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DNA as a Next-Generation Biomonitoring Tool of Hospital Effluent Contamination

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Abstract: A DNA biosensor based on a modified gold electrode with a Au/cysteine/DNA matrix was developed for ultratrace determination of genotoxicity antibiotics. The modified Au/cysteine/DNA electrode was characterized by cyclic voltammetry and impedance spectroscopy methods. The interaction between immobilized DNA and genotoxicity antibiotics in hospital wastewater was investigated using differential pulse voltammetry (DPV) technology. Using this technique, ciprofloxacin and ofloxacin were detected in real time in the hospital wastewater (HW) of the Tunisian cities of Gabes, Tozeur, Sfax, and Gbeli. In addition, physicochemical parameters such as the chemical oxygen demand (COD), biological oxygen demand (BOD), and total organic carbon (TOC) of HW samples that may affect the nature of the samples were studied. Comet assay (single-cell gel electrophoresis) was performed to measure the capacity of xenobiotics to induce DNA damage. In our conditions, this test indicated that all tested wastewater was able to alter cell integrity and cause DNA molecular damage, and the most genotoxic effect was found in the wastewater of Gabes hospital. Results show that the concentrations of the two antibiotics reached 33 and 40 ng/mL in the hospital wastewater of Gabes and Tozeur, respectively. The DNA biosensor based on the modified gold electrode exhibited superb performance and offers a probable application for the detection of genotoxicity antibiotics in hospital wastewater. The level of genotoxicity is proportional to the concentration of antibiotics detected in hospital wastewater. We will explore the application of this model for continuous monitoring downstream of hospital discharge and wastewater treatment plants for effective control of the presence of genotoxic products.

Keywords: genotoxicity; antibiotics; hospital effluents; biomonitoring; DNA hybridization; immobilization; DPV; comet assay

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1. Introduction

In modern life, the number and consumption of pharmaceutical products are increasing. However, large quantities of these products are released into the environment and are accountable for aquatic pollution, including pollution of river environments, drinking water sources, and lakes.

Hospital effluents contain significant amounts of pathogenic organisms, micropollutants, and dangerous substances. Among them are medicinals, radionuclides, detergents, and disinfectants for medical purposes in a wide range of concentrations due to laboratory and research activities or medicine excretion, drug residues, metals (revealing and radiographic fixative), radioisotopes, benzene hydrocarbons, and colorants [1–3]. Pharmaceuticals are consistently employed in hospitals to treat diseases.

In fact, hospital effluents are generally discharged directly into the public sanitation network.

On the other hand, many studies have detected that the presence of antibiotics represents the main category, as their consumption increased by 36% between 2000 and 2010 [4].

After use, 5–90% of antibiotics can be excreted as metabolites or parent compounds [5,6]; therefore, the interaction of hospital pollutants with aquatic ecosystems increases the risk of existing substances that have a negative impact on the ecological balance of natural environments. Nasri et al. [1] have shown that this risk can be presented, in general, as the probability of occurrence of toxic effects after exposure of organisms to a dangerous product. Halling-Sor et al. [7] reported that the presence of pharmaceutical compounds in the aquatic environment, including potentially mutagenic substances, is suspected of involvement with other pollutants in the development of 49 certain cancers observed in recent decades [8,9].

Currently, electrochemical biosensors are receiving increasing attention in research areas, especially from the aspect of drug detection, for a variety of reasons such as their ease of use, high specificity, and sensitivity analysis [10]. Additionally, the sensitivity of analysis can be increased by merging when DNA is associated with nanostructure materials such as gold nanoparticles or protein [11]. In this study, a biosensor based on a gold electrode modified with a glutathione/AuNPs/cysteine/DNA matrix was developed to assess the level of drug contamination in the wastewater of four hospitals (Gabes, Tozeur, Sfax, and Gbeli). DNA damage due to the incorporation of contaminants was investigated using electrochemical measurements. The developed method is a practical and superb method to detect the ultratrace concentration of genotoxicity antibiotics in the matrix. Electrochemical investigation of antibiotic/DNA interactions can provide a crucial analytical tool. It is low cost, sensitive, accurate, and enables rapid analysis of samples.

2. Experimental Section

2.1. Chemicals and Reagents

All antibiotic requirements were of highpurity (>90) and purchased from Sigma-Aldrich (Bornem, Belgium) and Witega (Adlershof GmbH, Berlin, Germany). Methanol, ethanol, and acetonitrile (HPLC grade) were obtained from Biosolve, (Valkenswaard, The Netherlands). Formic acid (98%) was obtained from Merck (Merck Millipore, Darmstadt, Germany). Ultrapure water ($\geq$18 MΩ cm) was obtained from the Milli-Q- Advantage System) (Millipore Corp., Bedford, MA, USA). LMP agarose and NMP agarose were purchased from Invitrogen (Thermo Fisher Scientific, Carlsbad, CA, USA). Sodium chloride, EDTA, TRIS, DMSO, S9 meld (postmitochondrial supernatant scrap), cytochalasin B, DAPI (DNA staining), acridine-orange, and TritonX-100 were from Sigma-Aldrich (Bornem, Belgium). NaOH pellets were from VWR (Radnor, PA, USA). The positive controls benzo(α)pyrene (B(α)P), 4-nitroquinoline 1-oxide (4-NQO), methyl methanesulfonate (MMS), and ethyl methanesulfonate (EMS) in toxicological tests were obtained from Sigma-Aldrich. (Bornem, Belgium). Cysteine (95%), glutaraldehyde (Glu, 25%), and acetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate was obtained from Fluka Chemie (Fluka ChemieGmbH, Buchs, Switzerland). All oligonucleotides and complementary DNA stock solutions were diluted with PBS (100 mM, pH 7.4) and stored at −20 °C until use. Working solutions were prepared using ultrapure water. PCR-amplified real samples were approved by the Department of "Autoimmunity and Down", Faculty of Pharmacy. Oligonucleotides were purchased from BioneerOligo Synthesis Report (Bioneer, BPS Bioscience, Biotools, Tunisia). Their base sequences are as follows: 5′NH_2-$(CH2)_6$-ATTTGTTCATGCCT-3′.

2.2. Sample Collection

Water sample collection was conducted in March 2016 from the wastewater of four hospitals (Gabes, Tozeur, Gbeli, and Sfax). All samples were collected in amber glass bottles, transported to the laboratory, and stored at −20 °C until further analysis.

2.3. Sample Extraction

A 100 mL volume of water samples was transferred to a propylene tube and spiked with the respective internal standards at a concentration of 60 ng mL^{-1}. Next, an Oasis® HLB cartridge was preconditioned with 6 mL of methanol and 6 mL of Milli-Q water. After vortexing for 10 s, about 2 mL min^{-1} of samples was transferred to the cartridge. The cartridge was then rinsed with 6 mL of Milli-Q water and desiccated under vacuum for 20 min. After elution with 12 mL of methanol, the analytes were evaporated to dryness at 40 °C under a slow stream of nitrogen. Each extract was dissolved in 1 mL of Milli-Q water. Afterward, a 10 mL aliquot of the supernatant was poured through a 1 µm PVDF filter, and an 8 µL aliquot was injected into UPLC-MS/MS.

2.4. UPLC-MS/MS Optimization

Analysis of antibiotics in hospital wastewater was achieved by UPLC-MS/MS. The UPLC-MS/MS method was carried out on an Acquity® sample, and a Waters solvent manager was linked to a XEVO TQ-MS triple quadrupole mass spectrometer (Milford, MA, USA) operating in positive electrospray ionization (ESI+) MS/MS mode. Chromatographic separation was performed using an Acquity UPLC® BEH C18 column (2.1100 mm i.d., 1.7 µm) from Waters (Milford, MA, USA). Mobile phases were composed of 0.1% ammonia in water (A) and acetonitrile (B).

The elution method was used as follows: 0–10 min: 1–99%, linear decrease to 1–99% B at 1–14 min, and re-equilibrate to initial conditions

The autosampler and column were held at 8 and 40 °C, respectively. A gradient program was executed to analyze the samples within 10 min. In this research, each sample was tested in triplicate and enriched with the respective SI at two levels of QC in order to assess the extraction efficiency of each different matrix.

Specificity, linearity, matrix effect, repeatability, reproducibility, limit of detection (LOD), and limit of quantification (LOQ) were validated according to the EU 657/202 standard and Tahrani et al. [12].

2.5. Electrochemical Measurements

All electroanalytical analyses were carried out using a potentiostat (PGSTAT 302 N) and an electrochemical cell containing a platinum wire as the counter electrode, a saturated KCl reference electrode of Ag/AgCl/KCl as the reference electrode, and the modified gold electrode as the working electrode (geometrical area of 0.031 cm^2). Electrochemical impedance spectroscopy analysis was carried out in the frequency range from 0.1 to 100,000 Hz. All electrochemical measurements were performed in a Faraday cage at room temperature (25 °C) to reduce light or electrical perturbation effects. Activation and modification of the working electrode by the ss-DNA probe were carried out as follows: prior to the modification step, the gold electrode was mechanically polished with alumina slurry, followed by rinsing with distilled water and sonication in acetone for 5 min. Then, the gold electrode was treated with piranha solution (H_2SO_4/H_2O_2, 1 : 3 v/v) for 1 min and rinsed with water. Afterward, the gold electrode was immersed in cysteine solution (5 mM) for 2 h in 0.1 M PBS (pH 7.4) to establish Au/cysteine SAMs. After that, before immobilizing the DNA probe, the Au/cysteine SAMs electrode was incubated in glutaraldehyde (GA) vapor for 1 h. The prepared Au/cysteine/GA SAMs-modified electrode was washed with PBS and incubated in the probe DNA (ss-DNA) solution for 1 h at room temperature.

2.6. Hybridization with Real PCR Samples

Real samples obtained from PCR amplification were denatured by heating at 95 °C for 5 min, followed by rapid cooling in an ice bath. The Au/cysteine/ss-DNA-modified electrode was immediately incubated for 20 min. Subsequently, the electrode was rinsed with PBS to remove the nonhybridized and adsorbed DNA target. The different steps outlining the preparation of the DNA biosensor are illustrated in Figure 1.

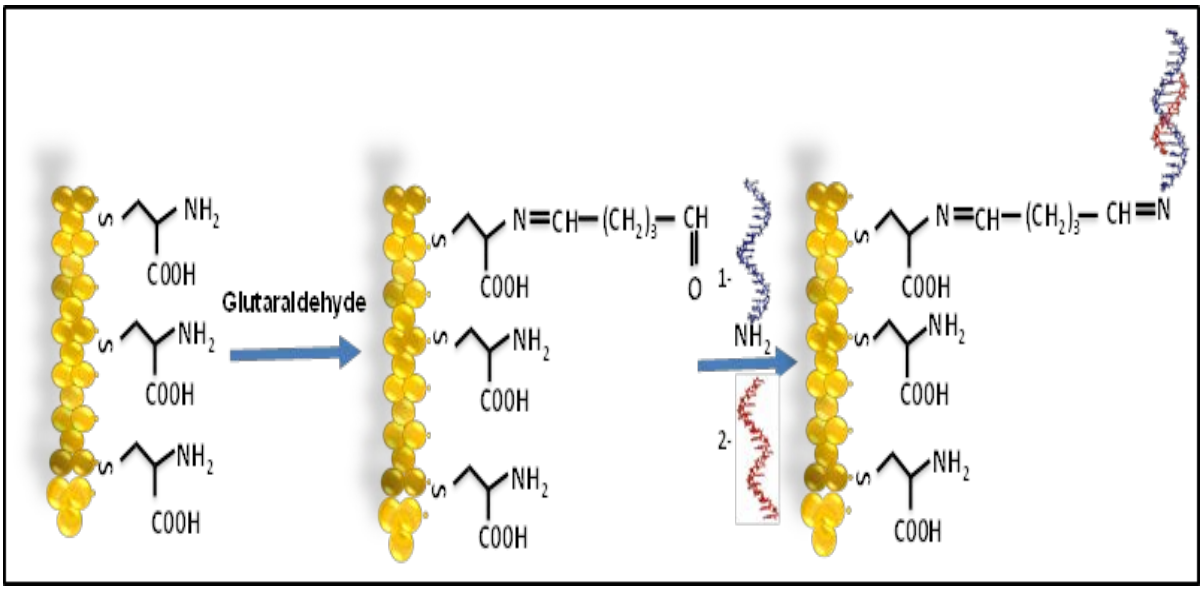

Figure 1. Procedure for the preparation of the Au/cysteine/DNA-modified electrode.

2.7. Comet Assay

The test was carried out according to regular procedures. First, 4 × 105 C3a cells were inoculated in a 24-well plate and incubated at 37 °C (5% CO_2) for 24 h. Then, the culture medium was removed, and the four dilutions of wastewater described above were added to the cells to be incubated under the same conditions for 24 h [13,14].

The next step consisted of spreading 75 µL of C3a cell suspension (0.8% low-melting-point (LMP) agarose) exposed to LMP on a glass slide previously covered with 1% NMP (melting-point agarose). Cells were lysed overnight using lysis buffer (2.5 M NaCl; 100 mM EDTA; 10 mM TRIS). Unwinding of the alkaline DNA was carried out by incubating for 40 min in electrophoretic buffer (0.3 M NaOH, 1 mM EDTA) in a horizontal electrophoresis unit (20 min, 1 V/m, 300 mA). Then, the slides were rinsed with Tris neutralization buffer (0.4 M, pH 7.5)

DAPI (fluorescent dye) was used to stain DNA, and slides were analyzed with an Axio Imager.Z2 (Zeiss) fluorescence microscope equipped with Metasystems' Metacyte and Metafer 4 software (version 3.8.5) to quantify DNA damage (Altlussheim, Germany). Ethyl methanesulfonate (EMS) (0.5 mM) was used as a positive control, and an unexposed control cell was used as a negative control. For each concentration, two different gels were made with the analysis of 50 cells per gel. In addition, the percentage of comet tail DNA was used as a measure of DNA damage.

3. Results and Discussion

3.1. Electrochemical Biomonitoring

The prepared Au/cysteine/GA/DNA electrode was characterized by electrochemical analysis such as voltammetric and impedance methods in aqueous PBS solution (0.1 M) using $[Fe(CN)6]^{3-}$/$[Fe(CN)6]^{4-}$ (5 mM) as a redox probe with a scan rate of 50 mVs^{-1}. Figure 2A displays the working electrode cyclic voltammograms before and after the modification process. The change in the electroactivity aspect of the solid/liquid interface during the modification process demonstrates the adsorption of the different deposit layers. The deposit of the cysteine layer enhances the conductivity of the modified electrode due to the fast charge transfer established at the solid/liquid interface. The conductivity increases then decreases after the DNA layer deposition. This behavior can be attributed to the flexible DNA sequences that reduce the charge transfer process. This change in conductivity surface during modification was confirmed using impedance spectroscopy measurements. Figure 2B displays the impedance spectra of the bare Au, Au/cysteine, and

Au/cysteine/DNA-modified electrodes. The evaluation of the conductivity of the surface electrode before and after modification by impedance was in good agreement with the voltammetry behavior. After the modification step by the cysteine layer, the charge transfer resistance of the Au/cysteine interface decreases. The presence of cysteine molecules as electron accelerator mediators reduces the charge transfer resistance, which enhances the charge transfer across the modified interface, leading to a more conductive surface.

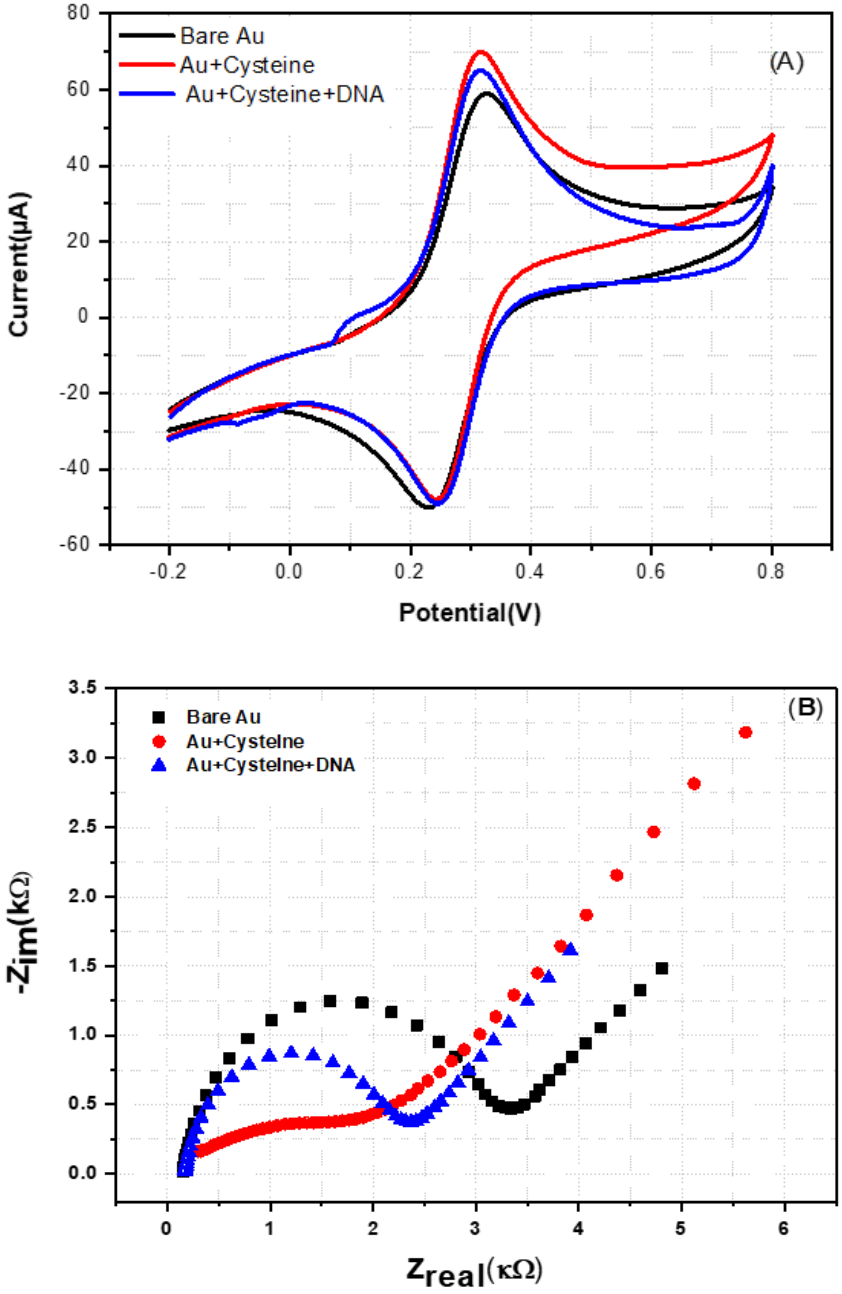

Figure 2. Cyclic voltammograms (**A**) and impedance spectra (**B**) of bare Au, cysteine/Au, and DNA/cysteine/Au-modified electrodes. Scan rate: 50 mV/s in 0.1 M PBS and 5 mM of ferric/ferrocyanide redox probe.

As a result, the adsorption of the DNA macromolecule layer enhances the charge transfer resistance, which can be due to the repulsive interaction between the negative redox probe species and the DNA sequence bases.

The electrochemical biomonitoring of the impact of water on the environment and human disease requires a suitable and rapid system for meticulous alerts [15,16]. In our work, the developed DNA system was applied for hospital wastewater characterization collected from different Tunisian regions. In fact, DNA has an electroactive aspect that can degrade when its nitrogen bases such as guanine, adenine, and cytosine are affected [17]. For this reason, we investigated DNA damage induced by hospital wastewater using differential pulse voltammetry methods. The constructed and optimized bioanalytical system was based on ss-DNA/cysteine as an electroactive matrix deposited on an Au electrode [18]. Differential pulse voltammetry (DPV) measurements showed a significant effect of hospital effluents on the hybridization process of immobilized DNA, and the binding reaction and interaction between immobilized DNA and wastewater samples were achieved by dipping the biosensor into different test solutions for different times.

In order to establish the optimal bioanalytical performance of the DNA sensor, the influence of the immersion times was studied. Moreover, the different hospital wastewater samples were analyzed with the differential pulse voltammetry method (DPV). Figure 3 shows the electrochemical behavior based on the differential pulse voltammetry signal of the modified electrode by DNA in contact with the water samples for 1, 2, and 3 h. A significant effect induced by the different water samples on DNA activity was obtained during an exposure time of 2 h, which was selected as the optimal duration.

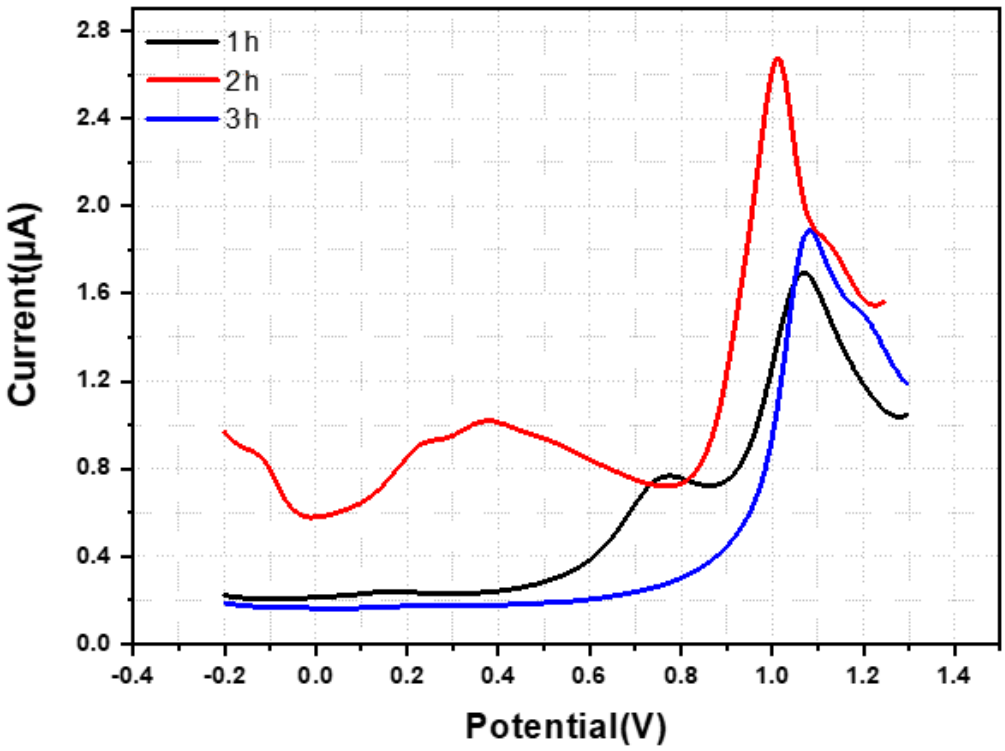

Figure 3. DPV of Au/cysteine/ss-DNA immersion in Sfax water sample for 1, 2, and 3 h.

The signature of the modified electrode was recorded before and after immersion in hospital wastewater samples. As shown in Figure 4, well-developed signals of single bases were determined at +0.8 and +1.103 V for guanine and adenine, respectively. After immersion, the observed signals of the DNA bases were perturbed. As a result, a noticeable increase in the signal intensity was observed for adenine. This behavior indicates the interaction of DNA with antibiotic drugs.

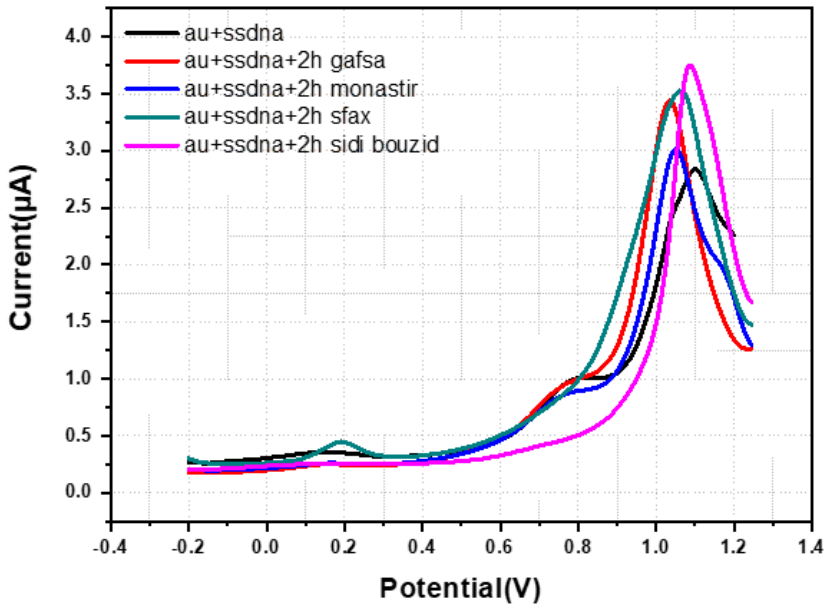

Figure 4. DPV of curves of Au/cysteine/ss-DNA before and after immersion in different real samples in acetate buffer (pH 4.8).

After the dipping process in the real wastewater samples, the ss-DNA/cysteine/Au-modified electrode based on single-stranded DNA was transferred to the target DNA solution, and the resulting DPV curves are illustrated in Figure 5. The decrease in the guanine peaks was suggested as a measure representing the degree of damage to this nucleobase and proposed as a screening test for environmental pollutants present in these samples. Moreover, we can observe an increase in the peak current of adenine, and the peak potential moves toward the positive direction. This result can be attributed to the interaction between ciprofloxacin and the double helix of ds-DNA.

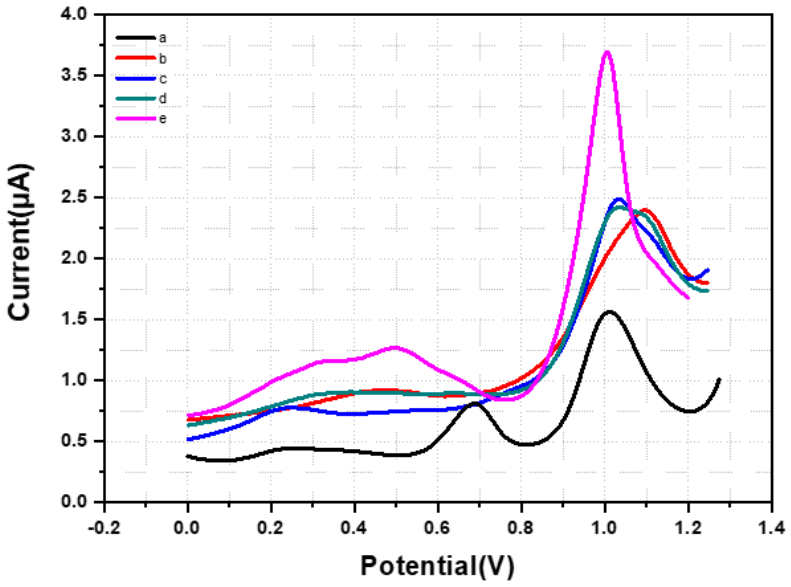

Figure 5. DPV of the hybridization-modified electrode before and after immersion in different water samples during 2 h in acetate buffer (pH 4.8).

This phenomenon shows that an interaction between DNA, ciprofloxacin, and ofloxacin exists. It was expected that antibiotics were electrostatically attached to the negatively charged phosphate backbone of DNA [19].

3.2. DNA Damage Measured with the Comet Assay

Our results showed that the different wastewater samples used in this study were able to induce DNA damage quantified by the comet assay. In fact, at the level of the Ca3 cells, the total score goes from 10% in the untreated cells to 15, 20, 25, and 40%, respectively, for cells treated with HWS, HWG, HWT, and HW Ga. Using ethyl methanesulfonate (EMS) as a positive control, this score increased significantly up to 75% compared to control cells and cells treated with wastewater from different hospitals.

In addition, the damage caused to DNA was detected more in the wastewater from Gabes hospital. This result was proved by the very high percentage of the total scores (<35%), as shown in Figure 6. The results show the potential genotoxicity of hospital wastewater (HW). In fact, Gabes hospital showed that a concentration-dependent increase in DNA damage was strongly induced ($p < 0.001$). The observed toxicity is related to antibiotics. Many recent studies have demonstrated the toxicity of hospital wastewater contaminated with antibiotics; these studies have found a relationship between exposure to antibiotics in wastewater and the levels of toxicity [20].

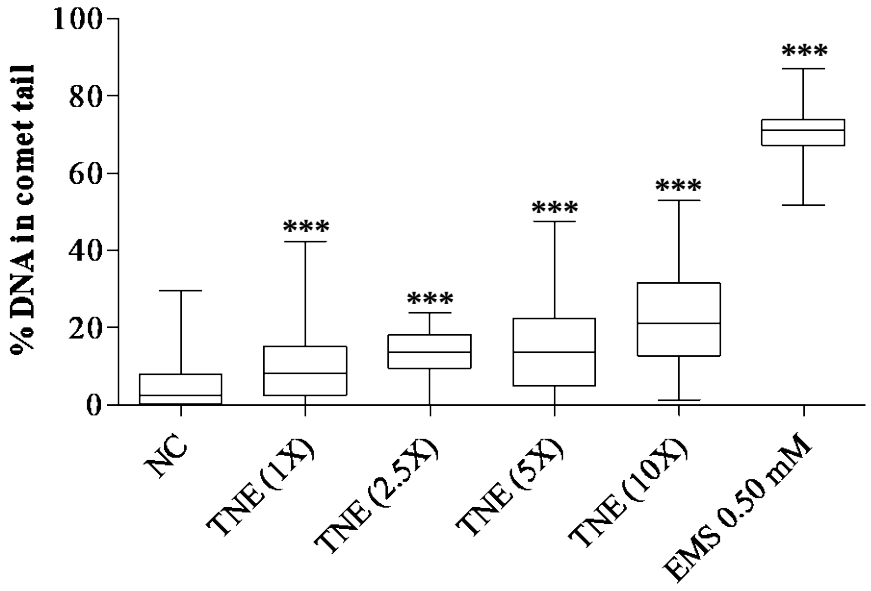

Figure 6. DNA damage expressed as % DNA in comet tail from C3a cells that were exposed to different hospital wastewater samples; HWS: hospital wastewater Sfax; HWG: hospital wastewater Gbeli; HWT: hospital wastewater Tozeur; HWGa: hospital wastewater Gabes; NC: negative control; EMS: ethyl methanesulfonate; ***: $p < 0.001$.

3.3. Physicochemical Analysis

The results of the physicochemical parameters of the seven wastewater samples taken from different hospitals in Tunisia are shown in Table 1. The obtained results indicate that the highest values of the BOD and TOC were 747 $\pm$ 2.12 mg L^{-1} and 562 $\pm$ 3.53 mg L^{-1}, respectively, observed in the wastewater collected from the hospital located in Monastir city.

Table 1. Determination of antibiotics in wastewater samples collected from four Tunisian hospitals.

HWPCP	Gbili	Tozeur	Gabes	Sfax
MES (mg/L)	14	117	91	790
COD (mg/L)	143	282	264	1000
BOD (mg/L)	128	164	172	242
TOC (mg/L)	104	132	138	176
NO-3 (mg/L)	<0.5	<0.5	3.6	5.7
SUR (mg/L)	2.4	9.4	8.5	<0.5
Turbidity (NTU)	27.5	273	145	272
PH	7.2	6.5	6.6	7.36
Organic matter	133	203.33	202.66	494.66
Nonbiodegradable matter	15	118	92	758
Biodegradability ratio	1.11	1.71	1.53	4.13

HW: hospital wastewater; PCP: physicochemical analysis.

Correspondingly, the pH values of all analyzed samples were always in the alkaline range from 7.06 ± 0.18 to 7.69 ± 0.22. It should be taken into account that the COD/BOD ratio provides an initial estimation of the potential biodegradability of the organic matter in a given effluent. The results demonstrated that the average ratios were between 2 and 4 in hospital wastewater samples. Simultaneous analysis of 56 antibiotics in the four hospital wastewater samples and the results are collected in Table 2.

Table 2. Determination of antibiotics in wastewater samples collected from four Tunisian hospitals using UPLC-MS/MS.

Antibiotics	Ciprofloxacin	Ofloxacin
Wastewater collected from Gabes hospital	33	40
Wastewater collected from Tozeur hospital	4.3	7.5
Wastewater collected from Sfax hospital	3.4	nd
Wastewater collected from Gbeli hospital	8.7	nd

Ciprofloxacin was found and quantified in hospital wastewater with mean concentrations of 33, 4.3, 3.4, and 8.7 mg L^{-1} in the hospital wastewater samples collected from Gabes, Tozeur, Sfax, and Gbeli, respectively. However, ofloxacin was detected in concentrations up to 40 and 7.5 mg L^{-1} only in the hospital wastewater samples collected from Gabes and Tozeur, respectively.

The electrochemical study was in good agreement with the physicochemical analysis. Therefore, we observe a good correlation between the toxicity effects of effluents on the immobilized DNA and the physicochemical parameters determined for each effluent. The evolution of DNA activity in different water samples varies with the contact time. In fact, a loss of immobilized DNA regular activity can be observed after three hours of immersion in the effluent water. Consequently, the developed electrochemical method can be successfully used for biomonitoring water in its different rejected forms, especially in the medical field.

Table 1 gives ratios between 1.11 and 1.71. In Gabes, Tozeur, and Gbeli, the effluents are biodegradable, while the effluent in Sfax is nonbiodegradable. According to Rejsek [21] and Hao et al. [22], an effluent is considered easily biodegradable if its COD/BOD5 ratio is less than 2, biodegradable when it is between 2 and 3, and nonbiodegradable when it is greater than 3. In fact, according to Wang et al. [23], conventional water quality indices and chemical analyses provide limited information about the potential toxic effects and the numerous formed substances that can jeopardize human health. In our case, the wastewater samples collected from Sfax, Gabes, and Tozeur hospitals were found to be the most polluted (in terms of physicochemical parameters) when compared to those collected from Gbeli hospital. Generally, pollutant load does not necessarily indicate toxicity. This has been well observed in our study and confirms the findings of Dellai et al. [24].

The present work showed that hospital effluents cause serious damage to DNA. This genotoxicity was detected by electrochemical methods. The effluent collected in Gabes city hospital (containing low concentrations of COD, BOD, NO_3, etc.) proved to be the most genotoxic.

3.4. UPLC-MS/MS Analysis

The concentrations of analytes found in hospital effluents were assessed utilizing UPLC-MS/MS. The results confirmed the emergence of several antibiotics in hospital wastewater, as shown in Table 2, and the chromatogram illustrates the intensity of each compound. It is clear that ciprofloxacin and ofloxacin have a higher concentration among other antibiotics; therefore, the DNA damage is attributable to the presence of ciprofloxacin and ofloxacin, which were detected in this effluent by UPLC-MS/MS. These findings were confirmed by the study of Ji et al. [20], who showed a relationship between effluents contaminated with antibiotics and the degree of toxicity observed.

The toxicity of pharmaceutical wastewater charged with antibiotics has been demonstrated in recent studies such as the research of Ji et al. [20], which demonstrated a correlation between antibiotic contamination of wastewater and the degree of observed toxicities.

The level of genotoxicity is proportional to the concentration of antibiotics detected. Indeed, the genotoxic effect is very high in the effluent collected from Gabes hospital in a dependent way, which showed the highest concentration of these two antibiotics at 33 and 40 ng mL^{-1} in Gabes and Tozeur wastewater of ciprofloxacin and ofloxacin, respectively.

4. Conclusions

In this work, a new matrix was developed based on Au/cysteine/DNA using the assembled approach on a gold electrode. The functioning of the new biosystem applied in the aqueous media was characterized by electrochemical measurements such as voltammetry and impedance spectroscopy methods. DPV measurements were able to quantify immobilized DNA damage induced by hospital effluent action perturbing the hybridization reaction. These electrochemical investigations were supported by physicochemical analysis. We are able to explore this model for continuous monitoring downstream of medical institution discharge and wastewater treatment plants for effective management of the presence of genotoxic substances.

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