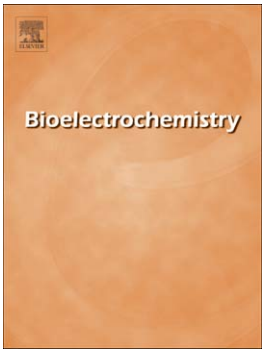

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Tania García-Mendiola, María Ramos Cerro, José María López-Moreno, Félix Pariente, Encarnación Lorenzo

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Dyes as Bifunctional Markers of DNA Hybridization on Surfaces and Mutations Detection.

Tania García-Mendiola [a,b], María Ramos Cerro [a], José María López-Moreno [a], Félix Pariente [a,b] and Encarnación Lorenzo [* a,b]

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

[b] Instituto Madrileño de Estudios Avanzados (IMDEA) Nanociencia

[*] e mail: encarnacion.lorenzo@uam.es

Abstract

The interaction of small molecules with DNA has found diagnostic and therapeutic applications. In this work, we propose the use of two different dyes, in particular Azure A and Safranine, as bifunctional markers of on-surface DNA hybridization and potent tools for screening of specific gene mutations directly in real DNA PCR amplicons extracted from blood cells. By combining spectroscopic and electrochemical methods we demonstrate that both dyes can interact with single and double stranded DNA to a different extent, allowing reliable hybridization detection. From these data, we have also elucidated the nature of the interaction. We conclude that the binding mode is fundamentally intercalative with an electrostatic component. The dye fluorescence allows their use as nucleic acid stains for the detection of on-surfaces DNA hybridization. Its redox activity is exploited in the development of selective electrochemical DNA biosensors.

Keywords: Dye-DNA interaction, redox indicator, DNA biosensor, gene mutation screening.

1.**Introduction**

Deoxyribonucleic acid plays a relevant role in living cells because it contains the genetic information that is essential to promote the biosynthesis of proteins through replication and transcription processes. Although DNA molecules are very stable and the genetic code is protected within the cell nucleus, the original (wild type) sequence may be altered for several reasons, such as, failures in the replication process, interaction with xenobiotics capable of binding to genomic material, light radiation and others. Sometimes, alterations in the original DNA sequence (mutations) lead to significant changes in the transcribed proteins and can cause serious hereditary diseases in the living organisms, included humans. From a clinical point of view, the detection of changes in the sequence of particular genes is essential in order to obtain an early diagnostic of hereditary diseases and to organize less aggressive and effective therapeutic solutions.

Interaction of small molecules with double stranded DNA takes place at the molecular level following three binding modes: electrostatic, groove binding and intercalative. Hence, studies of the structure, binding specificity, mechanism and dynamics of such interactions have attracted the continuous interest of chemists and biologists, because they may be helpful in the understanding of the action mode of some antitumor, antivirus and anti-patogenic drugs [1,2]. In general, planarity and charge delocalization are important features needed for efficient intercalators [3].

Safranine O and Azure A are important cationic dyes that belong to the phenazinium and phenothiazinium group, respectively. Both molecules present a planar structure (see Figure 1 of Supporting Information) that may enhance interaction with DNA via intercalation. Though the interaction of some phenazinium and phenotiazinium dyes

with double strand DNA (dsDNA) has been reported previously by others authors [4,5], as far as we know, no method for DNA sensing based on the bifunctional markers Azure A and Safranine has been reported. Whereas the interaction of these dyes with dsDNA has been well documented in terms of binding properties [4,5] similar systematic studies regarding the interaction with single strand DNA(ssDNA), and the potential selectivity for one of these forms, has not been yet reported.

Hybridization between complementary ssDNA sequences is the most specific bio-recognition system in nature. Unlike enzymes or antibodies, nucleic acid recognition layers can be easily prepared and are very stable. Such recognition layers add a new and unique dimension of specificity in the arsenal of electrochemical biosensors and can play a relevant role in point-of-care analysis. Indeed, the detection of specific DNA sequences or DNA mutations using electrochemical biosensors may greatly reduce the assay time and simplify the analytical protocols compared with traditional DNA sequencing methods. These fast monitoring approaches are needed for quick preventive action and early diagnosis [6-11]. However, hybridization detection, in an accurate and selective mode requires very selective electrochemical molecular probes, with different binding affinity to ssDNA and dsDNA, in particular for the detection of point mutations. The difference in duplex formation between mutated and wild-type sequences is relatively small, and not all the proposed hybridization-based assays are sensitive enough. Thus, there is still a great deal of interest in understanding and controlling the interaction of molecules with DNA, in order to design highly sensitive and selective molecular probes for DNA analysis.

In this work, combining spectroscopic and electrochemical techniques, we want to deepen the understanding of the mode of interaction of two small molecules, Safranine O and Azure A, with ss and dsDNA. This research stems from our efforts to study both

fundamental and practical aspects of the interactions between bifunctional molecular probes and DNA [12] in order to develop portable DNA-based sensing devices based on direct fluorescent or electrochemical measurements, without the need for labelling the target sequence. This proof-of-principle work has been finally demonstrated by developing biosensors able not only to detect DNA in a rapid and easy way, but also genetic mutations associated with human diseases, directly in real DNA 300 base pair PCR amplicons extracted from blood cells. As case study, sequences of *Helicobacter Pylori* and mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) [13] were chosen.

2. Experimental section

2.1. Chemicals

Potassium nitrate, sodium phosphate and sodium chloride were obtained from Scharlab Company. Azure A (AA), Safranine O (SAF) and all other chemicals used in this work were reagent grade quality, obtained from Sigma-Aldrich Co., and used as received without further purification. All solutions were prepared just prior to use. Water was purified with a Millipore Milli-Q-System (18.2 MΩ cm). Nuclease free solutions were used in DNA experiments. Double strand calf thymus DNA (ds-ctDNA, activated and lyophilized) was purchased from Sigma-Aldrich Co. ds-ctDNA stock solutions (nominally 1.0 mg/ml) were prepared in 0.1M phosphate buffer (PB) pH 7.0 solution. The DNA solutions gave a UV absorbance ratio (A_{260}/A_{280}) of about 1.9, indicating that the DNA was free of protein [14]. The concentration in base pairs (bp) of DNA was determined by using a molar absorptivity of 6600 M^{-1} at 260 nm [15]. Single strand calf thymus DNA (ss-ctDNA) was obtained by boiling in water capped vials containing ds-ctDNA (1.0 mg/ml) in 0.1M PB pH 7.0 for 30 minutes. To prevent spontaneous renaturation, this solution was subsequently quenched in an ice-bath. The resulting denatured DNA samples were stored frozen at -20ºC. Custom made synthetic oligonucleotides (25-mer) from the pathogen bacterium *Helicobacter Pylori* 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 [16]. The PCR samples consisted of thiolated wild type (WT11- SH, WT12- SH), wild type (WT11, WT12) and mutant (F508del, and SNP G542X) 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 polimerease chain reaction (PCR) are respectively listed in Table 1 and 2 of Supporting Information.

2.2. Apparatus

UV-Visible spectra were recorded in a double beam PharmaSpec UV-1700 series Shimadzu Corporation, operating from 200 nm to 800 nm in 1.0 cm quartz cells. Fluorescence spectra were conducted on a Cary Eclipse Varian spectrofluorimeter equipped with a peltier system to control the temperature inside the cuvettes. Andor Revolution DSD2 simple confocal microscope was used for on surface Dye-DNA localization. Sequencing of DNA samples was carried out using a Sanger Sequencher 3730xL. Arrays 36cm. POP 7. Applied Biosystems. The final concentration was determined by UV-vis molecular absorption spectrometry using a Thermo Scientific NanoDropTM 1000 Spectrophotometer (NanoDrop Technologies). PCR fragments were generated in a BIO-RAD thermocycler (DNA Engine Tetrad2. Peltier Thermal Cycler. BIO-RAD Laboratories Inc.). Electrochemical 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.

2.3. Procedures

2.3.1. Spectroscopic measurements

UV-visible absorption and fluorescence titrations were carried out at 10 µM of the dye while the concentration of ss and ds-ctDNA was varied from 0 to150 µM or from 0 to

250 µM in the case of SAF fluorescence titrations. The absorbance or the emission intensity was plotted *vs* DNA concentration in base pairs. The intrinsic binding constants, K_b, were determined using equation 1 [17,18].

$$[ADN]/ (\varepsilon_a - \varepsilon_f) = [ADN]/(\varepsilon_b - \varepsilon_f) + 1 / K_b (\varepsilon_a - \varepsilon_f) \quad \text{(Equation 1)}$$

Where ε_a is the molar absorptivity of the dye in presence of the different concentrations of ss or ds-ctDNA, ε_f and ε_b are the molar absorptivity for free and bound forms of the dye, respectively.

The magnitude of the hypochromism in the presence of DNA, has been correlated with the overall binding strength and was calculated as:

$$\text{Hypochromicity \%} = (\varepsilon_b - \varepsilon_f / \varepsilon_f) \times 100 \quad \text{(Equation 2)}$$

Where ε_f and ε_b are molar absorptivity for free and bound forms of the dye, respectively [19].

2.3.2. Electrochemical studies

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were carried out in 0.1M PB pH 7.0. 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. AuSPEs were electrochemically activated by placing a 50 µL drop of 0.1 M H_2SO_4 solution on their surface. Then, the potential was cycled (10 times) from -0.200 V to 1.20 V (*vs* Ag) at 100 mVs^{-1} and the electrodes were washed with deionized water.

2.3.3. Immobilization of DNA on AuSPEs.

10 µl of 2.0 mM ds or ss-ctDNA solution were drop- cast onto an *AuSPE* followed by air-drying. Afterwards, the resulting modified electrode was soaked in sterilized water for 30 min and rinsed with water to remove any un-adsorbed DNA. These modified electrodes are denoted in the text as ds-ctDNA/AuSPE or ss-ctDNA/AuSPE, respectively.

10 µL of 40 µM thiolated synthetic (HP1-SH) sequence or 10 µL of 2.5 ng μL^{-1} thiolated PCR samples (WT11-SH or WT12-SH) were transferred onto the AuSPE. Afterwards, the electrode was kept at room temperature for 24 or 120 hours, respectively. Then, it was soaked in sterile water for at least 30 min.

2.3.4. Sample preparation for confocal fluorescence microscopy measurements

ds or ss-ctDNA/AuSPE were immersed in 0.1M PB pH 7.0 containing 1.0 mM of AA or SAF and the potential was cycled (100 times) at 100 mV/s. Then, the electrodes were rinsed with sterile water, left to dry at room temperature and observed under the microscope to take the images.

2.3.5. Denaturation of PCR DNA samples

Thiolated PCR samples immobilized onto AuSPEs were denatured by immersing the resulting modified electrode in 6 M guanidine hydrochloride solution over 24 hours at room temperature [20]. Afterwards, the electrode was soaked with water over 3 hours. Non-thiolated PCR samples, used as analytes, were denatured by immersing them in boiling water (100 ºC) for 30 minutes followed by rapid cooling in an ice bath [21].

2.3.6. Hybridization and detection

AuSPEs modified with the capture probe (denatured thiolated PCR samples or thiolated synthetic oligonucleotides) were subsequently hybridized (1h 40 ºC) with 10 µL of the

analyte: denatured PCR (5.0 ng μL^{-1} wild type or mutated samples) or synthetic oligonucleotides (20 µM complementary, non complementary or SNP sequence). After the hybridization step, DNA modified electrodes were immersed in 0.1M PB pH 7.0 containing 1.0 mM of AA or 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, and differential pulse voltammograms were immediately recorded.

3. Results and Discussion.

3.1 Dye interaction with DNA

Spectroscopic studies

The interaction of SAF and AA with DNA was studied by UV-visible spectroscopy. The electronic spectra of both dyes in the absence and presence of increasing concentrations of single and double stranded ctDNA are presented in Figure 1. The absorption spectra of aqueous solutions of SAF and AA in 0.1M PB pH 7.0 solution, show quite similar broad and lowest-energy bands with absorbance maxima at around 516 nm and 630 nm, respectively. In the presence of DNA, the absorbance peak of SAF decreased about 42 % and 36 % at saturating concentrations (150 μM in base pairs) of ss and ds-ctDNA, respectively. This hypochromic effect is concomitant with a red-shift of 12 nm for ss-ctDNA and 9 nm for ds-ctDNA. Similar red-shifts are observed in the electronic spectra of Azure A in the presence of ss-ctDNA and ds-ctDNA. However, the hypochromic effect was less evident (18% and 11% of decrease in absorbance) than in the case of SAF. It should be also stated that the electronic spectra of AA in the presence of ss-ctDNA show one isosbestic point at 625 nm, suggesting the presence of two different dye species in equilibrium: the free and the DNA-bound dye. These DNA-induced spectral variations can be explained in terms of changes in the local polarity around the chromophore due to strong interactions with pyrimidine and purine bases [22]. The decrease in the local polarity of the ct-DNA environment compared with the pure buffer solution causes a decrease in the gap in energy between the HOMO and LUMO molecular orbitals of the dye, giving rise to the bathochromic effect. Thus, the hypochromic and bathochromic effects observed indicate that equilibrium binding processes are taking place [23]. In fact, molecule intercalation is generally characterized by a bathochromic shift concomitant with hypochromism, due to the stacking between

DNA-π and the molecule's π domain [19]. Hence, the results obtained point to a strong interaction between these dyes and ds-ctDNA and suggests that it is mainly through intercalation, which agrees well to with that reported by other authors [4,5].

Most of the existing literature concerning AA and SAF binding to DNA has focused on the elucidation of the mode of their interaction with ds-ctDNA. However, the present work is focused on understanding the mode of interaction with both ds and ss-ctDNA in order to establish the differences between them, which is fundamental, if one wanted to use these dyes as indicators of the hybridization event for the development of DNA biosensors. According to the data presented in Figure1(C, D), the hypochromic effect, for both dyes, is higher in the presence of ss-ctDNA than with ds-ctDNA. This fact, which is more evident in the case of SAF, suggests a strong interaction between the dye and the unpaired bases of ss-ctDNA. Hence, in order to elucidate the mode of such interaction, we have evaluated the effect of parameters that may strongly affect the mode of interaction, in particular, the ionic strength. The electronic spectra of the dyes, in the presence and absence of ds and ss-ctDNA, in 0.1M PB pH 7.0 supplemented with 0.4 M NaCl (Figure 2 of SI) were very similar to those depicted in Figure 1. This result suggests that the interaction of both dyes with DNA, is independent of ionic strength, which agrees well with intercalation as one would expect for ds-ctDNA. However, in the case of ss-ctDNA, intercalation by stacking between the base pairs should not be the main mode of interaction because DNA strands are separated. The large hypochromic effect observed in the presence of ss-ctDNA suggests a strong interaction between the dye and the individual bases, which may be due to hydrophobic interactions between the planar structure of the dye and the planar domains of the DNA bases, giving rise to adducts in which the dye is in close proximity to the pyrimidine or purine bases [24].

The absorption spectra changes observed were used to estimate the respective binding constants. From the plot of the [DNA]/ (ε_a- ε_f) *versus* [DNA] and according to Equation 1 (see experimental section), the respective intrinsic binding constants (K_b) were calculated (see Table 3SI). K_b values obtained for ds-ctDNA are comparable to those reported for other intercalating agents [4,23], such as methylene blue, [25] and [Cr $(H_2O)_2$ (naphen)]$^{2+}$ [26], but lower than those of the classical intercalators, like ethidium bromide [27]. Concerning K_b for ss-ctDNA, which has not been previously reported for these dyes, it is worth noting that values obtained are higher than those obtained for ds-ctDNA. It should be also noted that ionic strength does not affect K_b values in practical terms, which agrees well with a strong interaction between the dyes and the individual bases, due to the close proximity between them, and allows an electrostatic binding as the main mode of interaction to be discounted. This behavior was also reported in the literature for others phenothiazines such as methylene blue, which has a strong affinity for the free guanine bases present in ss-ctDNA and interacts in a different way with ss and ds-ctDNA [28, 29].

Figure1.

On the other hand, the absorbance spectra of AA in the presence of saturating concentrations of ds-ctDNA (150 µM in pair bases) show a strong dependence on temperature (see Figure 3B of SI). When the temperature of the solution increases from 25 to 80 ºC, the absorbance peak of the dye shows a significant hyperchromic effect concomitant with a blue-shift. At temperatures close to 80ºC, the AA spectrum is quite similar to that of the free dye in solution. This result suggests that there is a gradual liberation of the dye bond to DNA as soon as the double helical structure is denaturated by the temperature. Similar experiments carried out with saturating concentrations of ss-

ctDNA (150 µM in pair bases) show the same hyperchromic and blue-shift effect on the AA band for temperatures from 25 to 55 ºC. However, the absorbance of the dye does not reach the value obtained when the AA is in a DNA free solution, suggesting that the interaction with ss-ctDNA is stronger than with ds-ctDNA. From 55 to 80 ºC a hypochromic effect is observed which points to a different kind of interaction of this dye with ss-ctDNA and ds-ctDNA (see Figure 3C of SI). As a control experiment the absorbance spectra of AA as a function of the temperature was also carried out (see Figure 3A of SI). No changes in the absorbance spectra was observed.

SAF and AA show emission spectra with maxima centred at 590 nm and 658 nm, when they are excited at 520 nm and 632 nm, respectively. As in the case of absorption bands, we have exploited this property for studying their interaction with ds and ss-ctDNA. For this purpose the emission spectra of 10 µM SAF (A, C) or 10 µM AA (B, D) in 0.1M PB pH 7.0, in the absence and in the presence of increasing amounts of ds and ss-ctDNA were recorded (see Figure 4 SI). The addition of DNA gives rise to a gradual decay in the emission intensity, which is different for the two dyes studied. In both cases, higher fluorescence quenching is observed when dyes are bound to ss-ctDNA than to ds-ctDNA. This effect is more pronounced in the case of SAF. Fluorescence quenching is the result of a change in the environment around the dye or in its excited-state properties probably due to the presence of hydrophobic interactions between the dye and DNA. However, for molecules binding to DNA via an intercalative mode, either fluorescence enhancement [30] or quenching [26,31] has been reported.

The interaction strength between the dyes and DNA was quantified by using the Stern-Volmer equation [32]. The quenching constants (K_{SV}) were calculated from a plot of I_o/I *vs* [DNA], where I_o and I are respectively, the fluorescence intensity of the free and the

bound DNA dye. Values obtained (see Table 4SI) are similar to those reported for other intercalators, such as 9-anthylmethylammonium chloride (AMAC), N-ethyl-(9-anthryl)methylammonium chloride (N-Et-AMAC) and 3-(9-anthryl) propylammonium chloride (APAC) [34]. Thus, these results again suggest that intercalation seems to be the main interaction mode of both dyes with ds-ctDNA. In addition, from the fluorescence titration data, the ratio [dsDNA]/[Dye] was estimated to be 9 for AA and 7 for SAF(see Table 4SI) indicating that about 9 and 7 bases of dsDNA are involved on binding a molecule of AA and SAF, respectively. Moreover, the results described above and the calculated quenching constants confirm that exist a strong interaction exists with ss-ctDNA. A partial renaturation of the base pairs in the ss-ctDNA, during the interaction process, is not enough to explain such high binding constants (Table 4SI). This result should be interpreted by considering a specific interaction between these dyes and each individual base, resulting in close contact between them. In order to clarify this point, we performed confocal laser scanning microscopy (CLSM) to detect AA bound to ds or ss-ctDNA immobilized on a substrate. In this case, we used AA as a fluorescent DNA stain. Figure 2 shows the CLSM image obtained for on-surface ds-ctDNA and ss-ctDNA after staining with AA, as is described in the Experimental section. As can be seen, fluorescence emission of AA demonstrates that the dye is accumulated in the DNA after the interaction. Moreover, less fluorescence is observed on the ss-ctDNA than on the ds-ctDNA layer, corroborating, the stronger interaction between AA and ss-ctDNA. The differences in images A and B is also evidence of the hybridization event. Therefore, this dye can be employed as a hybridization molecular probe. As a control the same experiment was performed with a bare electrode AuSPE and no fluorescence was observed. This confirms that AA does not stain the bare AuSPE.

Figure 2.

Electrochemical behavior of Dyes at DNA-modified electrodes

Besides their interesting spectroscopic properties, AA and SAF are redox active molecules. Thus, in order to ascertain the interaction mode of AA and SAF with DNA and their potential application in the development of electrochemical biosensors, we studied their electrochemical behavior at DNA modified electrodes. For these studies, gold screen printed electrodes (AuSPEs) were employed as drop-on sensors in order to develop portable DNA sensing devices. Figure 3 shows the electrochemical behavior of AA and SAF, 1.0 mM in 0.1M PB pH 7.0, after cyclic potential scans at a bare AuSPE (curve a), a ds-ctDNA-modified AuSPE (ds-ctDNA/AuSPE) (curve b) and a ss-ctDNA modified AuSPE (ss-ctDNA/AuSPE) (curve c) in 0.1M PB pH 7.0. It can be seen that, at AuSPE (curve a), a pair of redox peaks, ascribed to the oxidation/reduction of the dyes, appear at a formal potential (Eº') value of – 0.688 and -0.402 V (*vs* Ag) for SAF and AA, respectively. The peak potential separation (ΔEp), at 100 mV/s, is 39 mV for AA, which is approximately that expected for a freely diffusing two-electron reversible redox process. In the case of SAF, a peak potential separation (ΔEp) of 66mV is observed, which is the expected for a freely diffusing one electron reversible redox process and is ascribed to one of the two electronic transitions of safranine [34] (see Scheme 1SI). At the ds-ctDNA/AuSPE (see Figure 3 A, C curve b), the Eº' shifted to -0.714 mV and -0.422 mV (*vs* Ag) for SAF and AA, respectively. At the ss-ctDNA/AuSPE (see Figure 3A and 3C curve c) Eº' shifted also to more negative values (-0.723 and -0.431, *vs* Ag, for SAF and AA, respectively). These results are consistent with a strong interaction between both dyes and the DNA immobilized on the electrode surface [21, 35] and agree well with those obtained by spectroscopic techniques,

confirming that the interaction with ss-ctDNA is stronger than with ds-ctDNA. From potential shifts ($\Delta E^{o\prime}$) measured, according to the Scheme (Scheme 2 SI) proposed by Bard and Carter [12] and using Equation (3), the ratio of the equilibrium surface-binding constants (K_{ox}/K_{red}) between ds ct-DNA and the dyes was estimated to be around 30 mV for SAF and 20 mV for AA. In the case of ss-DNA higher values (35 and 30 mV) were found.

$$E^{o\prime}_{free} - E^{o\prime}_{bound} = (0.059/n) \log K_{ox}/K_{red} \qquad \text{(Equation 3)}$$

where, $E^{o\prime}_{free}$ and $E^{o\prime}_{bound}$ represent the formal potentials of the dyes in solution and bound to DNA, and K_{ox} and K_{red} are the respective equilibrium binding constants to DNA for oxidized and reduced forms of the dye. The results obtained suggest that the oxidized form of the dyes interacts with the DNA backbone on the ds or ss-ctDNA/AuSPE more strongly than with the reduced form, which is characteristic of an interaction mode with some electrostatic component. This component must be higher in the case of ss-ctDNA.

In summary, we can conclude that the results obtained by spectroscopic and electrochemical experiments point to a strong interaction between the dyes and with solution or on-surface DNA, being stronger with ss-ctDNA than with ds-ctDNA. Moreover, the interaction seems to be by intercalation via an electrostatic component, this being more important in the case of ss-ctDNA. The extent of the different interaction of dyes with both forms of DNA has been exploited in the development of a DNA-sensing sensor.

On the other hand, a decrease in the peak current as well as a less reversible shape, which suggests severe limitations in the charge transfer kinetics, is observed after DNA electrode modification, in particular in the case of ds-ctDNA. Hence, it seems that the

dye molecule endures steric or electrostatic impediments to electronic transfer when the electrode is modified with DNA.

In order to determine the nature of the electrochemical process, the variation of peak current with the scan rate was studied. In the case of AuSPE, the peak current was directly proportional to the square root of the scan rate ($v^{1/2}$), as expected for a process controlled by diffusion. However, for the DNA modified electrodes the peak current was directly proportional to the scan rate, v, which is characteristic of a surface confined redox process. This result indicates that the dye is accumulated in the ds-ctDNA layer immobilized on the electrode surface. After repetitive potential cycles, there is an increase in the peak current at electrodes modified with DNA (Figures 3B, 3D and 5A, 5B of SI). Hence after each potential cycling the dye is accumulated on the DNA layer present at the electrode surface, in particular when the electrode is modified with ss-ctDNA. This result points again to a stronger interaction of the dye with the ss-ctDNA. If after repetitive cycling, the electrode is removed from the cell, rinsed with water and placed in electrolyte solution (0.1M PB pH 7.0) containing no dye, a pair of voltammetric peaks, quite similar to those previously obtained is observed. This indicates that the dye has incorporated to the DNA layer on the electrode surface (see Figure 6SI) [12].

In summary, we can conclude that the results obtained by spectroscopic and electrochemical experiments point to a strong interaction between the dyes and in-solution or on-surface DNA, and are stronger with ss-ctDNA than with ds-ctDNA. This difference in the interaction extend with both forms of DNA has been exploited in the development of a DNA sensing sensor.

Biosensor development

Based on the results described above, the use of AA and SAF as electrochemical indicators for application in DNA biosensing devices was evaluated. For this purpose in a first approach, a sequence of the *Helicobacter Pylori* was employed 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 study within the framework of developing an approach with broad applicability.

A unique 25-mer thiolated sequence of this bacterium (the probe: HP1-SH) was chemisorbed onto the AuSPE through the thiol group with a surface coverage around 70 70 pmol/cm^2. In the hybridization test, a complementary (HP2C), a non-complementary (HP2NC) and a SNP sequence (HP2SM), were selected as the target DNA. Changes in differential pulse voltammogram (DPV) peak currents of the dyes accumulated at the probe modified electrode before and after hybridization were obtained (Figure 7A and C of supporting information). Hybridization of the probe with the complementary sequence (HP2C) in the biosensor recognition layer resulted in a dramatic decrease (80%) in the DPV response, while virtually no change in current was obtained for the non-complementary (Figure 5SI A, C). This result agrees well with those above described for ct-DNA modified electrodes. Hybridization with the SNP target resulted in a significant but lower decrease (about 40%) in the peak current compared to that obtained when the probe was hybridized with its fully complementary sequence (Figure 7A and C of supporting information). This fact could be interpreted as an increase in the binding constant of the dyes with the distorted helix [36].

The changes observed in peak currents suggest that both hybridization and target sequence recognition can be achieved using AA or SAF as electrochemical indicators. The current response decreases in relation to the amount of the target sequence used over the range 0 to 20μM with a linear correlation coefficient of r = 0.996 (see Figure

7B and D of supporting information). The detection limit was calculated as three times the standard deviation divided by the slope of the calibration curve, and was determined to be 22.5 ± 0.1 nM of HP2C for SAF and 29.5 ± 0.3 nM of HP2C for AA, with a reproducibility of 94 % in both cases.

On the basis of above results, it can be concluded that both dyes are selective electrochemical indicators of the DNA hybridization event that allows not only DNA recognition but also SNP detection in a determined DNA sequence. Taking into account their selectivity and sensitivity they were applied to the detection of mutations associated with human diseases in real DNA PCR amplicons extracted from blood cells. In particular, mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) [37] associated with Cystic fibrosis (CF) were chosen as case for study. The most common mutant allele is the F508del [38], which is a three-nucleotide deletion causing the loss of a phenylalanine residue of the CFTR protein. The second most frequent is p.Gly542Stop (SNP G542X), which results in a truncated CFTR protein. SNP G542X belongs to those CFTR mutations that are common throughout the entire world. Sequencing of genes is the gold standard for identifying mutations. However, these methods have serious drawbacks as routine diagnostic tools, because of their labour intensity and cost. New, accurate and facile strategies for the detection of point mutations are therefore necessary. In this sense, a DNA biosensor can be an interesting alternative to classical gene assay due to its several advantages, in particular low cost and simplicity [6-11]. The biosensor was developed following the strategy depicted in Scheme 1. The mutation detection relies on the comparison of the voltammetric transduction of the hybridization reaction between the immobilized probe (obtained from denaturation of a thiolated wild-type PCR sample) and the target DNA (wild type or mutant) present in the sample. Two different thiolated capture probes, one per each

mutation to be analyzed, named WT11-SH and WT12- SH were obtained directly from thiolated PCR amplicons of exons 11 and 12, and were assembled on the electrode surface. After denaturation, they were hybridized with the corresponding PCR target, which comprise around 300 bp PCR amplicons of exons 11 and 12 of CFTR gene carrying F508del and SNP G542X mutations (see Table 2SI). The biosensor response was obtained from the corresponding DPVs of the AA or SAF accumulated on the ds DNA layer formed on the electrode surface after hybridization. As a control, wild type amplicons, WT11 and WT12 from exon 11 and 12 were used.

Scheme1.

Figure 4 shows the biosensor response for F508del and SNP G542X mutations using SAF and AA as redox indicators. The voltammograms show the same shape but different peak currents for mutated and wild type target DNA and confirm unambiguously the presence of mutation without the need for stringent conditions (i.e. formamide) usually used for such a purpose. Bar diagram of Figure 5 summarises the results obtained. Taking into account that the error associated with each measure is less than 5%, it can be concluded that the proposed screening method discriminates between wild and mutated samples. In addition, it is worth noting that the decrease in the current signal is slightly higher for SNP G542X than for F508del as a result of the major distorted DNA duplex formed after the hybridization with the three-nucleotide deletion mutation. The reproducibility was evaluated from the response of five different biosensors (prepared in the same manner) to either wild type or mutated target DNA. Reproducible signals were obtained with a relative standard deviation (RSD) of 5%, or less, in all cases.

The developed methodology, can be used as rapid and precise screening method of gene mutations as an alternative to the classical gene assay. These mutations can be used to trace generational inheritance patterns associated with specific diseases [39].

Figure 4.

Figure 5.

Conclusions

We have demonstrated the proof-of-principle of an electrochemical biosensor based on two redox indicators Azure A and Safranine as screening methods for detection of gene mutations. The fabrication and measuring protocols are simple, low cost, required short time for analysis consuming and can be easily adapted to the detection of other mutations. Only a specific DNA sequence (the probe) is required to modified the electrode surface. Hence, the method has a huge potential to greatly simplify the development of point-of-care molecular diagnostic analysis systems.

The interactions of both dyes with ss and ds-ctDNA were thoroughly investigated by absorption, emission and electrochemical studies. From these studies, it is clear that there is a strong interaction between these dyes and DNA, and they can be accumulated in an on-surface immobilized DNA layer. Using these findings as a point of departure and the fact that both dyes are fluorescent, they can be employed as markers of DNA hybridization on surfaces using confocal fluorescence.

Acknowledgements

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References

[1] Stoddart, J. F., From carbohydrates to enzyme analogues, Chem. Soc. Rev. 8 (1979) 85-142.

[2] Sutherland, I.O, Molecular recognition by synthetic receptors, Chem. Soc. Rev. 15 (1986) 63-91.

[3] Cao,Y, He,XW., Studies of interaction between Safranine T and double helix DNA by spectral methods, Spectrochim Acta Part A. 54 (1998)6 883-892.

[4] Paul, P., Kumar, S., Spectroscopic studies on the binding interaction of phenothiazinium dyes toluidine blue O, azure A and azure B to DNA, Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy.107 (2013) 303–310.

[5] Khan, A. Y., Saha, B., Kumar, S., Phenazinium dyes safranine O and phenosafranine induce self-structure in single stranded polyadenylic acid: Structural and thermodynamic studies, Journal of Photochemistry and Photobiology B: Biology. 132 (2014)17–26

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

[7] Mickelsen, S. R., Electrochemical biosensors for DNA sequence detection, Electroanal. 8 (1996)15-19.

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

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

[10] 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.

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

[12] Carter, M.T.; Rodriguez, M.; Bard, A., voltammetric studies of the interaction of metal-chelates with DNA .2. tris-chelated complexes of cobalt(III) and iron(II) with 1,10-phenanthroline and 2,2'-bipyridine, J.Am.Chem Soc. 111 (1989) (24) 8901-8911.

[13] Cox, T.K., Chakravarti, A., Buchwald, M., Tsui, L.C., Identification of the cystic-fibrosis gene - genetic-analysis, Science. 8(1989) 245(4922), 1073-1080.

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

[15] Doty, P., Rice S. A., the denaturation of desoxypentose nucleic acid, Biochim. Biophys. Acta. 16 (1955) (3) 446-448.

[16] García-Mendiola, T., Barreiro Martinez, T., Pariente, F., Molano, J., and Lorenzo. E., Screening of specific gene mutations associated with Human diseases, Electroanalysis. 26(2014) 1 – 11.

[17] Benesi, H. A., and Hildebrand, J. H., A Spectrophotometric Investigation of the Interaction of Iodine with Aromatic Hydrocarbons, J. Am. Chem. Soc. 71(1949)2703-2709.

[18] Meehan, T., Gamper, H., Becker, J. F., Characterization of reversible, physical binding of benzo[a]pyrene derivatives to DNA, J. Biol. Chem. 257 (1982) (17) 479-85.

[19] Bresling, D. T., Yu, C., Ly, D., Schuster, G., Structural modification changes the DNA binding mode of cation-substituted anthraquinone photonucleases: Association by intercalation or minor groove binding determines the DNA cleavage efficiency, Biochemistry. 36(1997) 34 10463-10473.

[20] Li, D., Zou, X., Shen, Q., Dong, S., Kinetic study of DNA/DNA hybridization with electrochemical impedance spectroscopy, Electrochem. Commun. 9(2) (2007) 191-196.

[21] 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 (8) (1995)774-777.

[22] Pyle, A. M., Rehmann, J. P., Meshoyrer, R., Kumar, C. V., Turro, N. J., Barton, J. K., Mixed-ligand complexes of ruthenium(II) - factors governing binding to DNA**,** J. Am. Chem. Soc. 111 (1989) (8) 3051-3058.

[23] Saha, I., Hossain,M., and Kumar,G.S., Base pair specificity and energetics of binding of the phenazinium molecules phenosafranine and safranine-O to deoxyribonucleic acids: a comparative study, Phys. Chem. Chem. Phys. 12(2010) 12771–12779.

[24] Cantor, C., Schimmel, P.R., Techniques for the study of biological function and structures, Biophysical Chemistry part II (1990) W. H. Freeman; San Francisco, 391-398.

[25] Zhang, L.Z.,Tang, G.Q. , The binding properties of photosensitizer methylene blue to herring sperm DNA: a spectroscopic study. Journal of Photochemistry and Photobiology B: Biology. 74(2004)119–125.

[26] Waring, M. J., Complex formation between ethidium bromide and nucleic acids**,** J. Mol. Biol. 13(1965) 269.

[27] Ambroise, A., Maiya, B. G., Ruthenium (II) complexes of redox-related, modified dipyridophenazine ligands: Synthesis, characterization, and DNA interaction, Inorg. Chem. 39(2000) 4256-4263.

[28] Erdem, A., Kerman, K., Meric, B., Salih Akarca, U., Ozsoz, M., Novel hybridization indicator methylene blue for the electrochemical detection of short DNA sequences related to the hepatitis B virus, Analytica Chimica Acta, 422(2000)139-146.

[29] Pournaghi-Azar, M.H., Hejazi, M.S.,Alipour, E., Differential pulse anodic stripping voltammetry of copper in dichloromethane: application to the analysis of human hair, Analytica Chimica Acta.570(2006)144-150.

[30] Chaires, J. B., Dattagupta, N., Crothers, D. M., Studies on interaction of anthracycline antibiotics and deoxyribonucleic-acid - equilibrium binding-studies on interaction of daunomycin with deoxyribonucleic-acid, Biochemistry. 21(1982)3933-3940.

[31] Kumar, C. V., Punzalan, E. H. A., Tan, W. B., Adenine-thymine base pair recognition by an anthryl probe from the DNA minor groove, Tetrahedron. 56 (2000) (36), 7027-7040.

[32] Cramb, D. T.; Beck, S. C., Fluorescence quenching mechanisms in micelles: the effect of high quencher concentration, J. Photoch Photobio. A. 134(2000) (1-2), 87-95.

[33] Wang, XM., Li, Y ., Gong, SJ ., Fu, DA., A spectroscopic study on the DNA binding behavior of the anticancer drug dacarbazine, Spectrosc. Letters. 35(2002) (6), 751-756.

[34] 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.

[35] Pang, D.W., Abruña, H.D., Micromethod for the investigation of the interactions between DNA and redox active molecules, Anal.Chem. 70 (1998) 3162-3169.

[36] 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

[37] Riordan, J.R., Rommens, J.M., Kerem, Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski J., Lok, S., Playsic, N.J, Correction, Science. 29,(1989)245 (4925) 1437-1437.

[38] Kerem, B., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M., Tsui, L.C., Identification of the cystic-fibrosis gene - cloning and characterization of complementary-DNA, Science. 8 245(1989) (4922), 1073-1080.

[39] 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.

Figure Captions

Figure1. Absorption spectra of 10 µM Safranine (A,C) and Azure A (B,D) in 0.1M PB pH 7.0 in the absence (curve a) and in the presence of increasing amounts: 75 (curve b), 125 (curve c), 140 (curve d) and 150 µM (curve e) of ds-ctDNA(A,B) or ss-ctDNA(C,D) in base pair.

Figure 2.CLSM image of a AuSPE modified with ds-ctDNA (A) and ss-ctDNA (B) after accumulation of AA (Magnification 20X).

Figure 3. Cyclic voltammograms of 1.0 mM SAF (A) or AA (C) in 0.1M PB pH 7.0 solution at a AuSPE (curve a), a ds-ctDNA/AuSPE (curve b) and a ss-ctDNA/AuSPE (curve c). Consecutive cyclic voltammograms of 1.0 mM SAF (B) or AA (D) in 0.1M PB pH 7.0 at a ss-ctDNA/AuSPE. Scan rate: 100 mV/s.

Scheme1. Scheme depicting the DNA biosensor.

Figure 4. DPVs of 1.0 mM SAF (A) or AA (B) in 0.1M PB pH 7.0 after accumulation in WT11-SH/AuSPE (A) or WT12-SH/AuSPE (B) before (curve a) and after hybridization with a F508del (A) or (B) mutated sequence (curve c) or a fully complementary sequence (curve b).

Figure 5. Peak current bar diagrams of the biosensor response before and after hybridization with a: a fully complementary sequence and mutated sequences: SNP G542X (C, D) and F508del (A, B) using SAF (A, C) and AA (B, D) as redox indicators.

Figure1.

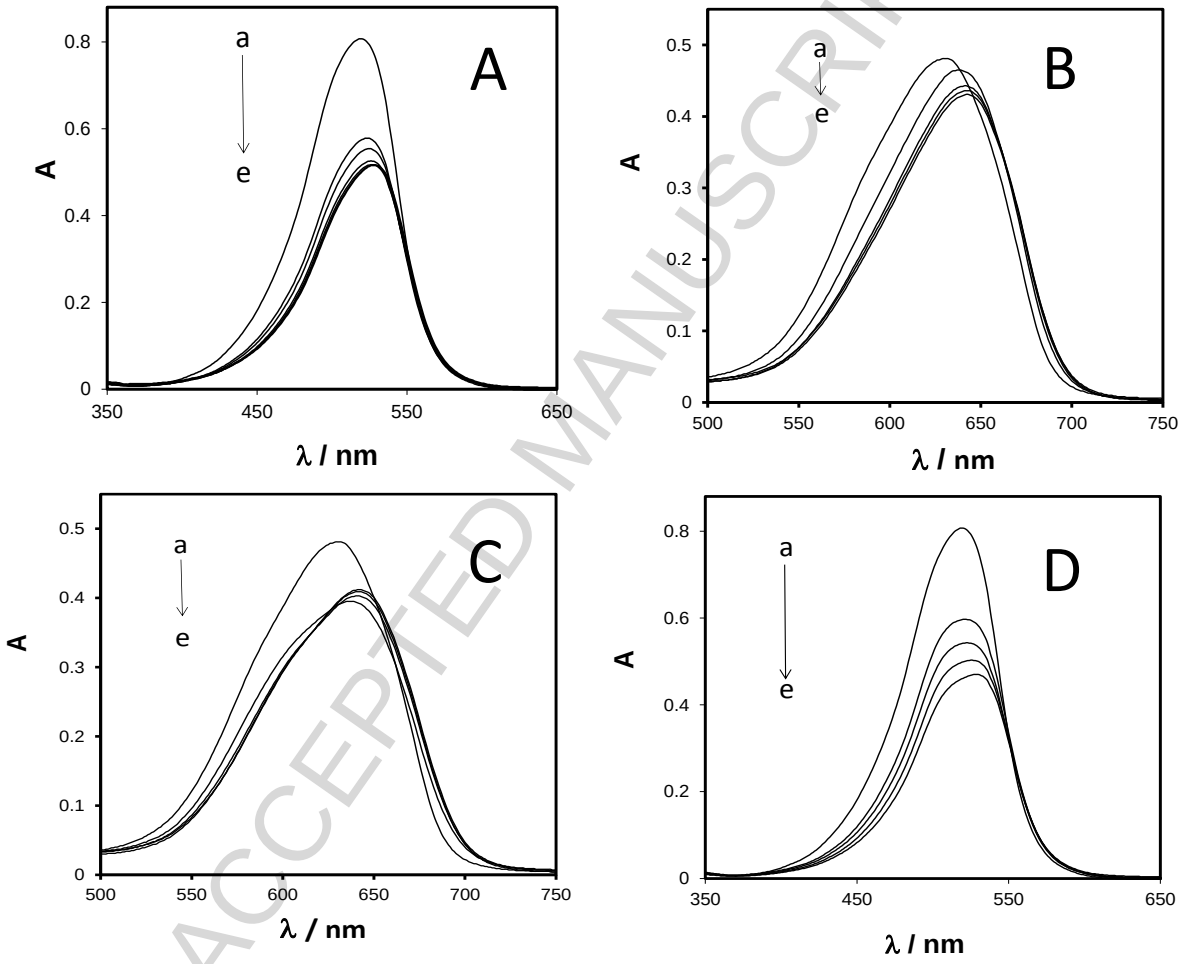


Figure 2.

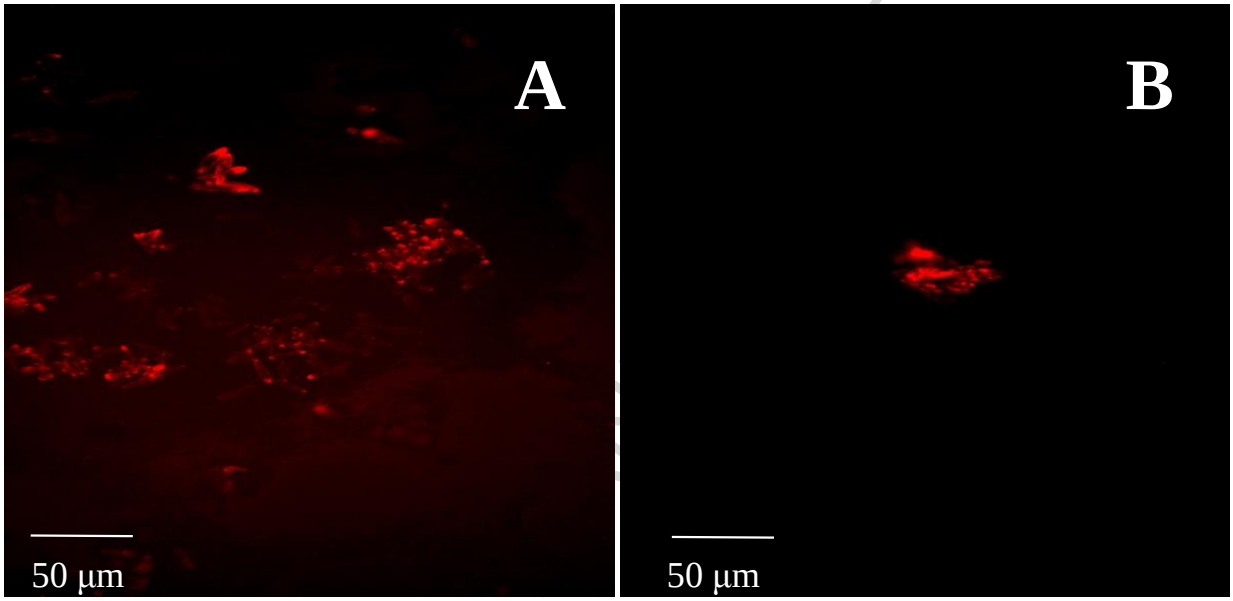
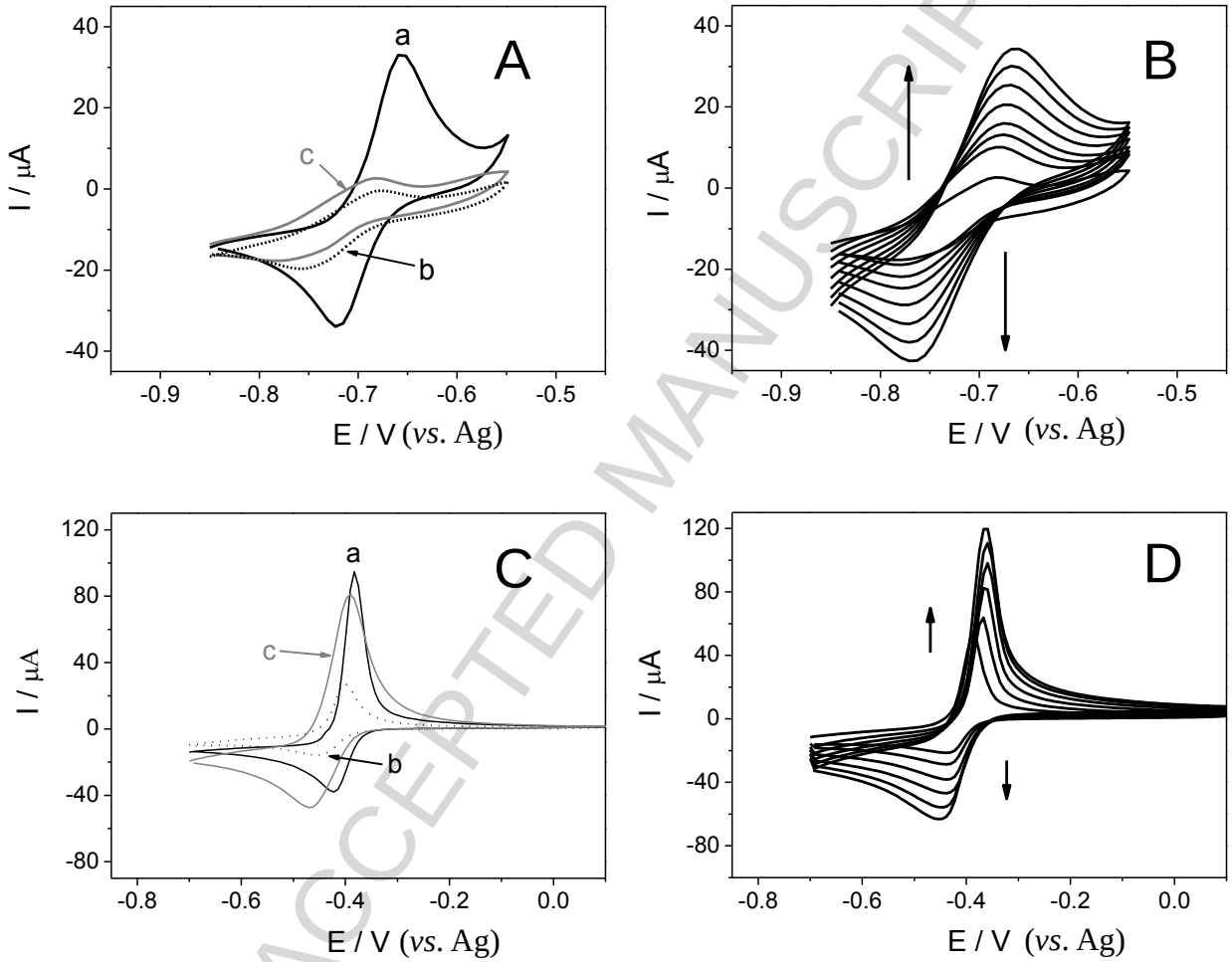


Figure 3.



Scheme 1

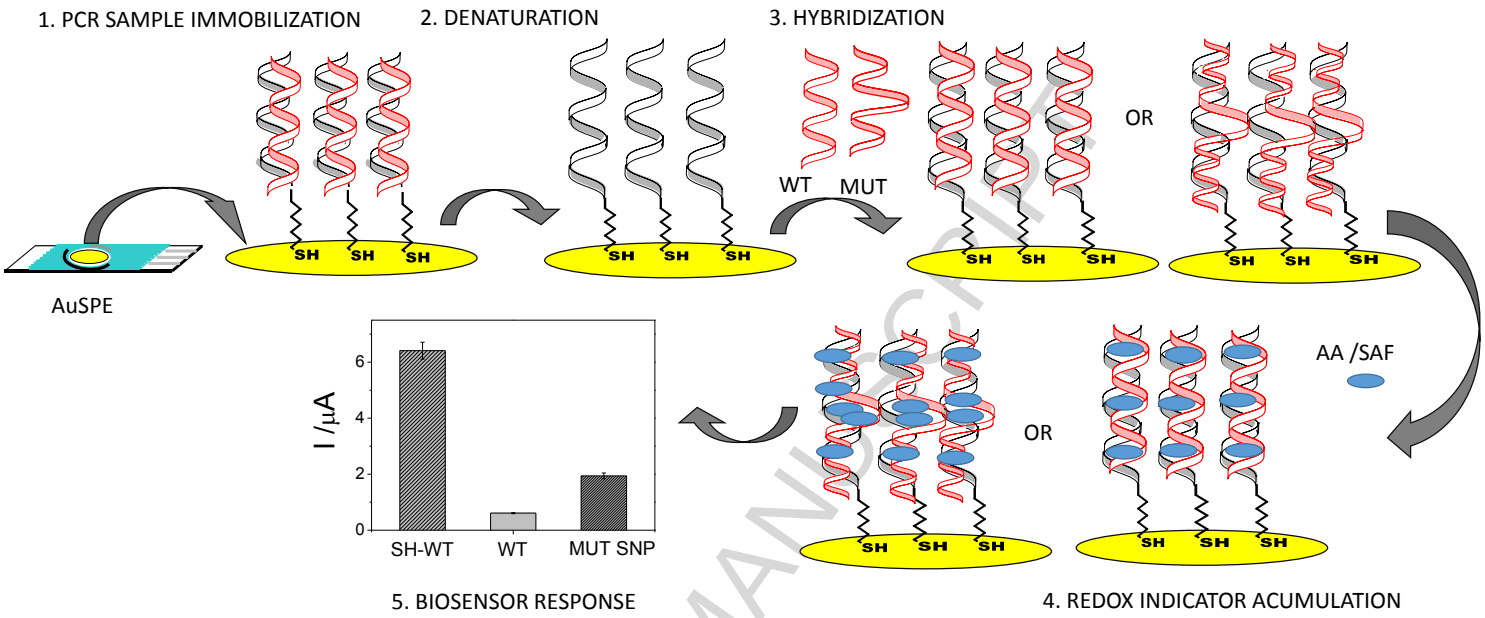


Figure 4.

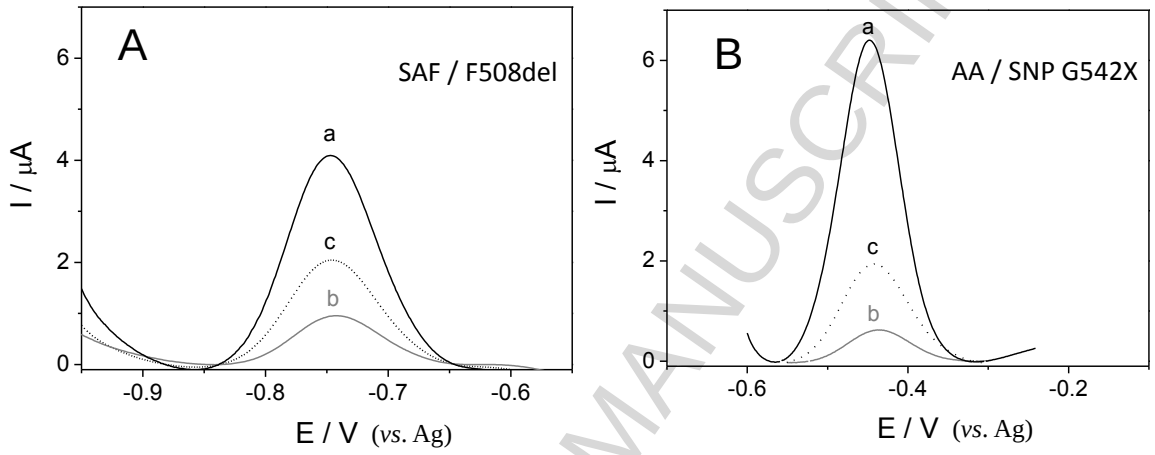
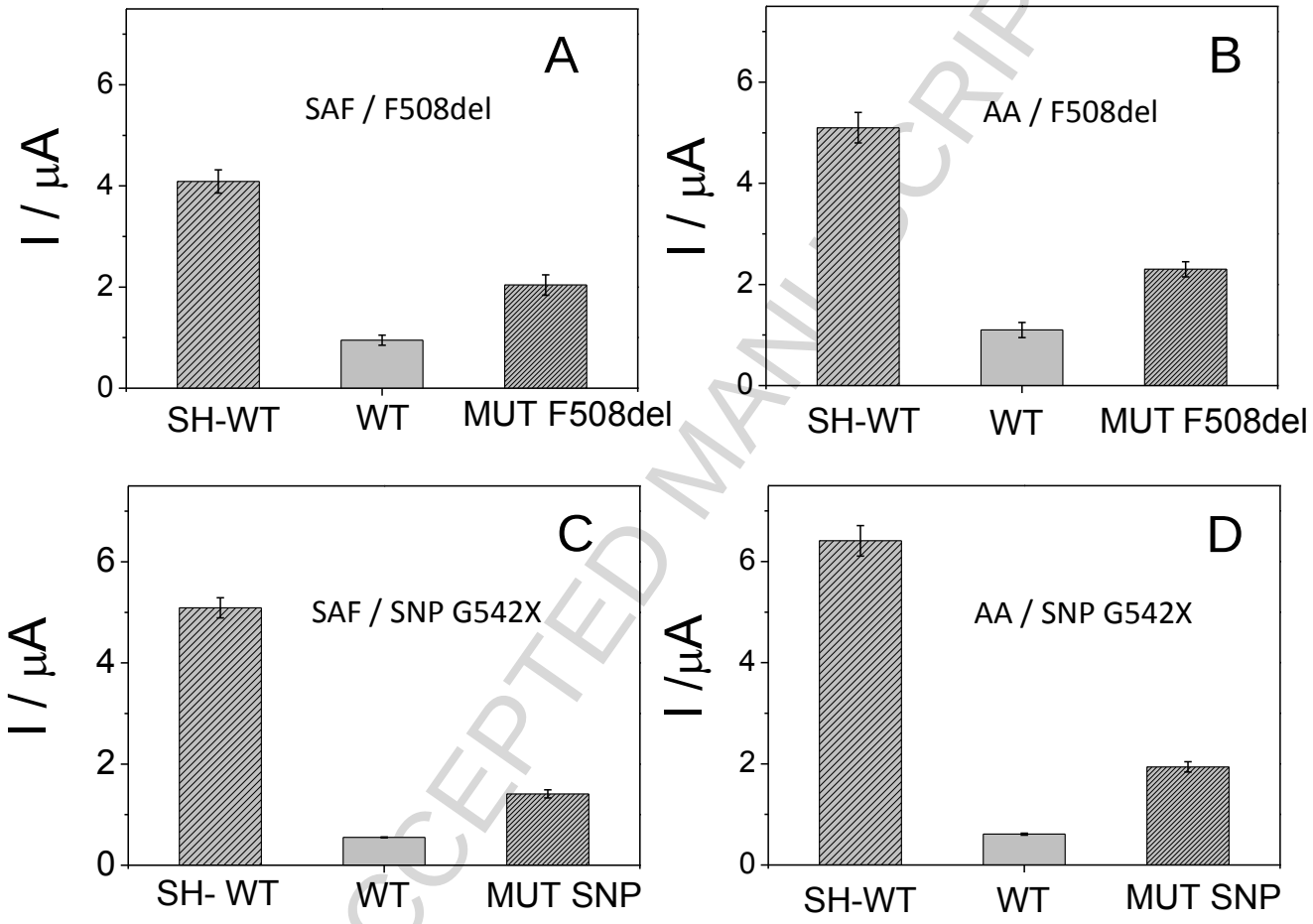
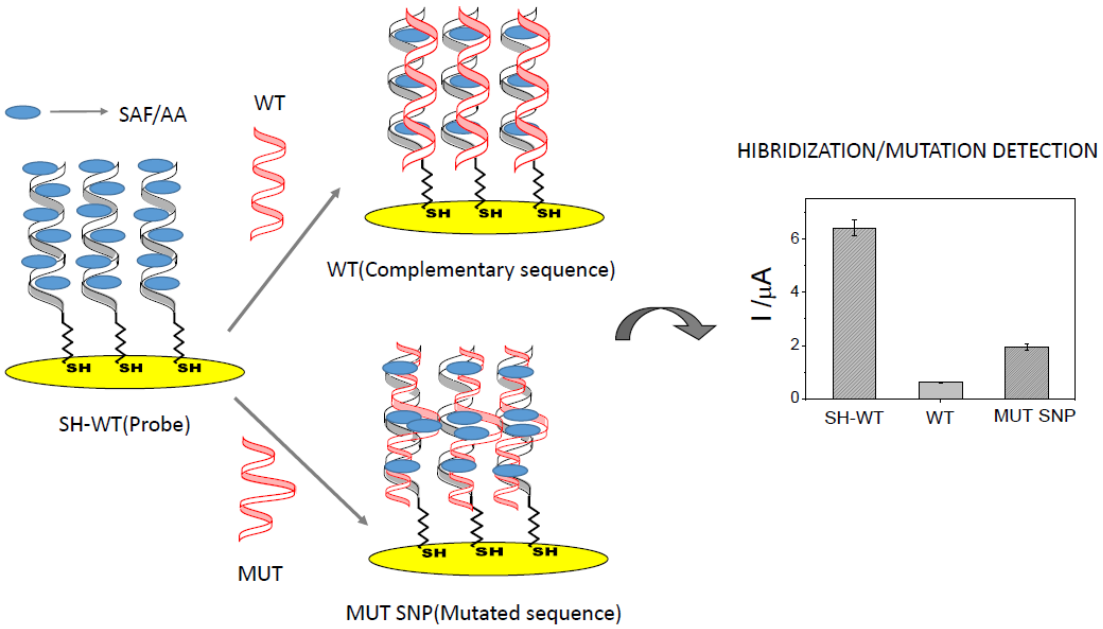


Figure 5.





Graphical abstract

Highlights

Interaction of Safranine and Azure A with DNA is demonstrated

Peak current decreases were seen after DNA hybridization for both dyes

Electrochemical DNA biosensors development using the dyes as redox indicator

Rapid gene mutations detection achieved for real DNA samples

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