correlated with MB concentration.

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