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DNA-based biosensors with external Nafion and chitosan membranes for the evaluation of the antioxidant activity of beer, coffee, and tea

Research Article

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Abstract: Novel electrochemical DNA-based biosensors with outer-sphere Nafion and chitosan protective membranes were prepared for the evaluation of antioxidant properties of beverages (beer, coffee, and black tea) against prooxidant hydroxyl radicals. A carbon working electrode of a screen-printed three-electrode assembly was modified using a layer-by-layer deposition technique with low molecular weight double-stranded DNA and a Nafion or chitosan film. The membrane-covered DNA biosensors were initially tested with respect to their voltammetric and impedimetric response after the incubation of the beverage and the medium exchange for the solution of the redox indicator $[Fe(CN)_6]^{3-/4-}$. While the Nafion-protected biosensor proved to be suitable for beer and black tea extracts, the chitosan-protected biosensor was successfully used in a coffee extract. Afterwards, the applicability was successfully verified for these biosensors for the detection of a deep degradation of the surface-attached DNA at the incubation in the cleavage agent (hydroxyl radicals generated via Fenton reaction) and for the evaluation of antioxidant properties of coffee and black tea extracts against prooxidant hydroxyl radicals. The investigation of the novel biosensors with a protective membrane represents a significant contribution to the field of electrochemical DNA biosensors utilization.

Keywords: *Electrochemical biosensors • Outer-sphere protective membranes • DNA damage • Reactive oxygen species • Antioxidant properties of beverages*

1. Introduction

External protective membranes have been numerously reported, particularly on enzyme biosensors, which prevent the biosensor surface from unwanted fouling and interferences. Typically, these are formed by polymers such as cellulose acetate, chitosan, and phospholipids, which are generally biodegradable, nontoxic, and effective barriers for small and large molecules [1]. In some instances, synthetic nonconductive and conductive polymers are utilized as well.

Chitosan is the most abundant basic cationic biopolymer structurally similar to cellulose [2]. The original biopolymer is not soluble in water and, after hydrolysis, its solubility depends on the amount of free amino groups in the chain. These amino groups (pK_a from 6.2 to 7.0) are completely protonated in acids, making chitosan soluble in acids such as acetic, nitric, hydrochloric, perchloric, and phosphoric [2-4]. Chitosan is considered one of the most valuable polymers for biomedical and pharmaceutical applications due to its biodegradability, biocompatibility, and nontoxicity and also due to its antimicrobial and

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antitumor properties. It is widely used in the preparation of nanoparticles, microspheres, hydrogels, films, and fibers for biomedical and pharmaceutical applications [5], for the immobilization of enzymes [6,7], and for the entrapment of DNA [8].

Nafion is a polymer with cation-exchange properties created by adding sulfonic acid groups into the bulk polymer matrix. Nafion combines the physical and chemical properties of the Teflon-based material with ionic characteristics. It is extremely resistant to a chemical attack. This means that Nafion does not release fragments or degradation products into the surrounding medium [9]. Negatively charged polymer Nafion is widely used to reduce the diffusion of small neutral or negatively charged interfering species such as ascorbic acid and uric acid. Nafion is biocompatible to enzymes since it has both hydrophilic and hydrophobic properties, is chemically inert and is subjected to relatively little adsorption of species from the solution [10].

It has been proven that around 2×10^4 DNA damaging events occur in every cell of the human body every day [11]. A significant portion of the damage is caused by reactive oxygen species (ROS). These include all partially reduced oxygen species, namely the superoxide anion radical ($\bullet O^{2-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet$OH) [12], and lead to diseases such as arteriosclerosis, hypertension, diabetes, and neurodegenerative diseases [13]. They are generated as by-products of respiration and constitute the major class of endogenous toxic agents in aerobic organisms. Biosensors based on an electrochemical transduction element and a nucleic acid biocomponent are already widely used for tests in medical, environmental, and food analysis. The detection of damage to the DNA belongs to one of the specific fields of analysis with DNA-based biosensors [14-16].

A combination of voltammetric and impedimetric DNA signals was used to obtain complex information on the type and degree of DNA damage [17]. Square-wave voltammetric (SWV) responses of the purine DNA bases and the thioridazine intercalator as well as cyclic voltammetric (CV) signals of the $[Fe(CN)_6]^{3-/4-}$ redox indicator were measured. Electrochemical impedance spectroscopic (EIS) data were obtained in the same solution within a rather simple scheme of the measurement. The damage to DNA was estimated according to an initial increase in the DNA bases responses at the helix opening followed by a decrease in DNA bases and intercalator responses at the degradation of DNA by breaks of its strands. The damage of the negatively charged DNA layer on the electrode was confirmed also by the change of the shift of the CV and EIS data for the DNA-covered electrode to those typical for the bare electrode without DNA [17].

Antioxidants are compounds which, at low concentrations, can protect biological substrates against an oxidation by the chelation of metal ions, inhibition of enzymes, elimination of hydrogen peroxide, or scavenging, trapping, and quenching of radicals [18]. Generally, there is no single antioxidant assay for food tests. The antioxidant activity/capacity may be broadly classified as the electron transfer- and hydrogen atom transfer-based assays [19]. Typical methods are: trolox equivalent antioxidant capacity, total radical-trapping antioxidant parameter, and ferric reducing-antioxidant power. By these methods, it is possible to determinate the antioxidant activity of fruits, vegetables, oils, and beverages [20].

Electrochemical (mainly voltammetric) methods provide the rapid, simple, and sensitive alternative in the analysis of bioactive compounds associated with the scavenging of the radicals as well as the antioxidant capacity itself. They are low-cost and usually do not require time-consuming sample preparation [21]. Recently, a differential pulse voltammetric (DPV) assay utilizing a glassy carbon electrode modified with multi-walled carbon nanotubes was used to evaluate the antioxidant capacity of coffee. This approach was based on the oxidation of hydroxycinnamic acids contained in coffee beans [22]. CV and SWV were also used to evaluate the antioxidant capacity of tea and to determine catechins in the samples of green and black tea [23,24]. Many of effective natural antioxidants present in plants, fruits, and their extracts have been evaluated by electrochemical DNA biosensors [25-27]. Similar to other assays based on the sensitivity of DNA to the presence of prooxidants, these biosensors utilize an ability of antioxidants to preserve the DNA structure under prooxidative conditions.

Beer, coffee, and tea belong to the most popular beverages in the world. They are rich in polyphenolic compounds exhibiting large antioxidant activity, which can be evaluated by the DNA biosensors as well [22,28-30]. This work proposes a fabrication of novel DNA-based biosensors with outer-sphere Nafion (NAF) and chitosan (CHIT) membranes for the evaluation of antioxidant properties of beverages (beer, coffee, and black tea) against prooxidant •OH radicals as the DNA cleavage agent generated *in situ* with Fenton's reagent. Using CV and EIS, an ability of the membrane-covered disposable biosensors, with a screen-printed carbon electrode (SPCE) as the transducer, to detect a deep degradation of the surface-attached DNA at the incubation in the

cleavage agent as well as a protection of the DNA layer by beverage components, when they are mixed with the cleavage agent, were investigated.

2. Experimental procedure

2.1. Reagents

Low molecular weight salmon sperm double-stranded DNA (dsDNA) (Product No. 31149) was obtained from Sigma–Aldrich, Germany. Its stock solution (0.5 mg mL^{-1}) was prepared in a 0.1 M phosphate buffer solution of pH 7.0 and stored at 4°C. Chitosan (from shrimp shells) of low viscosity (Product No. 50494) was obtained from Fluka, Germany. Its 0.5% (w/w) solution was prepared in 1% (v/v) acetic acid (99.8%, Lachema, Czech Republic) and filtered through a simple paper strip. The final chitosan (CHIT) solution was of pH 5.0. Nafion (NAF) (5% (w/w), Product No. 527084) was obtained from Sigma–Aldrich, Germany. Its 1% (v/v) stock solution was prepared by dilution in a 1:1 (v/v) ethanol:water mixture. An acetate buffer (AcB) (0.25 M containing 10 mM KCl, resulting pH 4.75) and a phosphate buffer solution (PBS) (0.1 M containing 10 mM KCl, resulting pH 7.0) were used as the main components of supporting electrolytes. The cleavage agent was prepared in PBS. Fenton reaction in the mixture of 0.24 mM H_2O_2 and 1 μM $[Cu(phen)_2](ClO_4)_2$ was used for the hydroxyl radicals generation. Other chemicals used (all obtained from Mikrochem, Slovakia) were of analytical reagent grade purity and were used as they were received. During the measurements, deionized, double-distilled water was used.

2.2. Apparatus

Voltammetric measurements were performed using the potentiostat Autolab PGSTAT-100 and the software GPES version 4.9.005 (Eco Chemie, Netherlands). EIS measurements were carried out on the Autolab using the impedance module FRA2 driven by the software FRA version 4.9.006 (Eco Chemie, Netherlands). A screen-printed three-electrode assembly (SPE) consisting of a carbon working electrode (SPCE) (25 mm^2 geometric surface area), a silver|silver chloride reference electrode (Ag-SPE|AgCl) (potential of 0.284 V *vs.* conventional Ag|AgCl|saturated KCl electrode) and a silver counter electrode (Ag-SPE) was obtained from Food Research Institute, Biocentrum, Modra, Slovakia. All measurements were performed in a 10 mL glass voltammetric cell.

2.3. Preparation of the biosensors

The SPCE surface was pretreated first by applying a potential of 1.6 V for 120 s and 1.8 V for 60 s in 10 mL of the AcB solution under stirring. This procedure was necessary to oxidize contaminants present on the carbon surface and to activate the electrode surface for the dsDNA immobilization [31]. The DNA biosensors with protective membranes, NAF/DNA/SPCE and CHIT/DNA/SPCE, were prepared by the application of 5 μL of dsDNA stock solution onto the SPCE surface and evaporation to dryness. Then, 5 μL of NAF or CHIT stock solution was deposited and evaporated.

The final biosensors were stable (possessing the signal values within the SPCE strip-to-strip repeatability) for several days, while keeping them in a dry atmosphere at a regular room temperature. Biosensors were prepared by a simple adsorption of the biocomponent immobilization. Both the NAF/DNA/SPCE and the CHIT/DNA/SPCE were not stable enough while in solution with leaching the modifier layers. Prior to taking a measurement, all working electrodes were immersed in PBS for 2 min and stirred to achieve equilibrium.

2.4. Preparation of the samples

Black tea extract: a tea bag was treated with deionized water at 98°C and leached for 4 min as indicated by the producer. Roasted black coffee extract: 2 g of ground coffee were weighed and treated with deionized water at 98°C. Both extracts were left to cool down to room temperature. The majority of CO_2 was released from 10 mL of lager beer by leaving the solution in the air for 15 min.

2.5. Procedures

Cyclic voltammetry (CV): the biosensor was immersed into 1 mM $[Fe(CN)_6]^{3-/4-}$ in PBS for 2 min under stirring. Cyclic voltammograms were recorded within a potential range from –0.8 to 1.0 V at a scan rate of 50 mV s^{-1} and a potential step of 5 mV.

Electrochemical impedance spectroscopy (EIS): the measurements were carried out in 1 mM $[Fe(CN)_6]^{3-/4-}$ in PBS at a polarization potential of 0.1 V (corresponding to a formal potential of the redox indicator used) in a frequency range of 0.1-5000 Hz (in 51 frequency steps) and an amplitude of 10 mV.

2.6. DNA damage and antioxidant activity evaluation

First, the CV and EIS measurements were performed with the DNA/SPCE, NAF/DNA/SPCE, and CHIT/DNA/SPCE in the PBS solution of 1 mM $[Fe(CN)_6]^{3-/4-}$ as described above. Then, after rinsing in deionized water, the same biosensor was incubated in the cleavage agent diluted with PBS (in a 1:1 (v/v) ratio) at an ambient temperature for a given time (2, 5, and 15 min) under stirring. After washing the electrode with deionized

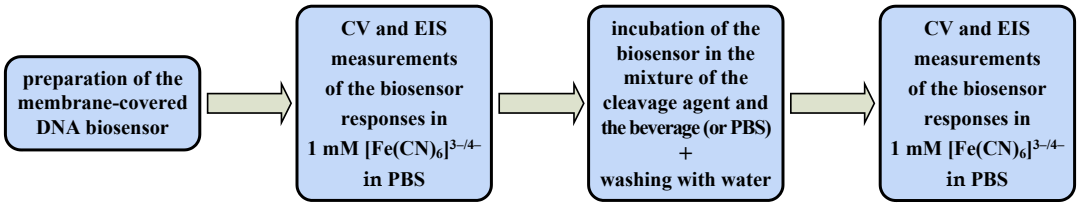

Figure 1. Working procedure used at the evaluation of the antioxidant activity of beverages.

water and the medium exchange for pure PBS, the CV and EIS measurements were performed again in 1 mM $[Fe(CN)_6]^{3-/4-}$ in PBS. The same approach was applied for the incubation of the biosensor in the mixture of the cleavage agent and the beverages (in a 1:1 (v/v) ratio). The diagram of the general working procedure is shown in Fig. 1.

The remaining portion of DNA (surv DNA) after the incubation of the biosensor in the cleavage agent was expressed as the normalized biosensor responses, which were calculated according to the Eqs. 1-3:

$$\Delta I = \frac{I_{\text{surv DNA}} - I_{\text{SPCE}}}{I_{\text{DNA}} - I_{\text{SPCE}}} \times 100 \quad (1)$$

$$\Delta(\Delta E_{\text{p}}) = \frac{\Delta E_{\text{p, surv DNA}} - \Delta E_{\text{p, SPCE}}}{\Delta E_{\text{p, DNA}} - \Delta E_{\text{p, SPCE}}} \times 100 \quad (2)$$

$$\Delta R_{\text{et}} = \frac{R_{\text{et, surv DNA}} - R_{\text{et, SPCE}}}{R_{\text{et, DNA}} - R_{\text{et, SPCE}}} \times 100 \quad (3)$$

where $I_{\text{surv DNA}}$ and I_{DNA} are the CV anodic currents of the 1 mM $[Fe(CN)_6]^{3-/4-}$ redox system measured for the DNA/SPCE, NAF/DNA/SPCE, and CHIT/DNA/SPCE biosensors at the peak potential obtained at the electrodes without DNA (*i.e.*, at 0.19 V for the SPCE, at 0.35 V for the NAF/SPCE, and at 0.16 V for the CHIT/SPCE), at which the I_{SPCE} values were obtained. Analogously, ΔE_{p} is the CV cathodic to anodic peak potential separation, and R_{et} is the electron transfer resistance at EIS.

The DNA damage experiments were repeated three times, and the CV and EIS measurements were repeated three times for each DNA damage experiment (the confidence intervals were calculated, and the error bars were constructed for the significance level α = 0.05). The relative standard deviation (*RSD*) of the data obtained during the repeated measurements (n = 3) was about 5% for I, 5% for ΔE_{p}, and 3% for R_{et}. The reproducibility of the responses obtained at the bare SPCEs as well as those covered by dsDNA and the external membrane layer was lower than 10% (*RSD*, n = 3). This was the reason why the normalized values of the biosensor responses were used.

3. Results and discussion

3.1. Effect of biosensor protective membranes

Three types of biosensors, simple DNA/SPCE and polymer membrane-covered electrodes, NAF/DNA/SPCE and CHIT/DNA/SPCE, were tested with respect to their response after incubation in the beverage (beer, coffee, and black tea) for 2, 5, and 15 min. The biosensors were rinsed with deionized water, transferred to the 1 mM $[Fe(CN)_6]^{3-/4-}$ solution, and the CV and EIS measurements were performed. The maximum CV anodic peak current (I_{p}) and resistance to the electron transfer (R_{et}) were evaluated (Fig. 2).

The values of I_{p} for the DNA/SPCE decreased with the time of the electrode incubation, and the corresponding increase in the values of R_{et} was observed. This observation can be described regarding the adsorption of high molecular weight substances in beverages leading to the sensor surface fouling. To eliminate this undesirable influence of substances present in beer and extracts of coffee and black tea on the biosensors, two types of external protective membranes, namely Nafion and chitosan films, were prepared and tested. The amount of polymer deposited on the DNA/SPCE surface was optimized with respect to biosensor response values. The polymers used differed in their charge in the medium of pH 7.0. With the negatively charged Nafion film, the CV response to the negatively charged indicator $[Fe(CN)_6]^{3-/4-}$ was diminished compared to the simple DNA/SPCE; with the positively charged chitosan, the indicator current response was higher than that of the DNA/SPCE due to the preconcentration of $[Fe(CN)_6]^{3-/4-}$ into the polymer.

The final NAF/DNA/SPCE and CHIT/DNA/SPCE biosensors showed that the fouling caused by substances present in the beverages was diminished. Compared to the simple DNA/SPCE, the response of the biosensors with the outer-sphere membranes exhibited a lower dependence on the time of the incubation of the beverage when the NAF/DNA/SPCE was used for beer and the black tea extract and when the CHIT/DNA/SPCE was used for the coffee extract. Based on these results, the NAF/DNA/SPCE biosensor was further used for the tests conducted in beer and black tea and the CHIT/DNA/SPCE biosensor that was used for the tests in coffee.

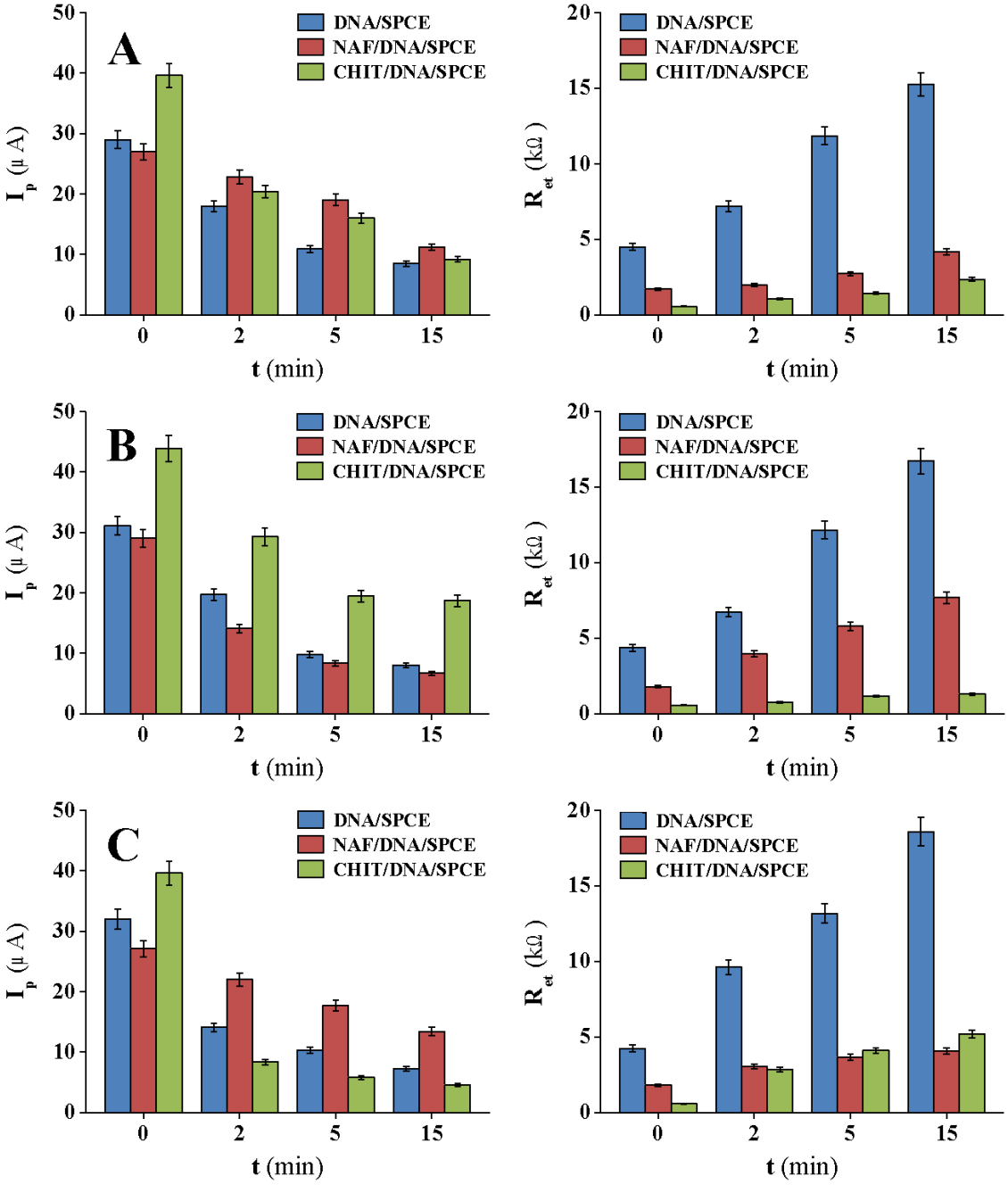

Figure 2. Comparison of the DNA/SPCE, NAF/DNA/SPCE, and CHIT/DNA/SPCE biosensor responses after the treatment in (A) beer, (B) coffee and (C) black tee extracts for a given incubation time.

3.2. Detection of the DNA degradation using the biosensors with the external membrane

For a deep DNA degradation, hydroxyl radicals were generated via the Fenton reaction by the mixture of Cu(II) ions and H_2O_2. The damage to DNA was evaluated using the complex approach given by a combination of CV and EIS measurements [17]. The biosensors were treated by the cleavage agent for 2, 5, and 15 min, and the CV and EIS measurements were performed after the medium exchange (Fig. 3). An increase in the CV current of the redox indicator and a decrease of the peak potential separation indicate a deep degradation of the DNA layer of the electrode surface. The DNA damage shows that the originally negatively charged DNA backbone was nicked and the remaining DNA was leaching from the electrode surface. The CV picture

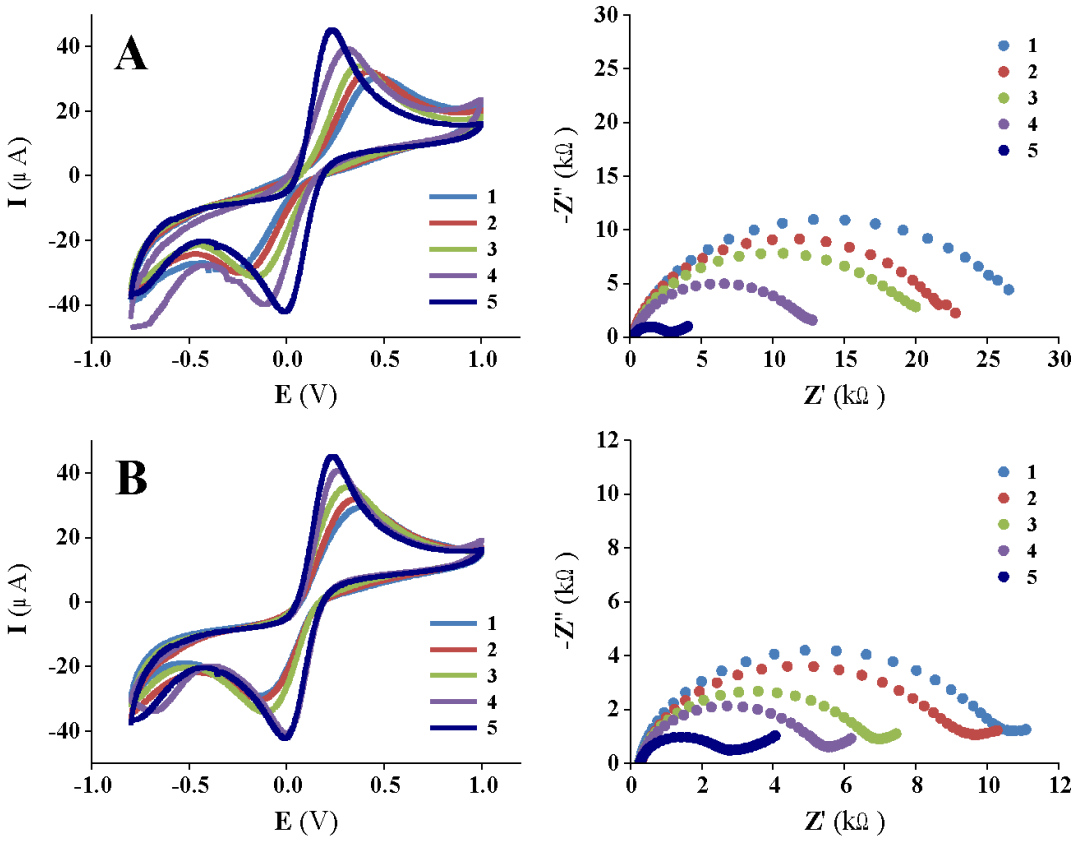

Figure 3. DNA damage detection at the (A) NAF/DNA/SPCE and (B) CHIT/DNA/SPCE. CV and EIS plots of 1 mM $[Fe(CN)_6]^{3-/4-}$ in PBS recorded before (curve 1) and after the incubation of the biosensor in the cleavage agent producing hydroxyl radicals for 2 min (curve 2), 5 min (curve 3), and 15 min (curve 4) followed by the medium exchange. Curves 5 correspond to the bare SPCE.

became better developed. Similarly, EIS measurements showed a decrease of the semicircle radius of the Nyquist plot curves due to the DNA degradation, which was coupled with the lowering of the electron transfer resistance.

The normalized values of CV data and the electron transfer resistance (R_{et}) were calculated as illustrated in the Experimental part (paragraph 2.6.). Table 1 summarizes the normalized responses obtained with the Nafion- and chitosan-protected biosensors. The comparison of these data with the normalized responses obtained for the DNA/SPCE without a protective membrane confirms similar ability of the biosensors with the polymer external membrane to detect the survived portion of DNA, *i.e.*, the damage to DNA.

3.3. Evaluation of the antioxidant activity of beverages

The antioxidant activity of the black tea and coffee extracts was evaluated using the biosensors with protective membranes effective against surface-active components. The antioxidant activity was investigated regarding the incubation of the biosensor in the mixture of the cleavage agent and the beverage. Using the normalized current and electron transfer resistance data obtained with the NAF/DNA/SPCE for black tea and the CHIT/DNA/SPCE for coffee, a higher portion of remaining DNA was found when compared to that detected after the incubation in the cleavage agent without addition of the beverage (Fig. 4). This is valid for the measurements at 2, 5, and 15 min of the biosensor incubation which indicate the ability of beverages to capture the reactive oxygen species for the given time. Of course, the DNA degradation is progressive with time in both the absence and presence of the beverage. The antioxidant ability of the black tea and coffee extracts was comparable.

4. Conclusion

A fouling of the DNA/SPCE biosensor surface in beer, coffee, and black tea was detected. Using the biosensors with outer-sphere protective membranes of Nafion (NAF) and chitosan (CHIT), their sensitivity to the detection of immobilized DNA and its deep degradation was higher

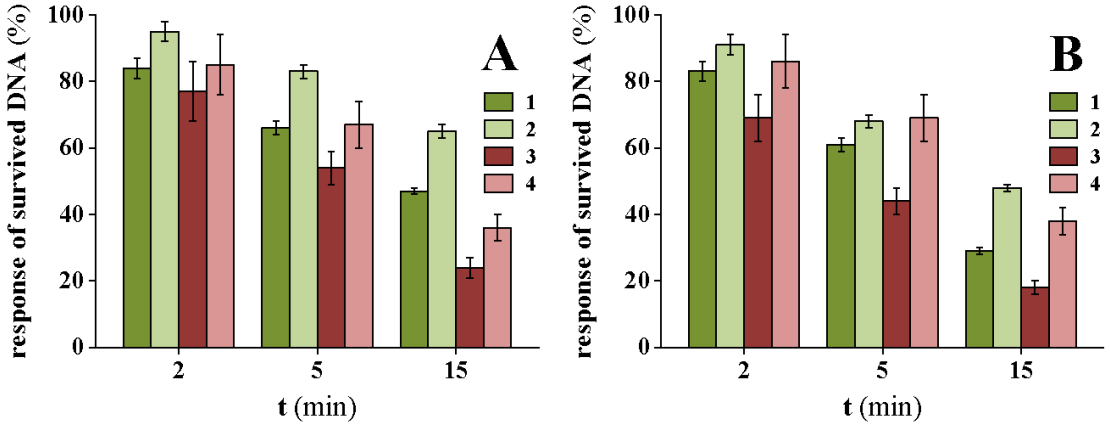

Figure 4. Antioxidant activity of black tea (A) (measured at the NAF/DNA/SPCE) and coffee (B) (measured at the CHIT/DNA/SPCE) evaluated by the comparison of the biosensor responses after the incubation in the 1:1 (v/v) mixture of the cleavage agent and PBS (1 and 3) and in the 1:1 (v/v) mixture of the cleavage agent and the beverage (2 and 4) for a given time. Biosensor responses: (1) and (2) ΔI, (3) and (4) ΔR_{et}, respectively.

Table 1. Comparison of the DNA/SPCE, NAF/DNA/SPCE, and CHIT/DNA/SPCE biosensor responses obtained after the incubation with the •OH cleavage agent for a given time.

Biosensor	t [min]	Normalized biosensor response [%]		
		ΔI	$\Delta(\Delta E_p)$	ΔR_{et}
DNA/SPCE	2	82 ± 4	87 ± 3	63 ± 9
	5	59 ± 4	73 ± 6	56 ± 8
	15	30 ± 2	37 ± 5	24 ± 6
NAF/DNA/SPCE	2	84 ± 3	79 ± 4	86 ± 9
	5	66 ± 2	58 ± 3	54 ± 5
	15	47 ± 1	36 ± 2	34 ± 3
CHIT/DNA/SPCE	2	83 ± 3	87 ± 4	69 ± 7
	5	61 ± 2	67 ± 3	44 ± 4
	15	29 ± 1	16 ± 1	18 ± 2

when compared to that of DNA/SPCE. While the NAF/DNA/SPCE biosensor was proved to be suitable for beer and black tea extracts, the CHIT/DNA/SPCE biosensor was successfully used in a coffee extract. Both Nafion and chitosan were obtained commercially, and the protected biosensors were easily prepared. With these biosensors, the DNA degradation was detected under simple *in vitro* conditions, and the antioxidant activity of selected beverages was evaluated.

The present study demonstrates the efficiency of the external biosensor membranes, known mostly for the enzyme biosensors, as they were applied to the DNA-based biosensor. The approach used in this experiment could be useful for the development of similar simple, inexpensive, and effective tools for warning tests and evaluations of beverages and environmental samples with complex matrices.

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