A vinyl sulfone clicked carbon-dot-engineered microfluidic paper-based analytical device for fluorometric determination of biothiols

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ABSTRACT:

A microfluidic paper-based analytical device integrating carbon-dot (CDs) is fabricated and used for a fluorometric off-on assay of biothiols. Vinyl sulfone (VS) click immobilization of carbon dots (CDs) on paper was accomplished by a one-pot simplified protocol that uses divinylsulfone (DVS) as a homobifunctional reagent. This reagent mediated both the click oxa-Michael addition to the hydroxyl groups of cellulose and ulterior covalent grafting of the resulting VS-paper to NH_2-functionalized CDs by means of click aza-Michael addition. The resulting cellulose nanocomposite was used to engineer an inexpensive and robust microfluidic paper-based analytical device (μPAD) that is used for a reaction-based off-on fluorometric assay of biothiols (GSH, Cys and Hcy). The intrinsic blue fluorescence of CDs (with excitation/emission maxima at 365/330 nm) is turned off via the heavy atom effect of an introduced iodo group. Fluorescence is turned on again by the displacement of iodine by reaction with a biothiol. The increase in fluorescence is related to the concentration over a wide range (1 to 200 μM for GSH, and 5-200 μM for Cys and Hcy, respectively), and the assay exhibit a low detection limit (0.3 μM for GSH and for Cys and 0.4 μM Hcy, respectively). The method allows for rapid screening and can also be used in combination with a digital camera readout.

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1. Introduction

Microfluidics is nowadays a flourishing technology embraced by most engineering disciplines including chemical sensing and biosensing.[1] In this field, microfluidics has become a common and powerful designing tool for the fabrication of (bio)sensors, analytical devices that miniaturize laboratory process aimed to the detection of a specific target by converting and amplifying a (bio)molecular recognition event to a dateable semi-quantitative or quantitative signal. Microfluidic (bio)sensing devices,[2] with their micrometer-scaled features, offer exceptional capabilities to integrate multiple functions or analytical steps (sample transfer, mixing, separation, and signal output) in a sole unit with derived benefits such as the need of small sample volume, low material consumption and cost, fast runaround time, and a facile automatization.

Albeit traditional microfluidic (bio)sensing devices are typically made from silicon and glass, because of the geometrical precision and inertness for reuse that these materials provide, and polymer materials, recent trends have moved the interest to cellulose paper as an appealing analytical platform.[3] The attractiveness of paper in this domain lies on its intrinsic natural porous structure, a physical characteristic that enables paper for spontaneous capillary-based fluidic transport of sample and reagents and also confers the benefits of reagent storage, mixing, flow control, and multiplex analysis.[4] Moreover, paper is earth-abundant, inexpensive, flexible, biodegradable, lightweight and a conveniently processable and chemically active matrix that permits its functionalization,[5] usually intended to enhance the original cellulose properties and the incorporation of novel functionalities to improve the sensing of the resulting engineered devices. For these reasons, microfluidic paper-based analytical devices (μPADs),[6] while retaining the miniaturization and integration characteristics of their counterparts, are also powerful tools for point-of-care testing (POCT) as they accomplish the ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users) criteria outlined by the World Health Organization (WHO) for POCT.[7] Therefore, μPADs have emerged as a leading alternative for the development of portable and disposable diagnostic devices aimed to high-tech applications.[8]

In order to provide conventional μPADs with higher versatility and multifunctional performance, the integration of diverse emerging functional nanomaterials into paper has been implemented to μPADs in the last decade expanding their potential.[9] Hybrid cellulose composites incorporating nanoparticles (NPs) has shown great potential for the fabrication of sophisticated (bio)chemical assay that exhibit excellent selectivity and sensitivity, and rapid response.[10] Nobel metal (Au and Ag), bimetals (including semiconductor quantum dots -QDs-) and metal oxides nanoparticles (NPs) as well as carbon nanomaterials (graphene, carbon nanotubes and carbon dots -CDs-) have been successfully incorporated into paper leading to NP-based paper (bio)chemical sensors. These devices have a well-designed nano-architectures with the nanomaterials being normally used to construct the sensing interfaces where serves as static signal reporters or as carriers for loading additional signal reporters.

Although the detection methods commonly used in (bio)sensing have been integrated into μPADs,[11] optical techniques are still prevalent because of their sensitivity and operational simplicity. Among the different

methods used to detect the optical response signal emitted from paper-based devices,[12] fluorescence spectroscopy in its numerous variations have attracted enormous interest and is nowadays a powerful tool.[12] As the majority of (bio)analytes do not exhibit fluorescence, the detection and quantification of these compounds using a fluorescent assay would require the mediation of fluorescent probes and for this purpose selected NPs are paradigmatic optical signal reporters. In this respect, QDs has been a rational choice for the preparation of fluorogenic paper nanocomposites because of their inherent and excellent optical properties such as a large Stokes shift, high fluorescence efficiency, broad excitation spectra, photobleaching resistance, narrow, symmetric, and tunable emission spectra, and simultaneous excitation of multiple fluorescence colors. However, substantiated concerns about the toxicity of these heavy-metal NPs have turned the attention to emergent CDs[13] as a valuable alternative to perform as fluorescent reporters in paper-based sensors because their low toxicity and other distinctive advantages: convenient synthesis, low toxicity, prominent biocompatibility, high resistance to photobleaching, colorful photoluminescence, high chemical resistance, high water solubility, easy functionalization, and low cost.[14] For this reason, in the actual state-of-the-art of paper-based (bio)chemical sensors, CDs-modified cellulose is becoming an appealing platform that, in the particular case of μPADs, have been applied in few reported cases.[15,16]

In this paper, we describe the vinyl sulfone (VS) click immobilization of CDs on paper by a one-pot simplified protocol that uses divinylsulfone (DVS) as a homobifunctional click reagent that allows the click oxa-Michael addition to the paper surface and ulterior covalent grafting of the resulting VS-cellulose to NH_2-functionalized CDs by means of click aza-Michael addition. Besides the well-known use of DVS as a cross-linking reagent of diverse (bio)polymers,[17] including cellulose with different purposes, the DVS-mediated immobilization of biomolecules, particularly enzymes, to cellulose is also a proven methodology.[18] However, the implementation of click VS click coupling for engineering CDs-based fluorogenic cellulose platform is described herein for the first time. As a proof-of-concept of the applicability of the novel platform, we fabricate an engineered fluorogenic CDs-based μPAD, and we demonstrate its applicability for a reaction-based off-on analytical evaluation of biothiols.

2. Materials and methods

2.1. Materials and reagents

L-Glutathione (GSH); L-cysteine (Cys); DL-homocysteine (Hcy); L-serine (Ser); L-histidine (His); L-galactose (Gal); L-lysine (Lys); L-arginine (Arg); L-asparagine (Asn); D-mannose (Man); ethanol; ethanolamine; triethylamine (TEA); dichloromethane (DCM); sodium hydrogenphosphate; sodium dihydrogenphosphate; sodium hydrogencarbonate; sodium carbonate; anhydrous citric acid (CA); iodoacetic acid (IAA); divinyl sulfone (DVS); branched polyethylenimine 25 kDa (25kPEI); N,N-dicyclohexylcarbodiimide (DCC); N,N-diisopropylethylamine (DIPEA) were purchased from Sigma-Aldrich (www.sigmaaldrich.com). NH_2-functionalized CDs were prepared following a slightly modified reported hydrothermal synthesis starting form citric acid (CA) as the carbon source[19] (see ESM). All reagents were

of analytical-reagent–grade unless stated otherwise. Whatman grade 1 chromatography paper from Sigma-Aldrich was used to manufacture the μPAD. All aqueous solutions were made using reverse-osmosis type quality water (Milli-RO 12 plus Milli-Q station from Millipore, conductivity 18.2 MΩ·cm).

2.2. Apparatus, instruments and software

The fluorescence intensity of the functionalized paper analytical device was measured with a Sony Cyber-shot DSC-HX 300 digital camera (Sony, Tokio, Japan) after illumination with an UV transilluminator 220 V (MUV 21 series). A script of MATLAB was used to analyze the region of interest (ROI) of the image along with the Color Space Converter. Fluorescence spectra were obtained using Cary Eclipse UV-Vis fluorescence spectrophotometer. A pH meter (Crison micro pH 2000) was utilized to measure the pH values of aqueous solutions. The analytical devices were fabricated on paper using a craft-cutting technique as a cost-efficient, simple and reproducible process using a 12 W CO_2 laser engraver (Rayjet, www.rayjetlaser.com). The size, morphology and characterization of the CDs were examined by high resolution transmission electron microscopy (HRTEM) using a FEI TITAN G2 microscope operating 200 kV. The XPS spectra measurements were performed on an X-ray photoelectron spectroscopy (Kratos Axis ultra-DLD) for characterization of the functionalized paper analytical device and CDs. The maps of the paper by elemental mapping scanning transmission electron microscopy (STEM) were carried out using scanning transmission electron microscopy Zeiss SUPRA 40 VP. All of these studies were carried out at the Centre of Scientific Instrumentation (University of Granada, Spain).

2.3. Preparation of CDs-paper: VS-click immobilization of CDs

The chemical immobilization of CDs into cellulose paper was carried out in a two-step procedure that use DVS as a homobifunctional cross-linker (Scheme 1). In detail, disks (3 mm-diameter) of Whatman grade 1 filters were first produced using a cutting technique that was optimizing using a sheet paper (150 mm-diameter) for the production of 120 replicas. In a first step, twenty paper disks (3 mm-diameter) were first immersed into a buffer (3 mL, 0.5 M Na_2CO_3, pH 12) and, then, DVS (180 μL) was added. The mixture was shacked with a stirrer (250 rpm, 25 ºC, 12 h). The resulting **VS-paper** disks were washed with 1:1 ethanol/water and drying at room temperature. In a second step, the activated **VS-paper** disks were deposited into a phosphate buffer (1 mL, 10 mM, pH 7.4) containing **NH_2-CDs** (5 μL, $1.72x10^{-5}$ mg·mL^{-1}), and shacked with a stirrer (250 rpm, 25 ºC, 6 h). The resulting **CDs-paper** disks were washed with 1:1 ethanol/water and drying at room temperature. To block the unreacted free VS groups, the pieces of paper were shacked (10 min) with a mixture of ethanolamine (100 μL), and trimethylamine (20 μL) in water (3 mL). Finally, the papers were washed with 1:1 ethanol/water and dried under vacuum at room temperature and stored in a dry environment at 4 °C in the dark until use.

2.4. Preparation of CDs-I-paper

The **CDs-paper** disks were immersed into a solution of DCM (30 mL) containing IAA (3.72 mg·mL^{-1}), DCC (4.12 mg·mL^{-1}) and DIPEA (2.58 mg·mL^{-1}). The vessel with the mixture was covered with an aluminum foil

to protect from the light and was orbital shook (6 h). After this time, the functionalization of the paper disks was estimated to be complete, and the resulting **CDs-I-paper** disks were washed with 1:1 ethanol/water and stored in a plastic box cover with aluminum foil to protect them from the light.

2.5. Image capture and processing

Cellulosic disks were imaged with a digital camera after reacting with standards or bioanalytes containing samples (Fig. S2). The optimized setting conditions used to photograph the devices were: ISO 1250, shutter speed 1/80 s, aperture value f/3.2 and focal length of 4 mm. The ISO parameter was investigated (Fig. S3) by increasing the fluorescence intensity from 400 to 4000 ISO because of the sensitivity of the camera increase. The imaging position of camera was also considered because of its effect on the imaging results, which should not be overlooked. Paper was placed under a UV lamp using a homemade accessory. This chamber has nine holes which let the fluorescence intensity of the paper through the box when we make a picture with a digital camera avoiding that the light from the lamp interferes in the measurement (Fig. 1). The UV source was used at 365 nm wavelength and 220 W. For the analysis of **CDs-paper** the fluorescence intensities of the captured images were converted to grey scale using a new MATLAB function to obtain the region of interest (ROI) that contains the relevant analytical information in a circular area of 3.4 mm diameter. This MATLAB function has been developed to automatically detect the circular zones of the image, to establish a subarea of each detected circle containing the main pixels with relevant analytical information (this allows to discard the pixels of the edges and those corresponding to possible shadows of the image) and to calculate the grey scale of each circular subarea.

2.6. Analytical protocol

Biothiols determination were carried out, under the optimized conditions by using a **CDs-I-paper** disk or a μPAD device. For **CDs-I-paper,** the disk was immersed into a sample of the analyte (GSH, Cys or Hcy, 1 mL, 1:10 phosphate buffer, pH 7.4, 10 mM) under dark conditions and a nitrogen atmosphere for 30 min to ensure complete reaction. After this time, the disk paper was washed with Milli-Q water, dried under vacuum at room temperature, and the measurement performed as indicated in previous section. All of the measurements were performed at least three times and the standard deviation was plotted as the error bar. For the μPAD device, a solution (5 μL) of different concentrations of GSH, Cys or Hcy (ranging from 1-500 μM) was dropped onto the sampling area. After flowing of the solution by capillarity towards the **CDs-I-paper** disk the free iodine liberated in the reaction was removed after 30 min of reaction by washing with MilliQ water (3 x10 μL), and the measurement performed as indicated above. The procedure employed for the detection of biothiols in human urine was the same descript for standard solutions of GSH, Cys or Hcy.

3. Results and discussion

3.1. Paper selection for CDs immobilization

The selection of the paper substrate to be used was envisaged as the first step. According with the sensing functionality expected for the intended μPAD, the optimal material selected should meet two criteria: a low fluorescence background and a homogeneous distribution of fluorescence in the detection area. With these requirements, a series of commercial quantitative filter papers of different filtration (6 types from 1 to 30 μm pore size, and an 80-100 $gr \cdot m^{-2}$ basis weight) and 3 types of Nylon membranes (1240, 1242, 1254, PC1, 1244, Whatman 1, Nytran SPC, Biodyne Plus, Biodyne C, respectively) were tested for their fluorescence properties (see Fig. S4). The data indicated that standard Whatman grade 1 filters show the lower fluorescence profile being selected as the optimal material for their engineering.

3.2. Fabrication of CDs-immobilized cellulose (CDs-paper)

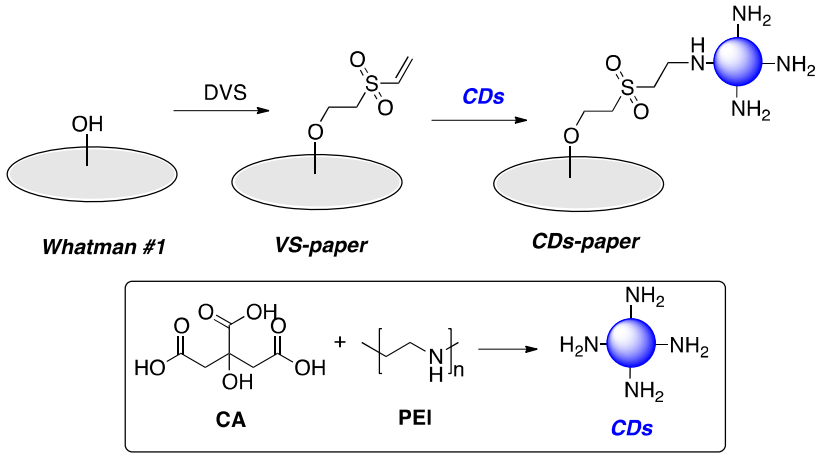
The engineering of CDs-modified paper sensors requires CDs immobilization to the cellulose surface as one of the cornerstone steps to enable sensing applications. For this purpose, adsorption and covalent grafting are the most commonly used approaches. The adsorption of CDs on paper is mediated by the electrostatic and Van der Waals forces being a successful, simple and inexpensive methodology to functionalize cellulose. However, preliminary studies for the immobilization of CDs by adsorption on standard Whatman grade 1 paper does not work as CDs are dragged by the solutions used causing an irregular distribution of the fluorescence as demonstrated by an assay based on the quenching effect of Cu^{2+} [20] (Fig. S5).

Taking into account this finding, the covalent immobilization of CDs was envisaged as an alternative pathway. The chemical functionalization by covalent bonding provides better binding of CDs to the paper surface regardless of the major number of steps usually required and the plausible detrimental of the paper properties. Because of these drawbacks, the chemical conjugation approach of CDs has been scarcely used up to the present.[21-23] From the panoply of methodologies developed for the modification of cellulose in diverse scenarios,[5,24] the synthetic strategies applied on the reported cases dealing with CDs combine the complementary reactivity of the abundant hydroxyl groups on cellulose with that of the surface functional groups of CDs (mainly carboxylates and amines). However, due to the inertness of the hydroxyl functions, a pre-activation step is required for the construction of cellulose-CDs covalent bridges. For CDs-modified cellulose oxidation of the hydroxyl groups for their transformation in more reactive carbonyl or carboxyl groups is the reported strategy that was followed by the formation of amide bridge with the CDs through common EDC/NHS reagents.[21-23] With this background, developing efficient methods for the covalent functionalization of CDs-cellulose composite materials is of utmost importance for engineering smart sensors.

In this context, the click-chemistry philosophy offers already unexplored possibilities for its implementation in the fabrication of novel CDs-modified cellulose materials. Owing to the remarkable attributes of simplicity, modularity, robustness and high conversion under mild reactions conditions, click chemistries have found significant utility in material science, including NP modification,[25] but a more limited application in the surface functionalization of cellulose. From the toolbox of click reactions, the prevalent Cu(I)-catalyzed azide-alkyne cycloadditions (CuAAC) and also thiol-ene reactions are the protocol most often employed for accessing clicked-cellulose materials.[26,27] However, the introduction of the corresponding clickable

groups, in both of the mentioned approaches, is a multi-step process that, despite the efficiency of the click assembly, discourages its application. Considering the bulk nature of cellulose paper and in order to find simple click alternatives for its modification that also precludes any purification step, we envisaged the Michael-type addition of vinyl sulfone (VS), a less considered click reaction, as a valuable option to solve these problems because VS chemistry can perfectly combine the inherent functionality (hydroxyl for cellulose and amino for CDs) of the two assembling units.

With this background, we designed a reliable and straightforward one-pot two-step procedure for the fabrication of CDs-immobilized paper **CDs-paper**- (Scheme 1). For our purpose, divinylsulfone (DVS) is the reagent of choice as it can behave as a homobifunctional click reagent that will allow, first, its click oxa-Michael addition to the paper surface by reaction with the hydroxyl groups, and, later, the covalent grafting of the resulting reactive **VS-paper** to NH_2-functionalized CDs by means of a click aza-Michael addition. Among the broad panoply of aminated CDs described in the literature, NH_2-functionalized CDs were prepared following a reported hydrothermal synthesis (180 ºC, 10 h) that use citric acid (CA), as the carbon source, and the polyamine branched polyethylenimine 25kDa (25kPEI), as a N-doping agent and surface modifier.[19] The anchoring of the CDs to the surface of cellulose paper was, then, performed by a reliable and simple sequence of two steps: first, VS functionalization of disks papers -**VS-paper**- by immersion into a buffered (pH 12) DVS solution, and, then, covalent click conjugation of the NH_2-functionalized CDs by an additional immersion of the **VS-paper** into a buffered (pH 7.4) solution of the CDs. Effectiveness of the immobilization was assessed by the quenching of the fluorescence mediated by Cu^{+2} [20] (Fig. S6) and by the physical characterization of the resulting **CDs-paper** (see below).

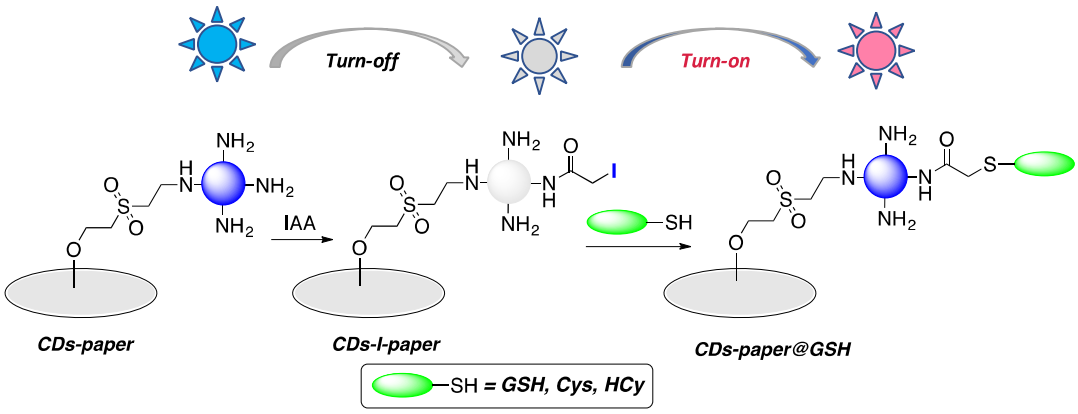


Scheme 1. VS-based click immobilization of CDs onto paper: Preparation of clicked **CDs-paper**

3.3. Evaluation of the sensing capabilities of CDs-paper

To prove the sensing capabilities of the fabricated fluorogenic clicked **CDs-paper** in view to later engineering μPADs that use the immobilized CDs as signal reporters, the biothiols glutathione (GSH), cysteine (Cys), and homocysteine (Hcy) were chosen as bioanalytes of interest because they play an important role in cellular functions and maintaining the biological redox homeostasis. GSH is the most plentiful intracellular thiol and

plays a vital role in maintaining biological homeostasis by acting as an essential endogenous redox regulator.[28] Abnormal level of cellular GSH is considered to be sign of many diseases, like liver damage, cancer, Alzheimer's and cardiovascular diseases. Deficiency of Cys is usually associated with leukocyte loss, hematopoiesis decrease, skin lesions etc. Furthermore, abnormal levels of Hcy can induce a risk factor for cardiovascular and Alzheimer's diseases.[29,30] Although great efforts in fluorescent probes have been devoted to detect these biothiols selectively and efficiently in biological samples, the contributions based on CDs[31-33] or in paper-based analytical devices[34,35] are limited. CDs-based biothiols sensors are usually fluorescence "off–on" devices that exploit a strategy based on the fluorescence quenching of the CDs by a sacrificial quencher (normally a metal ion: Au^{3+}, Ag^{+}, Cu^{2+}) and subsequent fluorescence revival by a reaction-based process with GSH, Cys or Hcy. However, in the context of our work the reported photochemical heavy-atom effect [36] exhibit by halogens (Cl, Br and I) that determines quenching of fluorescent probes (turn off) is a particularly appealing because of the easy halogen derivation of NH_2-functionalized CDs by reaction with monohalo-acetic acid. Later halogen nucleophilic displacement by a thiol-containing derivative elicits a significant fluorescent increase (turn on), strategy that have been applied in the design of CDs fluorescence probes for the detection of biothiols.[33] On this basis, the **CDs-paper** was reacted with iodoacetic acid (IAA) being obtained the corresponding **CDs-I-paper** by a straightforward immersion protocol (Scheme 2). The absent of fluorescent emission observed (turn-off) for this halogen-modified material confirms the role of iodine as a quintessential fluorescence quencher confirming, furthermore, the chemical functionalization of the paper, in concordance with our expectative (see below). With the **CDs-I-paper** in hands, the recovery of the fluorescence intensity (turn-on) was evaluated and confirmed when the -SH group of GSH, Cys or Hcy causes nucleophilic substitution, and release of iodine anions.



Scheme 2. Fabrication of fluorogenic off-on CDs-paper and its application in biosensing of biothiols (GSH, Cys, Hyc)

3.4. Characterization of CDs and functionalized papers

The CDs and the functionalized papers were characterized by means of techniques currently used in the characterization of CDs, namely, high-resolution transmission electron microscopy (HR-TEM), energy

dispersive X-ray spectroscopy (EDX), and X-ray photoelectron spectroscopy (XPS). First, HR-TEM images of the CDs (Fig. S1A) were obtained to attain information about their size and morphology. The presence of lattice planes with an interplanar distance around 3.22 nm indicates a high crystallinity for the CDs. CDs size are distributed between 2-5 nm, with a 3 nm average particle size (Fig. S1B), and the NPs show a circular shape, in concordance with the literature.[19] Elemental analysis by energy dispersive X-ray spectroscopy (EDX) were next performed to know the composition of CDs. The spectrum shows the presence of carbon, nitrogen, and oxygen atoms (Fig. S1C) and also a peak corresponding to uranium atoms, because of the use of uranyl acetate for the dye of CDs to improve their contrast for HRTEM analysis. Furthermore, the elemental composition of the CDs was also performed by XPS surface analysis. The results indicated the presence of carbon, oxygen and nitrogen in the CDs surface.

To attain information about the successful covalent click immobilization of CDs in cellulose paper, their iodine derivatization and their reactivity with biothiols, the surface of the corresponding paper disks was also characterized. To this aim, unmodified cellulose Whatman grade 1 paper was first characterized by XPS (Fig. S7). As expected, the obtained data for the elemental composition merely indicate the presence of carbon and oxygen atoms. After DVS treatment, the resulting **VS-paper** exhibit a typical doublet S_{2p} peak (168.0 eV) which is attributed to the sulfone group. In addition, the atomic quantification shows 8.25% S atoms corroborating the successful VS functionalization of the cellulose fibers (Fig. S7A). After VS-click immobilization of CDs, the XPS spectrum of **CDs-paper** show a predominant N_{1s} peak (400 eV), indicative also of the successful covalent anchoring of CDs (Fig. S7B). For the **CDs-I-paper** obtained after reaction with IAA, a I_{3d} peak (620 eV) confirms the incorporation of I atoms onto the cellulose with a 0.64% value. (Fig. S7C). After reaction of this I-functionalized cellulose with GSH for its determination, an 4.89% increase of S atom was quantified, data that confirms the substitution of iodine by the sulfur atom of the thiol group of GSH (Fig. S7D). The spatial distribution and compositional zonation of chemical elements in the samples of the different functionalized-papers was also elucidated by an element mapping performed by energy-dispersive X-ray spectroscopy (EDS) (Fig. S8). For **VS-paper**, the main constituents are C, O, and S and the mapping show a uniform distribution of S atoms on the paper surface (Fig. S8A). In addition, the elemental mapping for the **CDs-I-paper** reveals iodo signals after the IAA treatment surface (Fig. S8B).

Finally, fluorescence emission of **CDs-paper** and **CDs-I-paper** was evaluated (Fig. S9). In the first case, for a 330 nm wavelength excitation, the fluorescence spectrum of **CDs-paper** shows a peak with a maximum emission wavelength of 450 nm. By the contrary, this peak disappears for **CDs-I-paper** after functionalization of the CDs by IAA.

3.5. Fabrication of the µPAD

The microfluidic paper-based device (µPAD) designed to biothiols were engineered by using a design comprising four separate zones as shown in Fig. 1. The first zone is designed as a sampling and transporting paper-based area to move the biothiol (GSH, Cys or Hcy) sample toward the detection area. The second zone is the detection area where the CDs are immobilized covalently on a circular paper disk - **CDs-paper-**

previously prepared. The third zone is a paper-based transport channel to sweep along the free iodide produced after reaction of biothiol with the **CDs-I-paper**. The quarter area is a layer of Flexicel to be used as an absorbent pad to retain the free iodide. The different zones that make up the device were designed using Illustrator software (Adobe Systems) and the design exported as a FS file to the controller software of a desktop laser engraver. The different zones were aligned, assembled together and laminated with thermoplastic material (30 µ biaxially oriented polypropylene 30 film) with a clothes iron leaving a hole for sampling. Lamination improves the robustness of the paper device and also protects biothiols from atmospheric oxygen oxidation. The viability of the design is validated by a 95% rate of success.

(a) **(b)**

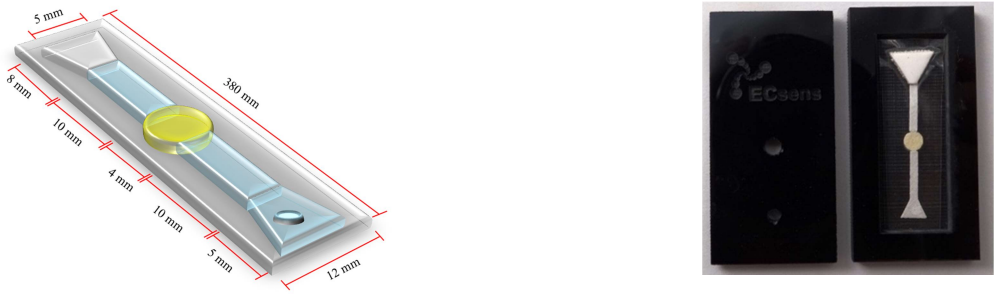


Fig. 1 Microfluidic paper-based analytical device for biothiols. **(a)** Representation of µPAD indicating dimensions. **(b)** Picture of the µPAD for biothiols determination inside of a homemade accessory

3.6. Optimization of method

To adjust the reactions and processes in order to ensure perfect performance of the µPAD device for the evaluation of biothiols, different variables involved in the engineered steps were studied and optimized by using GSH: (a) paper substrate; (b) concentration of reagents; (c) pH; (d) reaction time of the two last reactive steps (iodine derivatization of immobilized CDs and reaction with GSH). Respective data and Figures are given in the Electronic Supplementary Material. The following experimental conditions were found to give the best results: (a) Whatman 1 paper substrate; (b) 1.72×10^{-6} mg·mL^{-1} was′+ chosen as the optimal CDs concentration and the 120 mM concentration was selected as optimal for IAA; (c) The results indicate the value of pH 7.4 as optimal for further experiments; (d) for the iodine displacement by GSH the reaction is complete in shorter reaction times of 30 min, as deduced for the observed invariability of the fluorescence after this time, which indicated complete assembly of the GSH to the NPs.

3.7. Analytical characterization of µPAD for biothiols assay

As mentioned, the fluorescence intensity of **CDs-I-paper** is recovered (turn-on) when the biothiols cause nucleophilic substitution and release of iodine anions. The increase of fluorescence can be quantified by correlating the grey intensity of the paper with the concentration of biothiols present in the sample (Fig. 2A). This determination was performed following the described analytical protocol and by taking a photography of

the µPAD after the reaction with the different biothiols using a digital camera and a homemade accessory under UV lamp (wavelength of excitation = 365 nm) (Fig. 1B). The data show that the fluorescence intensity and the concentration of the biothiols exhibit a linearized logarithmic dependence according with the equation $I = a \cdot \log[\text{biothiol}] + b$ (Fig. 2B) where a is the intercept and b is the slope. The fluorescence intensity of the blank was subtracted for each of the concentrations studied.

The analytical performance characteristics of the µPAD are included in Table 1. A calibration plot (12 standards with 3 replicates obtained with a new device each time) was performed to obtain the calibration function. The limit of detection (LOD) of the device was then calculated for each biothiol (0.3 µM, 0.3 µM and 0.4 µM for GSH, Cys and Hcy, respectively) by using the standard criteria from a relative standard deviation (3.43%) of the blanks (n=18). Furthermore, the quantification limit was calculated for each biothiol being obtained values (0.9 µM, 1.2 µM and 1.3 µM for GSH, Cys and Hcy, respectively) that are in the range of the normal values observed for biothiols in biological fluids. The repeatability (RSD), obtained using 18 different µPAD working at 200 µM GSH, is 5.4%, an acceptable precision considering the measuring system used. The lifetime of the device was also studied for 28 days by keeping a set of µPAD at room temperature in dark using a desiccator, and regularly checking their response to a 100 µM GSH concentration. The fluorescence intensity observed was constant during the studied period.

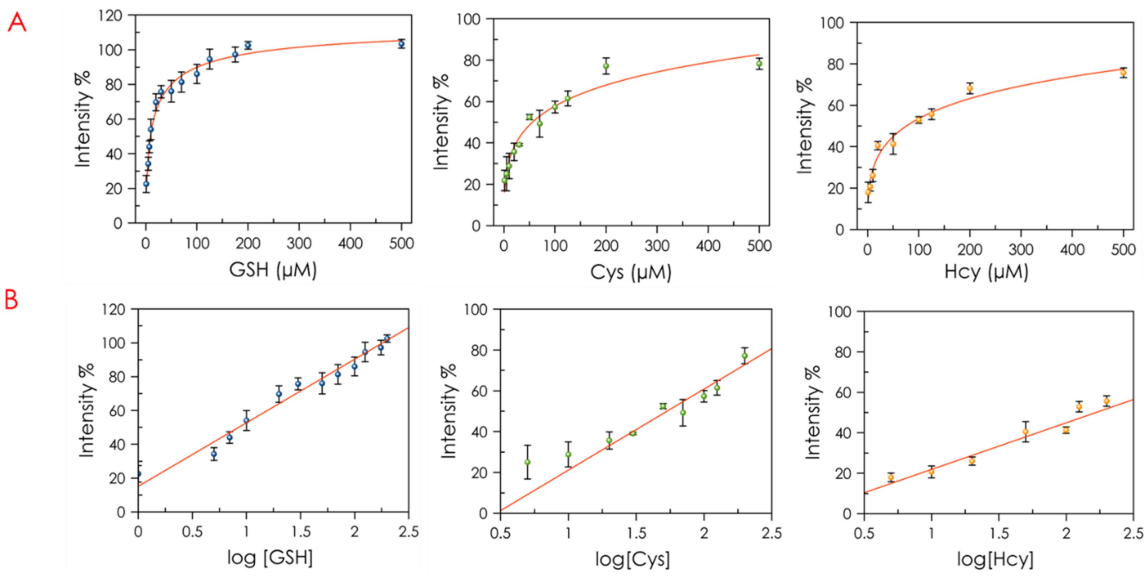


Fig. 2 (A) Dependence of the intensity of the fluorescence with the biothiols concentration. (B) Linearization of calibration functions for biothiols. Data points are the mean of 3 assays

Table 1. Analytical characteristics of µPAD for GSH, Cys and Hcy determination

Analytical parameter	Value		
	GSH	**Cys**	**Hcy**
Measurement range (µM)	1-200	5-200	5-200
Slope (a)	35.8	39.7	23.0
Intercept (b)	29.3	18.5	1.14
LOD (µM)	0.3	0.3	0.4
LOQ (µM)	0.9	1.2	1.3

A comparison of the response of standards including a sole biothiol (5 µM Cys, Hcy and GSH) shows that Cys and Hcy induces modest fluorescence changes but lower than GSH for reasons that are unclear (Fig.3). Results are consistent with those observed by Wu.[43] Experiments between GSH in the presence of Cys and/or Hcy, by using solutions containing equal concentration (5 µM) of each biothiol, indicate that the fluorescent response of **CDs-I paper** is almost additive reflecting the individual contribution of each biothiol. On this basis, µPADs based on **CDs-I paper** are appropriate for the evaluation of the total concentration of biothiols present in a sample. However, the concentration of biothiols varies significantly among different biological fluids (whole blood, plasma, urine) where the prevalence of a specific biothiol over the others is observed (GSH in blood, Cys in plasma and urine). This fact determines that the total concentration in each biological is influenced mainly by the predominant biothiol, anyway this assay can be useful the determine the global content as an index of biothiols presents.

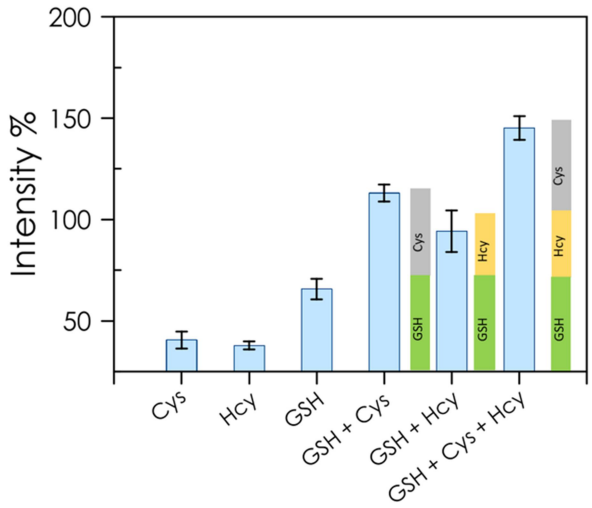


Fig. 3 Comparison of the response of 5 µM Cys, Hcy and GSH, 5 µM GSH in the presence of 5 µM Cys, 5 µM of GSH in presence of 5 µM Hcy and 5 µM GSH en 5 µM Cys and Hcy. Coloured bar indicates

theoretical values for the binary and tertiary systems obtained from the individual contribution of each biothiol according to the calibration function

A comparison of different reported methods to detect and quantify biothiols with the new method reported herein is presented in Table 2. The fluorescent systems collected in the Table shown attractive sensitivity, but they also exhibited some disadvantages. For example, g-C_3N_4-Ag^+ method present a good detection limit for GSH, Cys and Hcy and their detection in real samples is possible but the synthesis of reagent is difficult and the reaction time is longer than others.[37] On the other hand, PEI-AgNCs present a worse detection limit for GSH determination compared with others and the determination of Cys and Hcy present interference.[38] CdTe QDs-Hg^{2+} method exhibit a good linear range and detection limit for GSH, the reaction time is shorter than others but this methodology is designed only for cell.[39] In addition, some of the assays collected required in the use of Hg^{2+} for the construction of the sensor, which is harmful and should be avoided in routine analysis. While, the most important feature of the new sensing strategy developed is a good sensitivity for determination of GSH, Cys, and Hcy, a wide range and the reaction time than is shorter than other methods.

Table 2. Comparison between some selected assays for biothiols determination

Material	GSH		Cys		Hcy		Reaction time (min)	Application	Ref.
	LR (μM)	LOD (μM)	LR (μM)	LOD (μM)	LR (μM)	LOD (μM)			
g-C_3N_4-Ag^+	0.02-100	0.0081	0.02-100	0.0064	0.02-100	0.0096	45	Urine	[37]
PEI-AgNCs	0.5-6	0.38	0.1-10	0.042	0.1-10	0.047	30	Plasma	[38]
CDs/Hg^{2+}	0.5-10	0.008	0.5-10	0.0076	0.5-10	0.0069	12	Plasma	[40]
Mu-Hg^{2+}	0.1-40	0.01	0.5-30	0.02	0.5-50	0.04	5	Urine	[41]
Hg^{2+}-Au^+	0-0.25	0.0094	0-0.25	0.0083	0-0.25	0.0149	15	Serum	[42]
CdTe QDs-Hg^{2+}	0.6-20	0.1	2.0-20	0.6	-	-	5	Cell	[39]
CDs-I	1-200	0.3	5-200	0.3	5-200	0.4	30	Urine	This work

LR = Linear range, LOD = Limit of detection

3.8. Interferences

The effect of different compounds commonly present in urine (Ser, His, Arg, Asn, Gal, and Man) for their plausible interference in the detection of biothiols was next evaluated. The data (Fig. 4) shows that the **CDs-I-paper** exhibited excellent properties as a specific fluorescence and highly selective probe for biothiols over other amino acids, demonstrating their suitable application for the analysis of biothiols

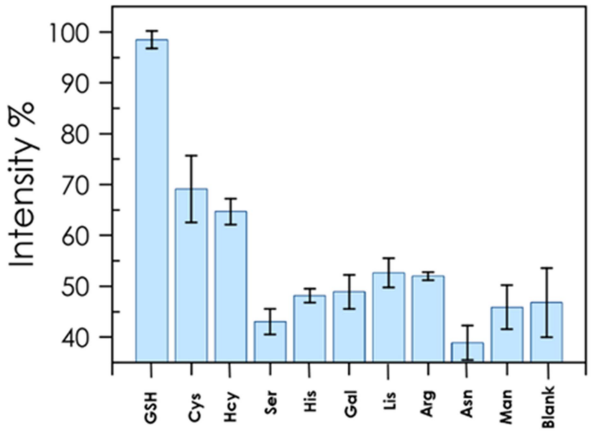


Fig. 4. Response of GSH, Cys and Hcy and interferences (100 µM)

3.9. Analysis of real samples

To evaluate the feasibility of the µPADs in the analysis of a real biological sample, **CDs-I-paper** was applied to determinate biothiols in urine. The samples analyzed were collected from anonymous healthy volunteers. All the samples were filtered by a 0.22 µm pore diameter filter then, the samples were diluted 1:10 with phosphate buffer (pH 7.4, 10 mM). The recovery experiment was carried out by spiking of 30 and 60 µM of standard GSH in the urine samples. The recoveries obtained are in the range of 98.6 - 111.5% and 100.2 - 112.0%, respectively. The results demonstrate that the method proposed in this work can be used for determination of biothiols in real samples (Table 3).

Table 3. Recovery study for GSH in urine samples

Sample	Concentration of GSH, µM		Recovery, %
	Amount added	Amount found	
Urine 1	30	29.8 ± 2.1	98.6
Urine 2		31.9 ± 2.8	106.4
Urine 3		33.9 ± 4.1	111.5
Urine 4		33.5 ± 1.7	110.6
Urine 1	60	60.5 ± 1.4	100.2
Urine 2		61.2 ± 2.7	102.0
Urine 3		67.4 ± 2.4	112.0
Urine 4		63.6 ± 4.2	105.0

4. Conclusions

In the present work, we have successfully engineered a novel, inexpensive and highly robust µPAD that behaves as a fluorescent sensing system for the determination of biothiols such as GSH, Cys and Hcy. The

device is based on the use of CDs as signal reporters that have been covalently immobilized on paper by VS click chemistry. The sensing is operative by turning off the intrinsic fluorescence of the CDs by exploiting the heavy-atom effect of their iodo chemical derivatization and the ulterior turning on by the displacement of this halogen by reaction with GSH, Cys or Hcy. The resulting increase of the fluorescence correlates accurately with the concentration of the different biothiols in samples. The μPAD provides a wide linear range (1 to 200 μM for GSH, and 5-200 μM for Cys and Hcy, respectively), and the assay exhibit a low detection limit (0.3 μM for GSH, and 0.4 μM for Cys and Hcy, respectively). The device allows the rapid screening of biothiols by the use of a UV lamp and a digital camera, avoiding specific instrumentation. In summary, the device shows great promise as a potent tool for clinical diagnosis and therapy related to GSH, Cys and Hcy.

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Compliance with ethical standards. The authors declare that they have no competing interests.

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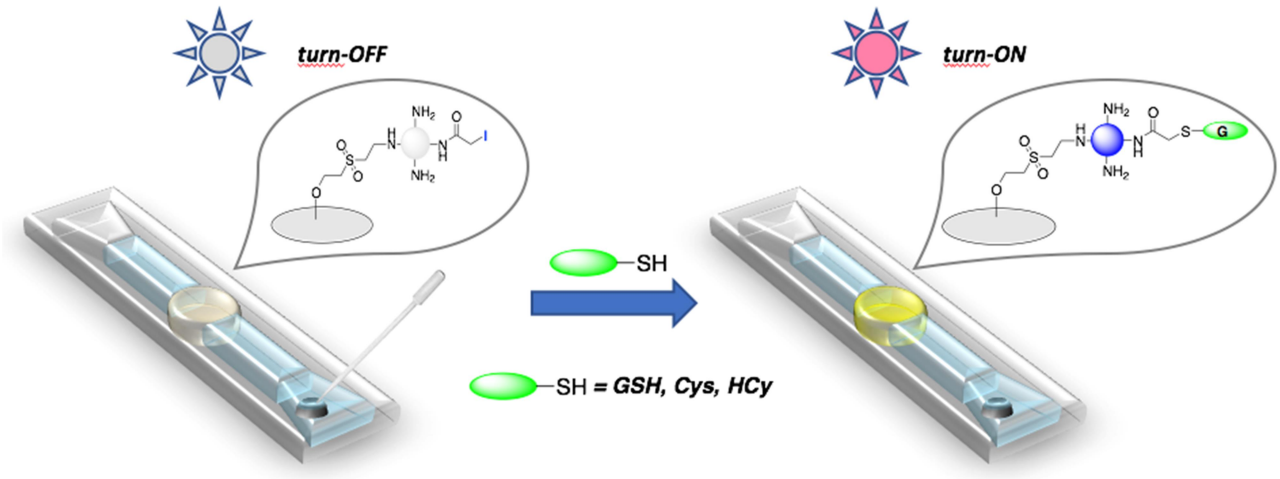
GRAPHICAL ABSTRACT



Schematic representation of a μPAD based on click immobilized carbon-dots and used for a reaction-based fluorometric off-on assay of biothiols. The intrinsic blue fluorescence of carbon-dots is turned off via the heavy atom effect of an introduced iodo group, and turned on by the displacement of this atom by reaction with a biothiol