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**ARTICLE** *in* ANALYTICAL CHEMISTRY · MARCH 2014

Impact Factor: 5.64 · DOI: 10.1021/ac500517h · Source: PubMed



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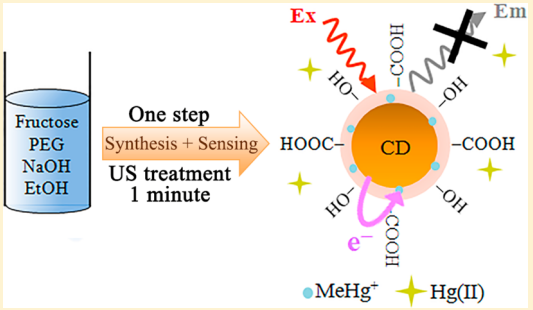
# In Situ Building of a Nanoprobe Based on Fluorescent Carbon Dots for Methylmercury Detection

Isabel Costas-Mora, Vanesa Romero, Isela Lavilla, and Carlos Bendicho*

Departamento de Química Analítica y Alimentaria, Área de Química Analítica, Facultad de Química, Universidad de Vigo, Campus As Lagoas-Marcosende s/n, 36310 Vigo, Spain

Supporting Information

**ABSTRACT:** A new fluorescent assay based on in situ ultrasound-assisted synthesis of carbon dots (CDs) as optical nanoprobes for the detection of methylmercury has been developed. Application of high-intensity sonication allows simultaneous performance of the synthesis of fluorescent CDs within the analytical time scale and the selective recognition of the target analyte. Microvolume fluorospectrometry is applied for measurement of the fluorescence quenching caused by methylmercury. The assay uses low amounts of organic precursors (fructose, poly(ethylene glycol), and ethanol) and can be accomplished within 1 min. A detection limit of 5.9 nM methylmercury and a repeatability expressed as a relative standard deviation of 2.2% ($N$ = 7) were obtained. CDs displayed a narrow size distribution with an average size of 2.5 nm as determined by electron transmission microscopy. To study the quenching mechanism, fluorescence, atomic absorption spectrometry, and Fourier transform infrared spectrometry were applied. Hydrophobicity of methylmercury and its ability to facilitate a nonradiative electron/hole recombination are suggested as the basis of the recognition event. A simple and green assay is achieved for quick detection of methylmercury without the use of tedious sample preparation procedures or complex and expensive instrumentation.




In the past several years, the development of fluorescent probes for the rapid and simple detection of a wide variety of analytes has attracted increasing attention. Organic dyes have been widely used as fluorescent probes, but these tend to have some limitations such as poor photostability, narrow excitation spectra, and broad absorption bands with red tailing. Recently, new fluorescent systems have been proposed to overcome these limitations. Among them, semiconductor nanocrystals, also called quantum dots (QDs), have been extensively applied for the detection of several ions and organic compounds.[1−3] The main properties of QDs are their broad absorption spectrum, narrow, symmetric, and tunable emission band, large quantum yields, and high photostability,[4] which account for the superior optical properties in comparison with those of conventional organic dyes. Nevertheless, the main drawbacks of QDs lie in their complicated and expensive synthesis routes and their composition, since toxic elements are commonly present in their structure.

As a new alternative to QDs, carbon dots (CDs) have recently been described by Xu et al.[5] The main properties of CDs include strong fluorescence, tunable color emission, and high photostability, but the main features that make them attractive are the absence of toxic elements and the ease of synthesis using simple, inexpensive, and ecofriendly carbon sources, such as glucose, sucrose, citric acid, or glycerol, among others.

In spite of the great potential of CDs for detection of chemical species, only a few works have reported the use of CDs as luminescent probes.[6] Concerning the analysis of metal ions, the most widely studied analyte is Hg(II),[7−10] which usually causes the fluorescence quenching of CD-based systems.

Mercury is a well-known environmental pollutant, its main organic form (i.e., methylmercury) being more toxic to living systems than inorganic species[11] due to its lipid solubility and ability to cross biological membranes. Methylmercury has been recognized as a potent neurotoxin that causes damage to the brain and nervous system, being particularly hazardous for pregnant women and infants.[12] Methylmercury is able to be accumulated and bioamplified in the human body through the food chain, so the main human exposure to methylmercury is linked to fish consumption.[13] Usually, complex hyphenated techniques based on separation techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC), or capillary electrophoresis (CE) coupled to a specific detector such as mass spectrometry (MS),[14] inductively coupled plasma mass spectrometry (ICPMS),[15] or atomic fluorescence spectrometry (AFS),[16] are required for methylmercury detection. In addition, implementation of extraction/preconcentration procedures is usually needed.[14,17−22] Nowadays, there is a need for simple and fast assays that can be

**Received:** February 6, 2014
**Accepted:** March 28, 2014

   dx.doi.org/10.1021/ac500517h | *Anal. Chem.* XXXX, XXX, XXX−XXX

applied for on-site analysis, focused on the sensitive detection of toxic metal species such as methylmercury. To date, no work regarding the detection of methylmercury using CD-based systems has been reported.

Several pathways have been reported for the synthesis of fluorescent CDs, including microwave treatment, thermal carbonization, acid dehydration, electrochemical methods, and ultrasonic treatment.[6,23] It is well-known that ultrasonic irradiation causes a variety of physical and chemical effects derived from acoustic cavitation and provides a unique interaction between energy and matter.[24,25] Until now, only three synthetic pathways have been proposed for the fabrication of fluorescent CDs by ultrasonic treatment.[26−28] In spite of the great advantages of using ultrasound energy for synthesizing CDs, the time required is too large (from 2 to 24 h), probably due to use of low-intensity ultrasonic devices (e.g., ultrasonic bath). In addition, the processes of synthesis, functionalization, and later interaction of CDs with the target analyte, to prepare the fluorescent probe, are very time-consuming. An alternative way to overcome this drawback would be to carry out in situ synthesis of CDs in the presence of the analyte to be detected. Therefore, the synthesis and interaction with the target analyte would occur almost at the same time.

Herein, we describe for the first time a fluorescent assay based on in situ ultrasound-assisted synthesis of CDs using D-fructose as the carbon source for the detection of methylmercury. Carbon dot growth under ultrasonic treatment in the presence of the target analyte extends the potential of CD-based systems as fluorescent probes. A sensing mechanism accounting for the selectivity of the assay toward methylmercury is outlined.

## EXPERIMENTAL SECTION

**Reagents and Chemicals.** Ultrapure water was obtained from an Ultra Clear TWF EDI UV TM system (Siemens AG, Barsbuettel, Germany).

D-Glucose, D-fructose, sucrose, glycerol, citric acid, bovine serum albumin (BSA), glutathione (GSH), and L-cysteine (Sigma-Aldrich, St. Louis, MO) and starch (Probus, Badalona, Barcelona, Spain) were tested as carbon sources.

Alcohols, including methanol (Prolabo, Paris, France), ethanol (Prolabo), 1-propanol (Sigma-Aldrich), 2-propanol (Merck, Darmstadt, Germany), and 1-butanol (Prolabo), were tried for the synthesis of CDs.

Poly(ethylene glycol) (PEG; $M_w$ = 200) was purchased from Sigma-Aldrich. NaOH, $HNO_3$, and HCl were obtained from Prolabo.

A 1000 mg $L^{-1}$ stock solution of $CH_3Hg^+$ was prepared from $CH_3HgCl$ (Riedel-de Haën, Seelze, Germany). Working solutions were prepared by dilution of the stock solution as required.

The following compounds were employed for assessing the selectivity of the sensing approach: $As_2O_3$ (Merck), $Bi_5O(OH)_9(NO_3)_4$ (Merck), $CaCO_3$ (Aldrich), $CaSO_4{\cdot}2H_2O$ (Merck), CdO (Aldrich), $CoCl_2{\cdot}6H_2O$ (Scharlau, Barcelona, Spain), ethylenediaminetetraacetic acid (EDTA) (Carlo Erba), $K_2Cr_2O_7$ (Prolabo), $KNO_3$ (Probus), $CuCl_2{\cdot}2H_2O$ (Merck), $FeCl_3{\cdot}6H_2O$ (Sigma), $HgCl_2$ (Prolabo), humic acid (Fluka), $MgCl_2{\cdot}6H_2O$ (Prolabo), NaCl (Sigma-Aldrich), $Ni(NO_3)_2{\cdot}6H_2O$ (Panreac, Barcelona, Spain), $Pb(NO_3)_2$ (Panreac), $SbCl_3$ (Carlo Erba, Italy), $Na_2SeO_3$ (Aldrich), and Sn (Probus). In addition, two organometals, i.e., methylcyclopentadienylmanganese tricarbonyl (MMT) and tetrabutyltin, purchased from Aldrich and Merck, respectively, were also tried as potential interfering agents.

The applicability of the method for $CH_3Hg^+$ detection in water and marine animal tissues was evaluated using the following certified reference materials (CRMs): DORM-3 (fish protein) and DOLT-4 (dogfish liver) from the National Council of Canada (Ottawa, Ontario, Canada) and BCR 464 (tuna fish muscle) and BCR 610 (groundwater) from the Community Bureau of Reference (Brussels, Belgium). The latter CRM was fortified due to the absence of methylmercury. In addition, recovery studies were performed with several real water samples.

**Apparatus.** A Sonopuls ultrasonic processor model HD 3200 (Bandelin electronic-GmbH & Co. KG, Berlin, Germany) with an output power of 200 W and a frequency of 20 ± 0.5 kHz was used for both CD synthesis and methylmercury extraction from fish. This system is equipped with an MS 72 titanium microtip of 2 mm diameter, which allows working with sample volumes in the range of 2−30 mL.

A Thermo Scientific NanoDrop 3300 fluorospectrometer was used to carry out fluorescence measurements. The technical specifications and operation mode have been outlined in earlier works.[29,30] Fluorescence measurements were performed at 517 nm after excitation at a wavelength of 470 nm using the blue LED. This portable instrument is well suited to field analysis since it only requires connection through a USB to a computer. UV− vis absorption measurements were performed using a NanoDrop model ND-1000 spectrophotometer.

Transmission electron microscopy (TEM) was performed with a JEOL JEM-1010 microscope operating at an acceleration voltage of 100 kV. The TEM samples were prepared by dropping the sample onto a carbon-coated copper grid.

Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet 6700 spectrometer within the range of 400−4000 $cm^{-1}$ with a resolution of 4 $cm^{-1}$.

To study the selectivity of the fluorescent assay toward methylmercury in the presence of Hg(II), a direct mercury analyzer DMA-1 (Milestone Srl, Sorisole, Italy) was used. This Hg analyzer does not require any sample preparation. Total Hg is directly converted into Hg vapor upon sample combustion followed by amalgamation and measurement by atomic absorption. Experiments were undertaken under the following instrument parameters: sample drying for 120 s at 200 °C, sample decomposition for 180 s at 750 °C, waiting for 30 s, and amalgam heating for 12 s.

**Sample Preparation.** The ultrasound-assisted extraction in marine animal tissues was carried out as follows: A 0.4 g sample of each CRM (BCR 464, DORM-3, and DOLT-4) was accurately weighed into polypropylene centrifuge tubes. Then 5 mL of 5 M HCl solution was added, and the suspension was sonicated at room temperature for 4 min at 12% sonication amplitude. After extraction, the supernatant was subjected to filtration through a PTFE syringe filter (0.45 μm pore size) and cleanup using C18 cartridges. Blanks were treated in the same way. Water samples were filtrated through 0.45 μm cellulose nitrate filters (Sartorius) and stored at 4 °C.

**Experimental Procedure for in Situ Synthesis of CDs and $CH_3Hg^+$ Sensing.** First, 40−45 mg of D-fructose was placed in a 7 mL glass vial. Then 1 mL of acidified water sample or standard (0.1 M HCl) was added, and D-fructose was dissolved. Later, 1.2 mL of PEG, 200 μL of 3.5 M NaOH, and 100 μL of ethanol were added to the mixture. Finally, the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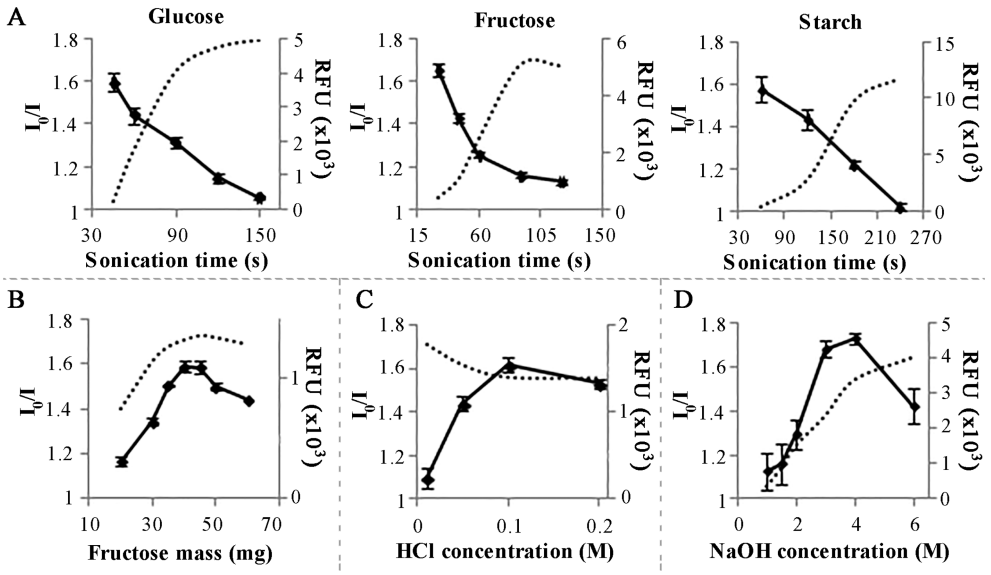


**Figure 1.** (A) Analytical response for the different carbon sources used as precursors of fluorescent CDs. (B) Effect of fructose mass (B). Effect of HCl concentration (C). Effect of NaOH concentration (D). The solid line represents the analytical response ($I_0/I$), whereas the dotted line indicates the intrinsic fluorescence intensity (relative fluorescence units, RFU) of CDs.

mixture was subjected to ultrasound irradiation for 60 s at 10% sonication amplitude with the ultrasonic probe directly inserted into the sample vial. The color of the bulk solution changed from clear to light yellow and then to brown. Lastly, when the solution reached room temperature, a 2 $\mu$L aliquot of the sample was withdrawn and placed onto the pedestal of the microfluorospectrometer to measure the fluorescence signal. A blank experiment was performed before each measurement.

## RESULTS AND DISCUSSION

**Ultrasonic Synthesis of CDs: Optimization of Experimental Parameters.** To evaluate the effects of the different variables involved in the CD-based assay for methylmercury detection and the interactions between variables, the $2_V^{5-1}$ factorial fractional design was applied for screening optimization.[31] This design allows assessment of the effect of 5 variables with 16 experiments. The significance of variable effects and their interactions were established by comparison with the experimental error, calculated as 2 times the average standard deviation of all experiments. Standardized effects are shown in Figure S-1 (Supporting Information). As can be observed, all studied variables had a significant effect, and no relevant interactions between them were found, so the effect of these variables was investigated in detail following the univariate approach.

**Selection of the Carbon Source and Concentration.** Since sonochemical synthesis of CDs has not been extensively studied to date, a preliminary evaluation of the different carbon sources to synthesize CDs with suitable optical properties was made.

In this sense, several carbon precursors were tested, including D-glucose, D-fructose, sucrose, starch, glycerol, citric acid, BSA, and GSH using 30 mg of each compound. Nevertheless, only glucose, fructose, and starch gave rise to fluorescent CDs. In the rest of the cases, no fluorescent signal was observed in spite of the fact that longer sonication times (up to 7 min) were employed. Given that the sonication time needed to form CDs varied for the different carbon sources, the formation kinetics were studied upon application of different sonication times. The results are shown in Figure 1A.

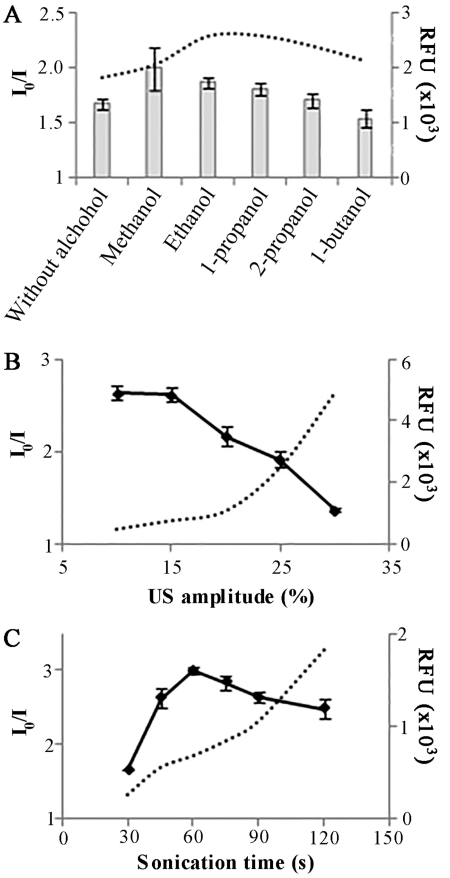


**Figure 2.** (A) Effect of the type of alcohol added. (B) Effect of the ultrasound amplitude. (C) Effect of the sonication time. The solid line represents the analytical response, whereas the dotted line indicates the intrinsic fluorescence intensity (relative fluorescence units, RFU) of CDs.


dx.doi.org/10.1021/ac500517h | Anal. Chem. XXXX, XXX, XXX−XXX

Table 1. Comparison of Analytical Characteristics of Different Methods for $CH_3Hg^+$ Detection

| method[a] | linear range | LOD (nM) | repeatability (%) | ref |
|---|---|---|---|---|
| HPLC−VG-AFS | | 5.1 | 1.6 | 16 |
| micro-HPLC−ICPMS | | 0.1 | 0.5−2.1 | 15 |
| SPME−ICPMS | 0−6.9 μM | 0.9 | 2.4 | 20 |
| SDME−HPLC−photometry | 23−556 nM | 4.6 | 5.3 | 21 |
| HS-SDME−ETAAS | 0−1.4 μM | 20 | 7.0 | 22 |
| DLLME−CE | 46−927 nM | 8.3 | 3.2 | 17 |
| DLLME−HPLC−ICPMS | 0.05−9.27 nM | 0.04 | 6.8 | 18 |
| DLLME−HPLC−DAD | 23−463 nM | 4.4 | 7.3 | 19 |
| SBSE−TD−GC/MS | 0.5−46.4 nM | 0.1 | | 14 |
| fluorene-based chemodosimeter (spectrofluorimetry) | 0−60 μM | 44.0 | | 35 |
| metal−organic framework (spectrofluorimetry) | 0−14 μM | 27.8 | | 36 |
| lysozyme-type VI-stabilized Au NCs (spectrofluorimetry) | 15−500 nM | 4.0 | | 37 |
| Rhodamine 6G-modified Au NPs (spectrofluorimetry) | 0.05−10 μM | 10 | | 38 |
| CD nanoprobe microfluorospectrometry | 23−278 nM | 5.9 | 2.2 | this work |

[a]Abbreviations: AFS, atomic fluorescence spectrometry; CE, capillary electrophoresis; CVG, chemical vapor generation; DAD, diode array detection; DLLME, dispersive liquid−liquid microextraction; ETAAS, electrothermal atomic absorption spectrometry; FI, flow injection; GC, gas chromatography; HPLC, high-performance liquid chromatography; HS-SDME, headspace single-drop microextraction; ICP, inductively coupled plasma; MS, mass spectrometry; NCs, nanoclusters; NPs, nanoparticles; SBSE, stir bar sorptive extraction; SDME, single-drop microextraction; SPME, solid-phase microextraction; TD, thermal desorption; VG, vapor generation.

Table 2. Analytical Results for the Detection of $CH_3Hg^+$ in Water Samples and Certified Reference Materials

| Water Samples | | | |
|---|---|---|---|
| sample | added $CH_3Hg^+$ concn (nM) | found $CH_3Hg^+$ concn (nM) | recovery (%) |
| river water | 0 | <LOD[a] | |
| | 46 | 43.6 ± 3.5 | 94.8 |
| | 116 | 111 ± 7 | 95.7 |
| | 232 | 219 ± 14 | 94.4 |
| tap water | 0 | <LOD | |
| | 46 | 42.3 ± 2.5 | 92.0 |
| | 116 | 104 ± 8 | 89.7 |
| | 232 | 214 ± 14 | 92.2 |
| sea water | 0 | <LOD | |
| | 46 | 45.7 ± 1.6 | 99.3 |
| | 116 | 119 ± 9 | 103 |
| | 232 | 234 ± 5 | 101 |
| BCR-610 | 23 | 20.9 ± 1.3 | 90.9 |
| Marine Animal Tissues | | | |
| CRM | certified value (mg/kg of $CH_3Hg^+$) | found value (mg/kg of $CH_3Hg^+$) | recovery (%) |
| BCR 464 | 5.50 ± 0.17 | 6.28 ± 0.47 | 114 |
| DOLT-4 | 1.43 ± 0.129 | 1.34 ± 0.084 | 93.7 |
| DORM-3 | 0.381 ± 0.060 | 0.369 ± 0.040 | 96.9 |

[a]LOD = detection limit.

As can be observed in Figure 1A (dotted line), CDs are synthesized in short sonication times using glucose or fructose, whereas longer times are needed to achieve the formation of fluorescent CDs from starch as the carbon source. It must be highlighted that, at the end of the reaction, the intrinsic fluorescence intensity of CDs synthesized from starch is nearly 3-fold higher than that for glucose or fructose, probably due to the large number of glucose units contained in its structure.

To evaluate the most suitable CD precursor for sensing $CH_3Hg^+$, the synthesis experiments were performed in the presence of $CH_3Hg^+$ and the fluorescence signal was monitorized at different sonication times. It was observed (Figure 1A, solid line) that the formation kinetics were not altered by the presence of $CH_3Hg^+$ but the fluorescence of CDs was quenched. With all CD precursors tested, the effect on the fluorescence intensity was higher with a decrease of the sonication time. In fact, sonication times of 40, 45, and 60 s for D-fructose, D-glucose, and starch, respectively, provided the best analytical responses ($I_0/I$). In view of the obtained results, fructose was selected as the carbon source for further experiments. It should be highlighted that the time required for the synthesis of fluorescent CDs is remarkably lower than that reported, i.e., 2−24 h.[26−28]

The effect of fructose mass was also studied. As can be noted in Figure 1B, this variable notably affects both the intrinsic fluorescence intensity of CDs and the analytical response in the presence of $CH_3Hg^+$. A D-fructose mass in the range of 40−45 mg provides both a suitable intrinsic fluorescence intensity of CDs (dotted line) and a good analytical response (solid line).

**Effect of pH.** Several studies have revealed that the pH of the medium strongly influences the fluorescence of CDs,[32,33] so the study of this variable is critical for the development of the fluorescent assay.

It must be mentioned that, although the final pH of synthesis must be strongly basic for CD formation, an acidic medium was used in the sample solution to ensure the stability of $CH_3Hg^+$ prior to the assay. Therefore, the HCl concentration was studied in the range of 0.01−0.2 M (Figure 1C). It was found that the analytical response increased with the HCl concentration up to 0.1 M and then a plateau was reached, so this concentration was chosen for further experiments.

As has been mentioned above, the presence of a basic medium is mandatory for the synthesis of fluorescent CDs. For this, 200 μL of NaOH was added to the medium before sonochemical treatment. The effect of NaOH concentration in the range of 1−6 M was studied (Figure 1D). A strong effect on the fluorescence of CDs was observed, a steady analytical response being found for NaOH concentrations in the range of 3−4 M. Probably, this effect is due to the deprotonation of groups at the CD surface leading to the repulsion of fluorescent CDs. A 200 μL sample of 3.5 M NaOH was injected into the synthesis medium to achieve optimal performance. Under those conditions, the final pH of the medium was ∼13.


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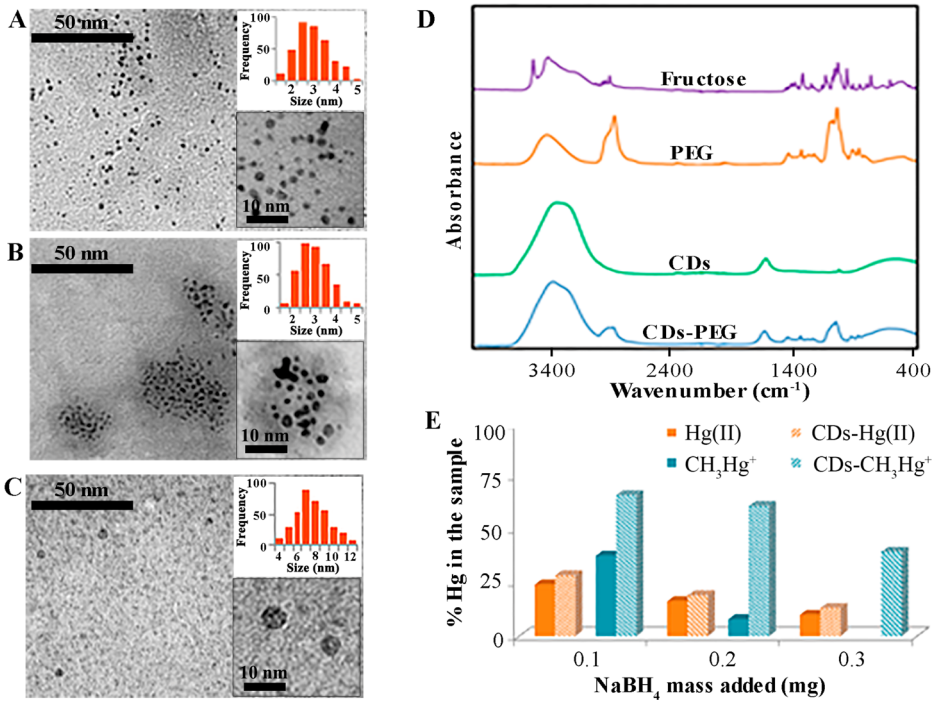


**Figure 3.** TEM images of CDs (A) prepared under optimized conditions, (B) prepared in the absence of PEG, and (C) prepared by application of ultrasound energy for 2 min. (D) FT-IR spectra for the different samples studied. (E) Volatilization experiments of Hg(II) and $CH_3Hg^+$ in samples containing fructose + PEG with and without ultrasonic treatment (the percentage of Hg remaining after hydridation is shown).

**Table 3. Comparison of the Main Characteristics of Synthesis Procedures for CDs**

| synthetic approach | precursor[a] | CD size (nm) | total time for synthesis | total volume (mL) | ref |
|---|---|---|---|---|---|
| microwave treatment | citric acid/1,2-EDA | 3 | 2 min + dialysis for 3 days | 10 | 39 |
| | glucose/$PEG_{200}$ | 5 | 2 min + dialysis overnight | 100 | 33 |
| hydrothermal method | EG/$H_2SO_4$ | 1−4 | 8 h | 30 | 8 |
| | BSA/$H_2SO_4$ | 1−2 | 2 h + dialysis | 4 | 32 |
| | citric acid/BPEI | 4−10 | 3 h | 10 | 40 |
| | lactose/Tris | 1.5 | 24 h + dialysis for 2 days | 40 | 41 |
| | $PEG_{200}$/NaOH | 5 | 6 h + dialysis | 60 | 9 |
| ultrasonic treatment (bath) | glucose/$NH_4OH$ | 10 | 24 h + dialysis | 100 | 28 |
| | glucose/NaOH | <5 | 28 h | 120 | 26 |
| | glucose/HCl | <5 | 10 h | 100 | 26 |
| | active carbon/$H_2O_2$ | 5−10 | 15 h | 75 | 27 |
| ultrasonic treatment (probe) | D-fructose/PEG/NaOH | 2.5 | 1 min | 2.5 | this work |

[a]Abbreviations: BPEI, branched poly(ethylenimine); BSA, bovine serum albumin; 1,2-EDA, 1,2-ethylenediamine; EG, ethylene glycol; PEG, poly(ethylene glycol); PVP, poly(vinylpyrrolidone); Tris, tris(hydroxymethyl)aminomethane.

**Effect of the PEG Concentration.** Addition of PEG was needed to achieve fluorescent CDs due to its effects as a passivation agent. The effect of the PEG concentration was studied in the range of 40−80% (v/v). We found that a concentration of 50% (v/v) is enough to achieve the best results, since the intrinsic fluorescence intensity remains constant at higher concentrations and a maximum analytical response is achieved. Therefore, 50% (v/v) PEG was employed for the rest of the experiments.

**Effect of Alcohol Addition.** The addition of organic compounds in sonochemical synthesis of metallic nanoparticles leads to an increase in the reaction kinetics due to the generation of a large number of secondary radicals.[34] For this reason, the effect of different alcohols (i.e., methanol, ethanol, 1-propanol, 2-propanol, and 1-butanol) on CD synthesis was studied.

Figure 2A shows the obtained results for the different straight-chain alcohols tested. An increase in the intrinsic fluorescence intensity of CDs as well as an improvement of the analytical response, except in the case of 1-butanol, were observed. The addition of methanol caused a significant uncertainty within replicates. A 100 $\mu$L sample of ethanol was added for further experiments since it provides suitable sensitivity and precision.

**Effect of the Sonication Amplitude and Time.** The sonication amplitude was also studied in the range of 10−30%. As can be observed in Figure 2B, the best analytical response is achieved at low sonication amplitude (10−15%), while higher values give rise to a decrease in the analytical response. On the other hand, the intrinsic fluorescence intensity of CDs decreases with a decrease of the sonication amplitude. The sonication time was studied in the range of 30−120 s. It can be observed that the intrinsic fluorescence intensity increases with


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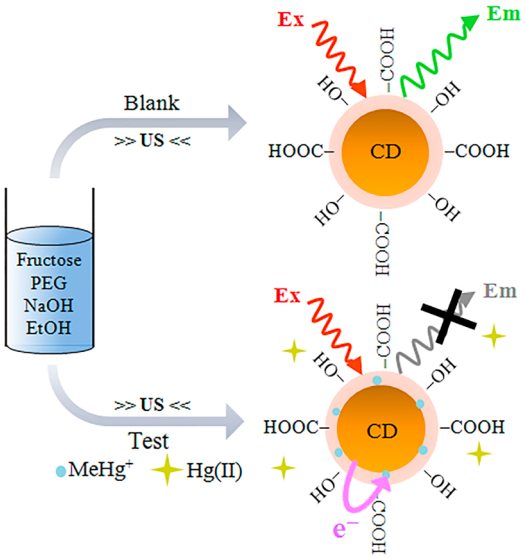


**Figure 4.** Schematic representation of the mechanism involved in the fluorescence quenching caused by the presence of $CH_3Hg^+$.

the sonication time whereas the best analytical response is achieved within 60 s of sonication time, which provides a fast method for synthesis and sensing of $CH_3Hg^+$.

**Study of Potential Interferences.** The effect of potential interferences (i.e., metal ions, organometals, salts, complexing agents, and natural organic matter) in the detection of $CH_3Hg^+$ in water samples was studied. The results of this study are shown in Table S-1 (Supporting Information).

None of the 16 metal species tested caused any noticeable interference effect at 0.5 mg/L (signal variation within ±20%). Salts and complexing agents typically present in water samples such as $MgCl_2$, NaCl, EDTA, $KNO_3$, $CaSO_4$, and $CaCO_3$ and humic acid (i.e., natural organic matter) did not cause any interference effect either. The absence of interference due to Hg(II) ions must be highlighted. Hg(II) did not cause any noticeable interference effect even at a concentration 250-fold higher than that of $CH_3Hg^+$, which makes the assay highly selective for methylmercury detection.

**Analytical Characteristics and Recovery Studies.** When CD synthesis is performed in the presence of $CH_3Hg^+$, a significant fluorescence quenching occurs and no spectral shift of the emission band is observed (Figure S-2, Supporting Information). The quenching effect is well described by the Stern–Volmer equation

$$I_0/I = K_{SV}[Q] + C$$

where $I_0$ is the fluorescence intensity of CDs synthesized in the absence of $CH_3Hg^+$, $I$ is the fluorescence intensity after quenching, $K_{SV}$ is the Stern–Volmer constant, $Q$ is the analyte concentration, and $C$ is a constant.

Analytical figures of merit were established under optimal conditions for the detection of $CH_3Hg^+$. The calibration curve was linear in the range of 23–278 nM $CH_3Hg^+$. The slope of the calibration curve, which represents the Stern–Volmer constant, was $1.1 \times 10^7$ $M^{-1}$. The limit of detection (LOD), estimated following the $3\sigma$ criterion, was 5.9 nM (~1.2 ppb). The repeatability of the analytical response (i.e., $I_0/I$), expressed as the relative standard deviation, was 2.2% ($N$ = 7), whereas the reproducibility studied as between-day precision was 4.1% ($N$ = 3). A comparison of several methods for the detection of $CH_3Hg^+$ is displayed in Table 1.

The figures of merit of our sensing approach for $CH_3Hg^+$ are comparable with or even better than those of methods using expensive and complicated chromatographic interfaces and/or preconcentration steps. In situ synthesized CDs using high-intensity sonication allow a simple and fast detection of $CH_3Hg^+$, where simple, safe, and inexpensive precursors are required (i.e., fructose, ethanol, and PEG).

**Analysis of Water and Fish.** The accuracy of the CD-based assay for the detection of $CH_3Hg^+$ was evaluated by analyzing different certified reference materials of water and marine animal tissues. Found contents in the marine tissues were in agreement with the certified values for $CH_3Hg^+$ in all cases (recoveries in the range of 93–114%), no significant differences being observed between the certified and experimental values ($t$ test, $p$ = 0.05). Several recovery studies were also performed using different real water samples (i.e., river, tap, and sea waters). Recoveries of $CH_3Hg^+$ in the water samples varied between 90% and 103%. The analytical results are shown in Table 2.

**Characterization of the CD-Based Nanoprobe.** The morphology and size of CDs synthesized by sonochemical treatment of fructose were studied following TEM measurements. Under optimized conditions, CDs synthesized by our proposed method are well dispersed and have a small size. The inset in Figure 3A shows the size histogram to prove that as-prepared CDs are narrowly distributed with an average size of 2.5 nm.

In addition, the main function of PEG is the stabilization of CDs, preventing their agglomeration and resulting in colloidal nanoparticles. This fact was verified by TEM analysis (Figure 3A–C). In the case of performing the synthesis in the absence of PEG (Figure 3B), the nanoparticle size was similar to that of CDs synthesized in the presence of PEG, but CD agglomeration was found.

Other experiments indicated that the sonication time had a great effect on the size of CDs (Figure 3C). In fact, the application of ultrasound for 120 s instead of 60 s led to the formation of CDs with an average size of 7 nm, i.e., nearly 3 times greater.

To determine the surface functional groups, different analyses by FT-IR were performed. As can be observed in Figure 3D, different bands were found for the studied species. The sharp band centered at ~3350 $cm^{-1}$ corresponds to the stretching vibrations of O–H groups, whereas the peak centered at ~2900 $cm^{-1}$ is typically associated with $sp^3$ C–H stretching vibrations of alkane groups. The low-intensity peaks in the 400–1450 $cm^{-1}$ range can be assigned to the bending vibrations of C–H bonds. In this region, a peak centered at ~1080 $cm^{-1}$ can be easily identified, which is attributed to the stretching vibrations of C–O. By comparing all FT-IR results, a new peak centered at 1643.4 $cm^{-1}$ was found, which may be assigned to C=O groups. This new peak may be due to the sonochemical treatment of fructose, which causes changes in its structure as revealed by the FT-IR spectra. All results suggest that the surfaces of CDs are surrounded by hydrophilic groups, which accounts for the great potential of CDs for sensing in aqueous samples.

Sonochemical synthesis of CDs has advantages over other synthesis methods (e.g., microwave treatment, thermal carbonization, acid dehydration, or electrochemical methods, among others), including fast reaction rates and the production of very


dx.doi.org/10.1021/ac500517h | Anal. Chem. XXXX, XXX, XXX–XXX

small CDs and, thus, higher surface-to-volume ratio. The main features of the most widely applied synthesis methods to date for CD formation are compared in Table 3.

In view of the data reported in Table 3, the reduced time required for the synthesis of CDs with a size near 2.5 nm must be highlighted. This reduced time allows one to use CDs for in situ sensing without the need for purification processes or additional studies on stability and storing conditions. Another relevant advantage of the proposed system resides in the possibility of performing it at room temperature, which makes it a safe synthesis method. Another remarkable feature is the good reproducibility of the synthesis procedure. In fact, no relevant changes in the optical properties of CDs were observed when they were synthesized on different days. A variation within ±1% in the intrinsic fluorescence intensity was observed, which is considerably lower than that obtained upon microwave treatment of carbohydrates, i.e., one of the most extended methods for synthesis of CDs.

**Investigation of the Sensing Mechanism.** To date, several CD-based systems have been developed for metal ion detection, Hg(II) ions being the most studied. Nevertheless, to the best of our knowledge, this is the first system based on the use of CDs that allows the selective detection of $CH_3Hg^+$. The main recognition mechanism reported in the CD-based systems for Hg(II) detection involves the electrostatic interaction between Hg(II) ions and CD functional groups.[8,9] Nevertheless, the latter interaction is unlikely to occur in our approach since mercury species are not ionized. At the pH of the assay (i.e., pH 13), the Hg species present should be $Hg(OH)_2$ and $CH_3HgOH$.[42]

To go in depth into the quenching mechanism involved and make an explanation for the high selectivity of the fluorescent assay toward $CH_3Hg^+$, several experiments were performed with solutions containing Hg(II) and $CH_3Hg^+$.

First, the possibility of losing Hg(II) by adsorption or volatilization was considered. Adsorption onto the vessel walls could occur when solutions of metal ions at trace level are stored. For testing potential losses by adsorption during the assay, alternative reaction vessels made of PTFE and quartz were used. The results showed no effect caused by the wall material on the analytical response, and the same behavior as in glass vials was found for both Hg species. Another process that could account for the different behavior of Hg(II) and $CH_3Hg^+$ in the CD-based assay is the volatilization of Hg species promoted by ultrasound. Ultrasound-promoted volatilization of Hg(II) has been reported from acid solutions in the presence of certain organic additives.[43] To study this process, solutions containing both Hg species were subjected to sonochemical treatment in the presence of all reaction components (i.e., D-fructose, PEG, NaOH, and EtOH), and the Hg remaining was quantified by a direct mercury analyzer. It was found that both species, Hg(II) and $CH_3Hg^+$, remained in the solution after the synthesis of CDs, which indicates that the selectivity of the assay cannot be ascribed to the loss of Hg(II) during the sonochemical treatment.

In view of the above results, other characteristics such as hydrophobicity of $CH_3Hg^+$ should be taken into account to explain the selectivity of the assay. Hydrophobicity facilitates the permeation of $CH_3Hg^+$ through the PEG coating, thus enhancing its further interaction with CDs. Therefore, $CH_3Hg^+$ is expected to interact with CDs more strongly than Hg(II) for fluorescence quenching to occur. This different behavior could be probed using hydridation with $NaBH_4$ and further analysis of the Hg remaining in the solution following the same approach as above. The weaker the interaction of Hg species with CDs−PEG, the easier the volatilization by hydridation. Hg analysis in the reaction media containing fructose, PEG, NaOH, and EtOH was carried out with and without sonochemical treatment to compensate for any matrix effects in the hydridation procedure.

As can be observed in Figure 3E, addition of 0.3 mg of $NaBH_4$ is needed to volatilize 90% of the initial Hg(II), regardless of whether CD synthesis takes place. This finding means that no interaction between CDs−PEG and Hg(II) exists. On the other hand, $CH_3Hg^+$ was volatilized by hydridation with 0.2 mg of $NaBH_4$ when no CD formation was carried out. However, when CD synthesis is performed in the presence of $CH_3Hg^+$, only 35% of the initial amount of this methylated Hg species can be volatilized, meaning that it becomes strongly attached to CDs−PEG. Once $CH_3Hg^+$ is attached to CDs, fluorescence quenching should occur. To gain insight into the quenching mechanism involved in the sensing of $CH_3Hg^+$, several studies were performed. First, emission and absorption spectra were obtained, and no shift of spectral bands was observed (Figure S-2, Supporting Information). This implies that neither changes of the CD composition nor formation of a complex occurs, so static quenching should be discarded. When dynamic quenching occurs, collisions between fluorophores and quenchers are involved, and these are influenced by the temperature and viscosity of the medium.[44] It was found that the calibration slope is affected by changes of these parameters, hence confirming that dynamic quenching is caused by collisions between CDs and $CH_3Hg^+$ when the latter passes through the PEG layer. Ultimately, fluorescence quenching occurs as a result of nonradiative electron/hole recombination through an effective electron transfer process, as has been suggested for other QD-based systems.[7]

Additional studies made with thiol-containing compounds support our proposed mechanism. One key feature of L-cysteine or GSH lies in its ability to form complexes with Hg-containing compounds through thiol groups. Upon addition of each compound prior to the synthesis of CDs, the complexation of $CH_3Hg^+$ with L-cysteine or GSH should prevent further interactions of $CH_3Hg^+$ with CDs. Indeed, it was found that no fluorescence quenching occurred when the CD synthesis was performed in the presence of $CH_3Hg^+$ and thiol-containing compounds. Nevertheless, when the addition of those thiols is carried out after synthesis of CDs in the presence of $CH_3Hg^+$, no recovery of the fluorescence is achieved, probably due to the strong entrapment of $CH_3Hg^+$. Therefore, thiol compounds are not able to remove it from the CD surface.

It was observed that the addition of $CH_3Hg^+$ to the reaction medium after CD synthesis caused only a weak fluorescence quenching (ca. 3-fold lower than that for direct synthesis in the presence of $CH_3Hg^+$) and complete recovery of the fluorescence occurred upon addition of L-cysteine or GSH. This finding indicates that $CH_3Hg^+$ is integrated within the CDs−PEG during sonochemical synthesis, and therefore, those thiols are not able to remove $CH_3Hg^+$ by complexation. This experiment confirms the results obtained above using hydridation with $NaBH_4$.

Thus, the selectivity of the assay can be ascribed to the different abilities of both Hg species to interact with CDs encapsulated in PEG. $CH_3Hg^+$, a hydrophobic species, should easily cross the PEG coating and come into contact with CDs,


dx.doi.org/10.1021/ac500517h | Anal. Chem. XXXX, XXX, XXX−XXX

this increased transport being facilitated by ultrasound. Hg(II), a hydrophilic Hg species, cannot interact in this way with CDs, and no fluorescence quenching should be expected. In contrast to other fluorescent assays involving CDs,[8,9] no interaction between Hg(II) and surface hydrophilic groups from PEG (i.e., −OH and −COOH) can occur under these conditions, as the Hg species present at the pH of this assay is $Hg(OH)_2$. The suggested mechanism involved in the recognition of $CH_3Hg^+$ is outlined in Figure 4.

## CONCLUSIONS

Integration of fluorescent CD synthesis and sensing within a single step allows an optical nanoprobe to be built, which has proved to be highly selective for $CH_3Hg^+$ detection at the nanomolar level. A recognition event based on the hydrophobicity of $CH_3Hg^+$ and its ultrasound-assisted permeation through the passivation coating made of PEG is proposed. The assay can be accomplished within 1 min and is well suited for field analysis with the use of a portable fluorospectrometer.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org/

## AUTHOR INFORMATION

### Corresponding Author

*Phone: +34-986-812281. Fax: +34-986-812556. E-mail: bendicho@uvigo.es.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

Financial support from the Spanish Ministry of Economy and Competitiveness (Project CTQ2012-32788) and the European Commission (FEDER) is gratefully acknowledged. I.C.-M. thanks the University of Vigo and V.R. thanks the Spanish Ministry of Education, Culture and Sports for predoctoral research grants. E. López and J. B. Rodríguez (CACTI, University of Vigo) are gratefully acknowledged for performing the FT-IR and TEM measurements, respectively.

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