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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.

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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.