Carbon nanodots based biosensors for gene mutation detection

Tania García-Mendiola[a,b,c], Iria Bravo[a,b], José María López-Moreno[a], Félix Pariente[a,b,c], Reinhold Wannemacher[b], Karina Weber[d,e], Jürgen Popp[d,e] and Encarnación Lorenzo[a,b,c]

[a] Departamento Química Analítica y Análisis Instrumental, Universidad Autónoma de Madrid, 28049, Spain

[b] Instituto Madrileño de Estudios Avanzados (IMDEA) Nanociencia, Faraday, 9, Campus UAM, Cantoblanco, 28049 Madrid, Spain

[c] Institute for Advanced Research in Chemical Sciences (IAdChem), Universidad Autónoma de Madrid, 28049, Madrid, Spain

[d] Leibniz Institute of Photonic Technology (IPHT), Albert-Einstein-Straße 9, 07745 Jena, Germany

[e] Institute for Physical Chemistry and Abbe Center of Photonics, Friedrich-Schiller-University Jena, Helmholtzweg 4, 07743 Jena, Germany

Keywords: Carbon nanodots, DNA electrochemical biosensor, detection of CFTR gene mutation

Abstract

An electrochemical DNA biosensor based on a carbon nanodots (CDs) modified screen-printed gold electrode as a transducer is reported in this work. CDs were synthesized by thermal carbonization of ethyleneglycol bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and characterized by different techniques (DLS, TEM, FTIR, Raman). The electrode surface modification was accomplished by drop-casting a suspension of CDs. SEM analysis and cyclic voltammetry were used to characterize the resulting modified electrode. Synthetic 25-mer or 100-mer DNA capture probes, capable to hybridize with a specific sequence of the pathogen *Helicobacter pylori* or the cystic fibrosis transmembrane regulator (CFTR) gene were attached to the CDs-gold surface. A 25-bases synthetic fully complementary sequence or a single nucleotide polymorphism to the DNA capture probe and a 373-bases PCR amplicon of exon 11 of CFTR containing a sequence complementary to the capture probe, were employed as target. The hybridization event was electrochemically monitored by using safranine as redox indicator, which selectively binds to double stranded DNA (dsDNA). A

detection limit of 0.16 nM was obtained for the 25-mer synthetic target DNA. The biosensor shows a very high reproducibility and selectivity, allowing to detect a single nucleotide polymorphism. It has been applied to the detection of F508del mutation in the CFTR gene.

Introduction

Nanomaterial-modified detection systems represent a chief driver towards the adaption of electrochemical methods for sensing applications, since nanomaterials enable functional tunability, ability to self-assemble, enhancement of surface area and novel electrical, optical and catalytic properties that emerge at this scale [1-3]. This results in tremendous gains in terms of sensitivity, selectivity and versatility [4].

Carbon dots (CDs) are defined as nanoparticles mainly composed of carbon, with a size below 10 nm [5-11]. These materials have attracted intense interest because their photophysical properties resembling in some respects those commonly found in semiconductor quantum dots (QDs), for example high photostability [5,12,13]. Besides, the CDs can be produced easily from a wide range of raw materials and excel by their robust chemical inertness and high solubility in aqueous media. Compared with traditional QDs, CDs have unique properties such as high biocompatibility, due to the absence of toxic metal ions, and do not require scarce elements or stringent, intricate, tedious, costly, or inefficient preparation steps [10,14]. This makes them promising potential substitutes for QDs in biolabeling, bioimaging, drug delivery, analytical sensing and photocatalysis.

A variety of preparative methods, such as acidic oxidation [15], microwave [16], ultrasonic [17], electrochemical oxidation [18], hydrothermal [19], supported synthesis [20], arc discharge [21], and laser ablation [22] methods, have been developed to synthesize CDs. Typically, CDs contain many carboxylic acid moieties at their surface, thus imparting them with excellent water solubility and the suitability for subsequent functionalization with various organic, polymeric, inorganic, or biological species. Based on distinct optical features, CDs have been employed as fluorescent probes for biological and environmental sensing [6]. However, these nanomaterials, unlike other carbon based nanomaterials, have not been widely explored as electrode modifiers for electrochemical biosensors development [23]; although it is well known that nanostructuring of the working electrode through the

application of nano-sized modifiers offers attractive new features, such as : (1) Nanostructured electrodes, especially those using carbon nanotube and graphene-based materials, exhibit faster electron transfer kinetics due to their extremely high conductivity along particular directions [24]. (2) The high surface area of nanostructured electrode modifiers can enhance the adsorption kinetics of analyte species. (3) Nanostructured materials can act as highly selective and tunable catalysts, due to their unique electronic or plasmonic structure. This is especially useful within detection systems using electrocatalysis [25]. (4) The surface chemistry of nanostructured systems can be tuned towards directing the assembly for particular capture probe or analyte species [26]. Such selective immobilization methods are enabled through nanomaterial modifiers and offer microarray-based pathways towards parallelized detection. The few works found in the literature reporting application of CDs in electrochemical sensors and biosensors are mainly focused on the electrocatalytic properties of this nanomaterial towards O_2 and H_2O_2 reduction, which was employed for glucose [27,28] or H_2O_2 [29] biosensing, or towards an analyte of interest such as dopamine [30] or 2,4,6-trinitrotoluene [31]. No results are reported concerning DNA biosensors. However, the detection of specific DNA sequences or DNA mutations using electrochemical biosensors may greatly reduce the assay time and simplify the analytical protocols compared to the traditional DNA sequencing methods. These fast monitoring approaches are needed for quick preventive actions and early diagnosis [32-37].

In the present work we have synthetized carbon dots by a thermal carbonization method [38] and we have used them to nanostructure screen-printed gold electrodes for the immobilization of unmodified oligonucleotides with the aim of developing a DNA biosensor. Other carbon materials, such as carbon nanotubes, have been previously employed in electrochemical DNA biosensors. However, these devices require chemically modified oligonucleotides, such as oligonucleotides with the 5′-aminogroup, which were covalently bonded to the carboxyl group of carbon nanotubes [39-42]. For the accurate and selective detection of hybridization we have used safranine, which is a very selective electrochemical molecular probe, with different binding affinity to single stranded DNA (ssDNA) and double stranded DNA (dsDNA) [43].

We have focused on (1) understanding how specific nano-sized modifiers may be applied to influence the DNA probe immobilization, since in this case thiolated probes are not required,

as well as on (2) the electron transfer event, because this is key to achieving gains in sensitivity, selectivity and versatility of the resulting detection system.

Materials and methods

Chemicals

Sodium phosphate and sodium chloride were obtained from Scharlab Co. Safranine O (SAF) and all other chemicals used in this work were reagent grade quality, and were obtained from Sigma-Aldrich Co., Water was purified with a Millipore Milli-Q-System (18.2 MΩ cm) and all solutions were prepared just prior to use. Double stranded calf thymus DNA (dsDNA) was also purchased from Sigma-Aldrich Co. dsDNA stock solutions (1.0 mg/ml) were prepared in 0.1 M phosphate buffer (PB) pH 7.0 solution. The DNA solutions UV absorbance ratio (A_{260}/A_{280}) was about 1.9, suggesting that the DNA was free of protein [44]. Using a molar absorptivity of 6600 M^{-1} at 260 nm [45] the concentration in base pairs (bp) of DNA was determined. Single stranded calf thymus DNA (ssDNA) was obtained by boiling in water capped vials containing dsDNA in 0.1 M PB pH 7.0 solution for 30 minutes. To prevent spontaneous renaturation, this reaction was subsequently quenched in an ice-bath. The resulting denatured DNA samples were stored frozen at -20ºC. Custom made synthetic oligonucleotides, 25-mer and 100-mer, from the pathogen bacterium *Helicobacter pylori* and the Cystic Fibrosis Transmembrane Regulator Gen, respectively, were supplied by Sigma-Aldrich Co. Genomic DNA was isolated from peripheral blood leukocytes from cystic fibrosis patients by standardized procedures (commercial Kit Purogene from Qiagen GmbH, Hilden, Germany) as we previously described [46]. The PCR samples consisted of wild type (WT) and mutated (F508del) sequences, validated by sequentiation methods carried out in the Medical and Molecular Genetics institute (INGEMM) of Madrid (Spain). Synthetic oligonucleotides and DNA samples sequences amplified by polymerase chain reaction (PCR) are respectively listed in Table 1 of SI.

Synthesis of CDs

CDs were prepared by thermal carbonization using ethyleneglycol bis-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) as the carbon source and

tris(hydroxymethyl)aminomethane (TRIS) as the surface passivation agent following the method previously described by Gaber Ahmed et al. [38]. Briefly, EGTA (2 mmol) and TRIS (8 mmol) were dissolved in deionized water (30 mL) and heated at 150 ºC on a hot plate until near dryness. A pale-yellow gel was formed. Then 1 mL of water was added and the previous procedure was repeated 5 times in about 30 min. The temperature was then increased to 180 °C until the pale-yellow gel turned to reddish-orange. Finally, the gel was dissolved in about 25 mL deionized water, filtered through 0.45 μm nylon filter and the solution purified by dialysis through dialyzer tube (MWCO, 3.5 KDa) for 3 days. The final solution was stored under 4 °C until use.

Experimental techniques

The Fourier transform infrared spectroscopy (FTIR) spectrum was recorded on a Bruker IFS60v Fourier-transform infrared spectrometer. Transmission electron microscopy (TEM) measurements were performed on a FEG S/TEM (Talos F200X, FEI) electron microscope. UV-vis absorption spectra were recorded on a double beam PharmaSpec UV-1700 series Shimadzu spectrophotometer operating from 200 nm to 800 nm. Fluorescence emission spectroscopy was carried out on a Cary Eclipse Varian spectrofluorimeter. UV-visible absorption and fluorescence emission spectra were performed in 0.1 M PB pH 7.0 solution in 1.0 cm quartz cells. Absorbance titrations were carried out at 60 μM of dsDNA in the absence and in the presence of increasing amounts of CDs from 0.5 to 5.5 μM in 0.1 M PB pH 7.0 solution. Fluorescence titrations were carried out at a 1.3 μM of the CDs while the concentration of dsDNA was varied from 0 to 750 μM.

Dynamic light scattering (DLS) measurements were performed at 25ºC using a VASCO Particle Size Analyzer from Cordouan Technologies.

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were carried out at room temperature with an Autolab PGSTAT 30 potentiostat from Eco-Chemie (KM Utrecht, The Netherlands) using the software package GPES 4.9 (General Purpose Elec. Experiments). Integrated screen-printed gold electrodes (4 mm diameter, AuSPEs) from DropSens S.L (Oviedo, Spain) that include a gold working, a silver pseudoreference and a gold counter electrode were used as electrochemical cells. The electrodes were connected

using a SPE Connector (DropSens S. L.) as interface. The parameters used in DPV measurements were: scan rate 10 mV/s; pulse amplitude 50 mV; pulse width 0.2 s. All the differential pulse voltammograms presented were baseline-corrected using the application included in GPES version 4.9 software. No changes in current or potential values were observed relative to data obtained without such application.

Raman spectroscopy measurements were carried out using a micro-Raman spectrometer equipped with a 632.8 nm line of He-Ne laser (WITec GmbH, Ulm, Germany). The laser power on the sample was 1 mW, the integration time, 0.5 s and a 50x objective lens was used. Raman samples were prepared by depositing 8 µL of a 277 µM CDs solution on a glass substrate and air drying.

Scanning electron microscopy (SEM) images were registered using a Philips XL30-FEG microscope. SEM samples were prepared by deposition of 10 µL of a 3.5 nM CDs solution onto gold AFM plates. These plates are glass substrates (1.1 cm x 1.1 cm) covered with a chromium layer (1–4 nm thick) onto which a gold layer (200–300 nm thick) was deposited (Arrandee Co. Werther, Germany). Prior to use, gold surfaces were annealed for 2 min in a gas flame in order to obtain Au (111) terraces. Samples were then air dried.

Zeta potential was determined at 25 ºC using a Zetasizer Nano ZS instrument (Malvern Instrument Ltd., Grovewood, Worcestershire, UK). Elemental Analysis measurements were carried out using a LECO CHNS-932 system. For Elemental Analysis, DLS and FTIR measurements a 277 µM CDs stock solution was directly used.

Preparation of the CDs modified screen-printed gold electrodes (AuSPEs)

AuSPEs were electrochemically activated by immersing them in a 0.1 M H_2SO_4 solution. Then, the potential was cycled (10 times) from -0.200 V to 1.20 V at 100 mVs^{-1} and the electrodes were washed with deionized water. Afterwards, the electrode was modified with 5 µL of the 3.5 µM CDs suspension and air dried for 24 hours. These modified electrodes are denoted in the text as CDs/AuSPEs.

Immobilization of DNA on CDs/AuSPEs.

For dsDNA or ssDNA immobilization, 10 µl of 2.0 mM ds or ssDNA in 0.1 M phosphate buffer (PB) pH 7.0 solution were drop-casted onto a CDs/AuSPE followed by air-drying

(dsDNA/AuSPE or ssDNA/CDs/AuSPE). Afterwards, the resulting modified electrode was soaked in sterilized water for 30 min and rinsed with water to remove any un-adsorbed DNA. In the case of DNA oligonucleotides 10 μL of 40 μM synthetic (HP1, WT) sequences were transferred onto the CDs/AuSPE. Afterwards, the electrode was kept at room temperature for 24 hours. Then, it was soaked in sterile water for at least 30 min.

Denaturation of PCR DNA samples

PCR samples were denatured immediately before use by heating at 100 ºC during 20 minutes followed by rapid cooling in an ice bath [47].

Hybridization and detection

CDs/AuSPEs modified with the capture probe (HP1/CDs/AuSPE or WT/CDs/AuSPE) were subsequently hybridized (1h 40 ºC) with 10 μL of the analyte: synthetic oligonucleotides (20 μM complementary, non-complementary or SNP sequence) or denatured PCR (5.0 ng μL^{-1} wild type or mutated samples). After the hybridization step, DNA modified electrodes were immersed in 0.1 M phosphate buffer (PB) pH 7.0 solution containing 1.0 mM of safranine (SAF) and the potential was cycled (100 times) at 100 mV/s. Then, the electrodes were rinsed with sterile water, placed in 0.1M PB pH 7.0 solution, and differential pulse voltammograms (DPVs) were immediately recorded.

Results and Discussion

Preparation and Characterization of carbon dots (CDs)

CDs were prepared by thermal decomposition of EGTA/TRIS as described in the experimental section. The corresponding size distribution histogram, obtained by measuring the average size of around 100 CDs by dynamic light scattering (DLS), indicates that these nanoparticles are monodisperse and have an average size of 3.4 nm ranging from 2 to 5 nm in diameter (Figure 1A of SI). Zeta potential is another important parameter related to nanoparticle stability or aggregation in a dispersion and can have significant implications on product performance since it is a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles. The zeta potential was found to be -26 ± 0.3 mV. This value according to the literature indicates that the CDs have a great stability. In fact, the potential zeta decreases until -8.1 ± 0.2 mV after 12 months of storage, which confirms their lack of tendency to aggregate.

The FTIR spectrum was used to investigate the surface functional groups of CDs. The hydroxide groups or water molecules give stretching bands at 3000 and 3454 cm^{-1} indicating the good water-solubility of CDs. The FTIR spectrum also shows the stretching vibrations of C=O at 1650 cm^{-1} and the –N-H stretching peak at 1543 cm^{-1}. Bands at 2800-2930 cm^{-1} and 1050-1120 cm^{-1} are assigned to the stretching vibrations of C-H and C-OH/C-O-C. These results indicate that CDs are surrounded by $–CONH_2$ and –OH groups (Figure 1B of SI). The optical properties of CDs were also studied by UV-vis absorption and fluorescence spectroscopy in order to characterize this nanomaterial. The UV-vis absorption spectrum shows a broad peak around 360 nm (Figure 1C of SI). The fluorescence spectrum shown in Figure 1D of SI reveals a narrow and symmetric emission band at 440 nm when excited at 260 nm

The TEM images (see Figures 2A of SI and 2B of SI) reveal that the as-synthesized CDs are mostly spherical. Fast Fourier Transform (FFT) analysis was used to measure the spacing of the planes (Figure 2C of SI). It was found to be 2.1 Å, which is characteristic of the CDs (100) plane. The diameters of the CDs are distributed in a narrow range, with an average value of 3.5 ± 0.2 nm. Elemental analysis confirms the composition of CDs. The results

obtained were: 55.10% C, 0.29% N and 10.17% H. From these data and taking into account the size of the CDs obtained by DLS, a concentration of 277 μM CDs was estimated. The synthesized CDs were also characterized by Raman spectroscopy. The Raman spectrum (see Figure 3SI) shows two peaks centered at 1330 and 1602 cm^{-1}, corresponding to the D band and G band, respectively, which are characteristic of graphitic carbon materials. The G band is attributed to the in-plane stretching vibrations of sp^2 carbon atoms and the D band is attributed to another in plane vibration of sp^2 carbon atoms in the presence of structural defects [48]. The intensity ratio I_D/I_G is an indicator of the crystallinity or disorder in graphitic systems. For the synthesized CDs the I_D/I_G ratio is ~1.22, indicating a disordered structure, probably due to the presence of functional groups from the precursors.

The results obtained with the different characterization techniques confirm the success of the CDs synthesis.

Electrochemical behavior of CDs gold screen-printed electrodes (AuSPEs).

For the development of the biosensing platform, the first step consisted in nanostructuring a AuSPE electrode with CDs (CDs/AuSPE). 5 μL of 1.5, 3.5, 7.0 μM CDs suspension were drop casted on the electrode surface, following the procedure described in the experimental section and depicted in Scheme 1. Best results concerning stability were obtained when 3.5 μM CDs suspensions were employed.

The resulting nanostructured electrode surface was analyzed by SEM. Figure 1 shows the SEM image of the CDs deposited on a gold surface at different magnification. It can be observed that the Au surface is partially covered by CDs, which can be clearly distinguished as spots randomly distributed on the gold surface. Moreover, the CDs show near spherical morphologies and homogeneous size. As a control, the SEM image of the unmodified gold substrate was recorded and it is shown in Figure 4 of SI.

Scheme 1.

Figure 1.

Nanostructuring of the AuSPE electrode with CDs must affect its voltammetric response. As can be seen in Figure 2, the characteristic cyclic voltammetric response of gold in 0.1 M sulfuric acid changes after electrode modification with CDs. The oxidation wave increases in current and shifts to more positive potential, from 0.81 V to 0.87 V. However, the sharp reduction peak decreases in current and shifts to less negative potential. This effect is consistent with a change in the different planes of the gold surface, after modification, being this change stronger in some planes than in others, indicating that a carbon/gold screen-printed electrode has been prepared.

Figure 2.

To further characterize these CDs modified electrodes, their cyclic voltammetry response in 0.1 M PB pH 7.0 solution was recorded (black curve of Figure 5 of SI). No redox response is observed in a wide potential window. Moreover, we have studied the electrochemical behavior of two different redox probes at the CDs modified electrode in 0.1 M PB pH 7.0 solution. In particular, safranine (SAF) and $K_3[Fe(CN)_6]$ were chosen for this purpose. For a positive redox probe, such as SAF, the cyclic voltammetry at CDs/AuSPE nanostructured electrode (Figure 5A of SI, red curve) shows a reversible redox couple ascribed to the oxidation/reduction of the dye in aqueous media, at a formal potential (E°) value of −0.694V. The peak potential separation of 76 mV is close to that expected for a freely diffusing one electron reversible redox process and is due to one of the two electronic transitions of SAF [49]. Compared with the response observed at bare AuSPE electrode (Figure 5A of SI, blue curve), the anodic current intensity is clearly enhanced. In addition, there is a decrease in the ΔE_p (from 97 mV to 76 mV). These effects can be explained by considering not only that CDs cause an increment of the relative surface area, but also that they can be involved in the oxidation and reduction process. In the case of the negative charge probe ($Fe(CN)_6^{3-}$) the shape of the cyclic voltammetric response at CDs modified electrodes (red curve of Figure 5B of SI) was similar to that observed at a bare gold electrode (blue curve of Figure 5B of SI) with a slight decrease in the ΔE_p. This fact suggests the absence of any kind of electrostatic repulsion between the probe and the CDs modified electrode.

Interaction of CDs with DNA.

CDs may exhibit interaction with DNA [3], which can be used for different applications. Hence, we focused our attention on studying this interaction by UV-vis absorption and fluorescence spectroscopy. The absorption spectrum of a solution containing 60 μM of double stranded calf thymus DNA (dsDNA) in 0.1 M PB pH 7.0 solution shows the characteristic band at 260 nm. Upon addition of increasing amounts of CDs (up to 5.5 μM), a significant increase in the absorption band is observed (See Figure 6A of SI). This effect may be ascribed to the interaction of electron pairs of oxygen atoms present in CDs with DNA bases forming hydrogen bonds. Although all the spectra are relatively similar in shape there is a shift to lower wavelengths due to the concentration changes, suggesting that there is an interaction between the DNA and CDs.

Interaction between DNA and CDs was also studied considering the fluorescence of CDs. In absence of DNA, exciting at 360 nm, the characteristic symmetric band at 440nm is observed (see black curve of Figure 6B of SI). Moreover, the CDs have a large Stokes shift, which is beneficial for the distinction of the target from the background signal in imaging [50]. The addition of DNA gives rise to a gradual reduction in the emission intensity with no evident shift in the emission maximum. The quenching of fluorescence emission observed for CDs upon binding to DNA is due to a charge transfer between CDs and DNA.

The interaction strength of CDs and DNA can also be quantified by using the Stern-Volmer equation, $F_0/F=1+K_{SV}[DNA]$. From a plot of F_o/F versus [DNA] the quenching constant (K_{SV}) was calculated to be $1.4\pm0.2 \times 10^4$ M^{-1}. This value is comparable to those reported for a number of ligands that interact stronger with DNA, such as (9-anthryl)methylammonium chloride (AMAC), N-ethyl-(9-anthryl)methylammonium chloride (N-Et-AMAC), and 3-(9-anthryl)propylammonium chloride (APAC) with K_{SV} values of 1.0×10^4, 1.2×10^4, and 1.4×10^4 M^{-1}, respectively [51].

Analytical Applications of CDs modified electrodes in DNA Biosensors

Considering the results discussed above, we evaluated the possibility of using the CDs modified gold electrode as a novel electrochemical transducer for applications in the development of DNA sensing devices. For this purpose, we employed a sequence of

Helicobacter pylori as a prototype system. *Helicobacter pylori* is a bacterium that can cause digestive illness and even stomach cancer. It has been chosen as a case of study within the framework of developing approaches of broad applicability.

A 25-mer sequence of this bacterium (HP1) was directly immobilized onto the CDs/AuSPE modified electrode as described in the experimental section. Based on the interaction between the DNA and the CDs, and according to the literature, oligonucleotides such as HP1 can be adsorbed by carbon based nanomaterials via π-π stacking interactions of the DNA bases and hydrogen bonding, while it must overcome electrostatic repulsion at the same time [52].

One of the strategies employed in the development of electrochemical DNA biosensors is the use of a redox probe for the hybridization detection. Different redox active molecules have been employed for this purpose. Among them, safranine (SAF) has been demonstrated to be a good hybridization detector since it interacts to different extent with dsDNA and ssDNA [43], giving very different voltammetric responses at dsDNA and ssDNA/CDs modified electrodes. Hence, in this work we have employed SAF as a redox indicator of the hybridization event in the CDs based DNA electrochemical biosensor developed. The dye is accumulated on the DNA layer present at the electrode surface by holding the potential at -0.75 V, potential cycling from -0.90 to -0.50 V and at open circuit. Best results were obtained by consecutive potential cycling (100 cycles). The cyclic voltammograms (CVs) of SAF accumulated on the CDs/AuSPE before and after modification with ssDNA and dsDNA were recorded (Figure 7 of SI). SAF does not accumulate in the CDs modified electrode (CDs/AuSPE) as is evident from the low signal obtained (black curve). However, at DNA modified electrodes (ds or ssDNA/CDs/AuSPE) a high peak current due to the oxidation/reduction of SAF accumulated on the DNA layer is observed, being this current higher for dsDNA than for ssDNA (see Figure 7 of SI), as it would correspond to an intercalative mode of interaction. To better discriminate between signal and current background and to reach a higher sensitivity, differential pulse voltammetry was employed in these studies.

For the biosensor development, the changes in Differential Pulse Voltammogram (DPV) peak currents of the dye accumulated at DNA probe modified CDs/AuSPE before and after hybridization with the DNA target were employed to detect the hybridization event. In the first hybridization test, a complementary (HP2C) and a non-complementary sequence

(HP2NC), as control, of the DNA probe were selected as the target DNA. The hybridization and labeling steps are described in the Experimental Section. DPVs, before and after hybridization, are shown in Figure 3. As can be seen, hybridization of HP1 with the complementary HP2C chain in the biosensor recognition layer resulted in a dramatic enhancement in the DPV response, whereas virtually no change in current was obtained for the non-complementary sequence (compare curves black and black dashed in Figure 3). The changes observed in peak currents suggest that both the hybridization process and the target sequence of the *Helicobacter pylori* DNA fragment can be recognized using this system. As a control, to assess the main role of CDs, the same set of experiments was carried out using gold and carbon screen-printed electrodes (CSPEs) without CDs. In both cases, there was no increase in the DPV response after hybridization with the complementary sequence and accumulation of SAF (see Figure 8 of SI).

As can be seen in Figure 4, the current response at -0.700 V increases upon increasing the amount of the target sequence used, with excellent correlation ($R^2 = 0.998$) over the range of 0.001 to 20 μM. The detection limit, calculated as the concentration corresponding to the HP1 signal plus 3 times the standard deviation, was determined to be 0.16 nM. Compared to the value obtained (22.5 nM of HP2C) in the same conditions using thiolated 25-mer *Helicobacter pylori* sequences as probes immobilized on bare AuSPEs without CDs, this detection limit is more than 100 times lower [43]. In addition, the reproducibility was evaluated using 5 different biosensors. A RSD of 5 % was obtained. The stability of the biosensor was also evaluated. For this purpose, different modified electrodes, prepared in the same manner (HP1/CDs/AuSPE), were used to detect the DNA target sequence of HP2C over a period of three months without losing the ability of detection.

To evaluate the selectivity of the biosensor a target sequence containing a single mismatch in the middle of the sequence (denoted as HP2SM) was employed under the same hybridization conditions for the perfectly matched sequence. One would expect that the hybridization of HP1with the single-mismatched target will give a distorted double-helix, which may interact with SAF in a different way. Based on this assumption, one would anticipate a different biosensor response when compared to hybridization with a fully complementary target sequence (HP2C). Figure 3 shows the DPV responses. As can be seen, there is a significant decrease in the peak current values obtained with the single-mismatched

sequence (grey dotted curve of Figure 3) compared to the response obtained after hybridization with the fully complementary sequence, HP2C (black curve of Figure 3). This diminution in the peak current could be interpreted as a decrease in the binding constant of SAF with the distorted helix.

Figure 3.

Figure 4

Screening of specific gene mutations associated with cystic fibrosis

In order to assess the broad applicability of the DNA biosensor developed, and since it is capable of detecting a single mismatch, it was also applied to the detection of gene mutations based on mismatches associated to human diseases in real DNA genomic samples. Sequencing of genes is the gold standard for identifying these mutations. However, these methods have serious drawbacks as routine diagnosis tools, because of their labor intensity and cost. DNA biosensors present advantages, in particular simplicity and low cost [34-37]. Therefore, we applied the developed DNA biosensor to the detection of mutations associated to cystic fibrosis (CF) in real DNA PCR amplicons extracted from blood cells. The more common mutation in the cystic fibrosis transmembrane conductance regulator gene (CFTR) associated to CF is the mutant allele F508del [53,54], which is a three-nucleotide deletion that causes the loss of a phenylalanine residue of the CFTR protein. This mutation was chosen as case of study in this work. Patients suffering CF present this mutation among others.

The biosensor was developed following the strategy depicted in Scheme 2. The mutation detection relies on the comparison of the voltammetric transduction of the hybridization reaction between the immobilized probe (a synthetic oligonucleotide of 100 bp complementary to the Wild type sequence) and the target DNA, which is a wild type (WT) or mutated (MUT) sequence. PCR samples comprising around 373 bp PCR amplicons of exon 11 of CFTR gene without the deletion (WT) from healthy people, or carrying the F508del mutation (MUT) from patients suffering the disease (see Table 1 of SI) were denaturized before hybridization with the probe. The biosensor response was obtained from the corresponding DPVs of SAF accumulated on the dsDNA layer formed on the electrode surface after hybridization. As a control, in addition to the wild type amplicon (WT), a non-complementary sequence (NC) was also used.

Figure 5A shows the bar diagram of the biosensor response for the F508del mutation, wild type (WT) and the non-complementary sequence (NC sequence). As can be seen, when the hybridization takes place with the mutated sequence the signal obtained is 1.5 higher than that obtained with the probe before the hybridization. However, when the hybridization takes place with the complementary sequence the signal is 2 times higher. Moreover, when the non-complementary sequences are used, the biosensor response is similar to that obtained with the probe, confirming that unspecific hybridization does not take place.

To avoid electrode-to-electrode variations and to have a better signal processing, we have plotted the percentage of normalized probe signal increment vs the DNA sequence used as target (see Figure 5B). The percentage of normalized probe signal increment was calculated by the ratio of the difference between the signal obtained before and after hybridization and the signal of the probe. As can be seen, the percentage is 100% after hybridization with the wild type sequence (healthy people), whereas this value decreases to 37.5% if the target is a sample carrying the mutation (CF patients). When no hybridization takes place, the signal increment is less than 10%. Considering that the error associated to each measure is less than 5%, it can be concluded that the proposed screening method discriminates between wild type and mutated samples, that is, from people with or without CF. The reproducibility was evaluated from the response of five different biosensors (prepared in the same manner) to either wild type or mutated target DNA. 95% of reproducibility was obtained in all cases.

The developed methodology can be used as rapid and precise screening method for the detection of gene mutations, without the need of previously modifying the DNA with an alkyl thiol group as an alternative to the classical gene assay. These mutations can be used to trace generational inheritance patterns associated with specific diseases [55]. In addition, the developed biosensor compares favorably to other DNA biosensors based on a similar method for the hybridization detection (Table 2SI), considering important analytical properties, such as sensitivity and detection limit [51,43,56-59].

Scheme 2.

Figure 5.

Conclusions.

We have developed a novel approach for electrochemical DNA biosensor development using disposable electrodes modified with CDs, synthesized by simple thermal carbonization of ethyleneglycol bis-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). These synthesized CDs are non-toxic, increase the performance of the electrochemical transductor of the DNA sensor and are very efficient for the immobilization of unmodified oligonucleotides keeping their hybridization capability. These biosensors are also useful for the detection of gene mutations in real human DNA extracted from blood cells. CDs based electrochemical sensors are expected to compete well with corresponding fluorimetric sensors [60], particularly in view of point-of-care applications.

Acknowledgements

This work has been supported by the Comunidad Autónoma de Madrid NANOAVANSENS (project No. S2013/MIT-3029) and Spanish Ministerio de Economía, Industria y Competitividad through projects Nos. CTQ2015-71955-REDT (ELECTROBIONET) and CTQ2014-53334-C2-1-R. E. L. thanks the Fulbright scholarship-Salvador de Madariaga program from Spanish Ministerio de Economía, Industria y Competitividad. I. B. gratefully acknowledges the FPI-2012 Grant from Spanish Ministerio de Economía y Competitividad. R.W. gratefully acknowledges support by the European Union structural funds and the Comunidad de Madrid MAD2D-CM Program (S2013/MIT-3007), as well as by the Spanish Ministerio de Economía, Industria y Competitividad through project No. MAT2015-71879-P.

References

[1] Katz, E., Willner, I.; Wang, J., Electroanalytical and bioelectroanalytical systems based on metal and semiconductor nanoparticles, Electroanal. 16 (2004) 19–44.

[2] Welch, C.M.; Compton, R.G., The use of nanoparticles in electroanalysis: a review, Anal. Bioanal. Chem. 384 (2004) 601–619.

[3] Milosavljevic, V.; Nguyen, H.V.; Michalek, P.; Moulick, A.; Kopel, P.; Kizek, R.; Adam, V., Synthesis of carbon quantum dots for DNA labeling and its electrochemical, fluorescent and electrophoretic characterization, Chem. Pap. 69 (2015) 192–201.

[4] Samanta, A.; Medintz, I., Nanoparticles and DNA – a powerful and growing functional combination in bionanotechnology, Nanoscale 8 (2016) 9037–9095.

[5] Baker, S.N.; Baker, G.A., Luminescent carbon nanodots: emergent nanolights, Angew. Chem., Int. Ed. 49 (2010) 6726–6744.

[6] Li, H.; Kang, Z.; Liu, Y.; Lee, S.-T., Carbon nanodots: synthesis, properties and applications, J. Mater. Chem. 22 (2012) 24230–24253.

[7] Shen, J.; Zhu,Y.; Yang, X.; Li, C., Graphene quantum dots: emergent nanolights for bioimaging, sensors, catalysis and photovoltaic devices, Chem. Commun. 48 (2012) 3686–3699.

[8] Zhang, Z.; Zhang,J.; Chen, N.; Qu, L., Graphene quantum dots: an emerging material for energy-related applications and beyond, Energy Environ. Sci. 5 (2012) 8869-8890.

[9] Zhu, S.; Tang, S.; Zhang; J.; Yang, B., Control the size and surface chemistry of graphene for the rising fluorescent materials, Chem. Commun. 48 (2012) 4527–4539.

[10] Cao, L.; Meziani, M.J.; Sahu, S.; Sun, Y.P., Photoluminescence properties of graphene versus other carbon nanomaterials, Acc. Chem. Res. 46 (2013) 171–180.

[11] Li, L.; Wu, G.; Yang, G.; Peng, J.; Zhao, J.; Zhu, J.J., Focusing on luminescent graphene quantum dots: current status and future perspectives, Nanoscale 5 (2013) 4015–4039.

[12] Wang, Y.; Hu, A., Carbon quantum dots: synthesis, properties and applications, J. Mater. Chem. C 2 (2014) 6921–6939.

[13] Chen, L.; Han, H., Recent advances in the use of near-infrared quantum dots as optical probes for bioanalytical, imaging and solar cell application, Microchim. Acta 181 (2014) 1485–1495.

[14] Li, L.L.; Ji, J.; Fei, R.; Wang, C.Z.; Lu, Q.; Zhang, J.R.; Jiang, L.P.; Zhu, J.J., A facile microwave avenue to electrochemiluminescent two-color graphene quantum dots, Adv. Funct. Mater. 22 (2012) 2971–2979.

[15] Liu, H.; Ye, T.; Mao, C., Fluorescent carbon nanoparticles derived from candle soot, Angew. Chem., Int. Ed. 46 (2007) 6473–6475.

[16] Zhu, H.; Wang, X.; Li, Y., Wang, Z.; Yang, F.; Yang, X., Microwave synthesis of fluorescent carbon nanoparticles with electrochemiluminescence properties, Chem. Commun. 45 (2009) 5118–5120.

[17] Li, H.; He, X.; Liu, Y.; Huang, H.; Lian, S.; Lee, S.T.; Kang, Z., One step ultrasonic synthesis of water-soluble carbon nanoparticles with excellent photoluminescent properties, Carbon 49 (2011) 605–609.

[18] Zheng, L.; Chi, Y.; Dong, Y.; Lin, J.; Wang, B., Electrochemiluminescence of water-soluble carbon nanocrystals released electrochemically from graphite, J. Am. Chem. Soc. 131 (2009) 4564–4565.

[19] Bourlinos, A.B.; Stassinopoulos, A.; Anglos, D.; Zboril, R., Karakassides, M.; Giannelis, E.P., Surface Functionalized Carbogenic Quantum Dots, Small 4 (2008) 455–458.

[20] Liu, R.L.; Wu, D.; Liu, S.; Koynov, K.; Knoll, W.; Li, Q., An aqueous route to multicolor photoluminescent carbon dots using silica spheres as carriers, Angew. Chem., Int. Ed. 48 (2009) 4598–4601.

[21] Xu, J.; Sahu, S.; Cao, L.; Anilkumar, P.; Tackett KN II, Qian, H.; Bunker, C.E.; Guliants, E.A.; Parenzan, A.; Sun, Y.P., Carbon nanoparticles as chromophores for photon harvesting and photoconversion, ChemPhysChem 12 (2011) 3604–3608.

[22] Hu, S.L.; Niu, K.Y.; Sun, J.; Yang, J.; Zhao, N.Q.; Du, X.W., One-step synthesis of fluorescent carbon nanoparticles by laser irradiation, J. Mater. Chem. 19 (2009) 484–488.

[23] Nguyen, H.V.; Richtera, L.; Moulick, A.; Xhaxhiu, K.; Kudr, J.; Cernei, N.; Polanska, H.; Heger, Z.; Masarik, M.; Kopel, P.; Stiborova, M.; Eckschlager, T.; Adam, V.; Kizek, R., Electrochemical sensing of etoposide using carbon quantum dot modified glassy carbon electrode, Analyst 141 (2016) 2665–2675.

[24] Sanghavi, B.J.; Sitaula, S.; Griep, M.H.; Karna, S.P.; Ali, M.F.; Swami, N.S., Real-Time Electrochemical Monitoring of Adenosine Triphosphate in the Picomolar to Micromolar Range Using Graphene-Modified Electrodes, Anal. Chem. 85 (2013) 8158–8165.

[25] Briones, M.; Petit-Domínguez, M.D.; Parra-Alfambra, A.M.; Vázquez, L.; Pariente, F.; Lorenzo, E.; Casero, E., Electrocatalytic processes promoted by diamond nanoparticles in enzymatic biosensing devices, Bioelectrochemistry 111 (2016) 93–99.

[26] Swami, N.S.; Chou, C.F.; Terberueggen, R., Two-potential electrochemical probe for study of DNA immobilization, Langmuir 21 (2005) 1937–1941.

[27] Li, H.; Chen, L.; Wu, H.; He, H.; Jin, Y., Ionic Liquid-Functionalized Fluorescent Carbon Nanodots and Their Applications in Electrocatalysis, Biosensing, and Cell Imaging, Langmuir 30 (2014) 15016−15021.

[28] Ji, H.; Zhou, F.; Gu, J.; Shu, C.; Xi, K.; Jia, X., Nitrogen-Doped Carbon Dots as A New Substrate for Sensitive Glucose Determination, Sensors 16 (2016) 630.

[29] Wang, Y.; Wang, Z.; Rui, Y.; Li, M., Horseradish peroxidase immobilization on carbon nanodots/CoFe layered double hydroxides: direct electrochemistry and hydrogen peroxide sensing, Biosens. Bioelectron. 64 (2015) 57–62.

[30] Huang, Q.; Hu, S.; Zhang, H.; Chen, J.; He, Y.; Li, F.; Weng, W.; Ni, J.; Bao, X.; Lin, Y., Carbon dots and chitosan composite film based biosensor for the sensitive and selective determination of dopamine, Analyst 138 (2013) 5417–5423.

[31] Zhang, L.; Han, Y.; Zhu, J.; Zhai, Y.; Dong, S., Simple and Sensitive Fluorescent and Electrochemical Trinitrotoluene Sensors Based on Aqueous Carbon Dots, Anal. Chem. 87 (2015) 2033–2036.

[32] Cattrall, R.W., Chemical Sensors, Chemistry Primers (1997) Oxford University Press, Oxford, UK.

[33] Mikkelsen, S.R., Electrochecmical biosensors for DNA sequence detection, Electroanal. 8 (1996) 15–19.

[34] Palecek, E.; Fojta,M.; Tomschik, M.; Wang, J., Electrochemical biosensors for DNA hybridization and DNA damage, Biosens. Bioelectron. 13 (1998) 621–628.

[35] Wang, J., Electrochemical biosensors: towards point-of-care cancer diagnostics, Biosens. Bioelectron. 21 (2006) 1887–1892.

[36] Miao, P.; Liu, L.; Nie, Y.; Li,G., An electrochemical sensing strategy for ultrasensitive detection of glutathione by using two gold electrodes and two complementary oligonucleotides, Biosens. Bioelectron. 24 (2009) 3347–3351.

[37] Wan, Y.; Zhang, J.; Liu, G.; Pan, D.; Wang, L.; Song, L.; Fan, C., Ligase-based multiple DNA analysis by using an electrochemical sensor array, Biosens. Bioelectron. 24 (2009) 1209–1212.

[38] Gaber Ahmed, G.H.; Badía Laíño, R.; García Calzón, J.A.; Díaz García, M.E., Highly fluorescent carbon dots as nanoprobes for sensitive and selective determination of 4-nitrophenol in surface waters, Microchim. Acta 182 (2015) 51–59.

[39] Daniel, S.; Rao, T.P.; Rao, K.S.; Rani, S.U.; Naidu, G.R.K.; Lee, H.Y.; Kawai, T., A review of DNA functionalized/grafted carbon nanotubes and their characterization, Sens. Actuators B 122 (2007) 672–682.

[40] Wang, S.G.; Wang, R.; Sellin, P.J.; Zhang, Q., DNA biosensors based on self-assembled carbon nanotubes, Biochem. Biophys. Res. Commun. 325 (2004) 1433–1437.

[41] Cai, H.; Cao, X.; Jiang, Y.; He, P.; Fang, Y., Carbon nanotube-enhanced electrochemical DNA biosensor for DNA hybridization detection, Anal. Bioanal. Chem. 375 (2003) 287–293.

[42] Zhu, Z., An Overview of Carbon Nanotubes and Graphene for Biosensing Applications, Nano-Micro Lett. 9 (2017) 25.

[43] García-Mendiola, T.; Ramos Cerro, M.; López-Moreno, J.M.; Pariente, F.; Lorenzo, E., Dyes as bifunctional markers of DNA hybridization on surfaces and mutation detection, Bioelectrochemistry 111 (2016) 115–122.

[44] Marmur, J., Procedure for isolation of deoxyribonucleic acid from micro-organisms, J. Mol. Bio. 3 (1961) 208–218.

[45] Doty, P.; Rice S.A., The denaturation of desoxypentose nucleic acid, Biochim. Biophys. Acta. 16 (1955)3.

[46] García-Mendiola, T.; Barreiro Martinez, T.; Pariente, F.; Molano, J.; Lorenzo, E., Screening of specific gene mutations associated with cystic fibrosis, Electroanal. 26 (2014) 1362–1372.

[47] Pang, D.W.; Qi, Y.P.; Wang, Z.L.; Cheng, J.K.; Wang, J.W., Electrochemical oxidation of DNA at a gold microelectrode, Electroanal. 7 (1995) 774–777.

[48] Ferrari, A.C., Raman spectroscopy of graphene and graphite: Disorder, electron–phonon coupling, doping and nonadiabatic effects, Solid State Commun. 143 (2007) 47–57.

[49] Heli, H.; Moosavi- Movahedi, A.A.; Jabbari, A.; Ahmad, F., An electrochemical study of safranin O binding to DNA at the surface, J. Solid State Electrochem. 11 (2007) 593–599.

[50] Liu, L.Q.; Li, Y.F.; Zhang, L.; Liu, Y.; Huang, C.Z., One-step synthesis of fluorescent hydroxyls-coated carbon dots with hydrothermal reaction and its application to optical sensing of metal ions, Sci. China Chem. 54 (2011)1342–1347.

[51] García, T.; Revenga-Parra, M.; Abruña, H.D.; Pariente, F.; Lorenzo, E., Single-mismatch position-sensitive detection of DNA based on a bifunctional ruthenium complex, Anal. Chem. 80 (2008) 77–84.

[52] Manohar, S.; Mantz, A.R.; Bancroft, K.E.; Hui, C.Y.; Jagota, A.; Vezenov, D.V., Peeling single stranded DNA from graphite surface to determine oligonucleotide binding energy by force spectroscopy, Nano Lett. 8 (2008) 4365–4372.

[53] Riordan, J.R.; Rommens, J.M.; Kerem, B.; Alon, N.; Rozmahel, R.; Grzelczak, Z.; Zielenski J.; Lok, S.; Playsic, N.J., Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA, Science 245 (1989) 1073–1080.

[54] Riordan, J.R.; Rommens, J.M.; Kerem, B.; Alon, N.; Rozmahel, R.; Grzelczak, Z.; Zielenski J.; Lok, S.; Playsic, N.J., Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Correction, Science 245 (1989) 1437–1437.

[55] Wilentz, R.E.; Chung, C.H.; Sturm, P.D.J.; Musler, A., Sohn, T.A.; Offerhaus,G.J.A.; Yeo, C.J.; Hruban , R.H.; Slebos, R., K-ras mutations in the duodenal fluid of patients with pancreatic carcinoma, Cancer 82 (1998) 96–103.

[56] Zhu, N.; Zhang, A.; Wang, Q.; He, P.; Fang, Y., Electrochemical detection of DNA hybridization using methylene blue and electro-deposited zirconia thin films on gold electrodes, Anal. Chim. Acta 510 (2004) 163–168.

[57] Niu, S.; Zhao, M.; Ren, R.; Zhang, S., Carbon nanotube-enhanced DNA biosensor for DNA hybridization detection using manganese(II)–Schiff base complex as hybridization indicator, J. Inorg. Biochem. 103 (2009) 43–49.

[58] He, P.; Dai, L., Aligned carbon nanotube–DNA electrochemical sensors, Chem. Commun. 3 (2004) 348–349.

[59] Aladag, N.; Ozkan-Ariksoysal, D.; Gezen-Ak, D.; Yilmazer, S.; Ozsoz, M., An Electrochemical DNA Biosensor for the Detection of the Apa I Polymorphism in the Vitamin D Receptor Gene Using Meldola's Blue as a Hybridization Indicator, Electroanal. 22 (2010) 590–598.

[60] Wang, G.L.; Fang, X.; Wu, X.M.; Hua, X.L.; Li, Z.L., Label-free and ratiometric detection of nuclei acids based on graphene quantum dots utilizing cascade amplification by nicking endonuclease and catalytic G-quadruplex DNAzyme, Biosens. Bioelectron. 81 (2016) 214–220.

Figure captions

Scheme 1. Preparation of nanostructured CDs/AuSPE electrodes.

Figure 1. SEM image of the synthetized CDs deposited onto a gold substrate at 300000x (A) or 100000x (B) magnification.

Figure 2. Cyclic voltammogram of AuSPE (black curve) and CDs/AuSPE (dotted curve) electrodes in 0.1 M H_2SO_4 solution.

Figure 3. DPVs response of HP1/CDs/AuSPE in 0.1 M PB pH 7.0 solution before (grey curve) and after hybridization with: a single mismatched, HPSM (grey dotted curve), a fully complementary, HP2C (black curve), and a non-complementary sequence, HP2NC (black dashed curve), after accumulation of SAF by consecutive potential cycling. Inset: Scheme of the hybridization process.

Figure 4. DPVs response of HP1/CDs/AuSPE in 0.1 M PB pH 7.0 solution after hybridization with different concentrations (from 1.0 nM to 20 μM) of the fully complementary sequence, HP2C, after accumulation of SAF by consecutive potential cycling. Inset: Calibration plot obtained. Error bars were estimated with the standard deviation of five different biosensors (n=5).

Scheme 2. Scheme of the DNA biosensor development.

Figure 5. A) Peak current bar diagrams of the biosensor response before (Probe) and after hybridization with: a fully complementary (Probe-WT), a mutated F508del (Probe-Mutated) and a non-complementary (Probe-NC) sequences using SAF as redox indicator. B) Signal processing of the biosensor response. Percentage of normalized probe signal increment with: mutated (CF patients), non-mutated (healthy people) and non-complementary sequence.