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Tests of lipid peroxidation products using the DNA based electrochemical biosensor

Adriana Ferancová[a]*, Katarína Beníková[a], Júlia Galandová[a], Ľudmila Sirotová[b] Ján Labuda[a]

[a] *Institute of Analytical Chemistry, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovakia*
[b] *Food Research Institute, Biocentrum, Kostolná 7, 900 01 Modra, Slovakia*

**adriana.ferancova@stuba.sk, jan.labuda@stuba.sk*

Abstract

In this work screen-printed carbon electrodes (SPCE) were modified with different nanocomposites: chitosan - multi-walled carbon nanotubes (CHIT-MWNT/SPCE), chitosan - single-walled carbon nanotubes (CHIT-SWNT/SPCE) and chitosan - carboxylated single-walled carbon nanotubes (CHIT-SWNT-COOH/SPCE) and further modified with DNA. Cyclic voltammetry and electrochemical impedance spectroscopy were used to characterize basic electrochemical properties of the prepared sensors and biosensors. For the lipid peroxidation tests, DNA was first incubated with malondialdehyde (MDA) and oxidized edible oils, then isolated and put onto the surface of CHIT-SWNT-COOH/SPCE. Adducts formation of MDA and lipid peroxidation products with DNA was found. These adducts inhibit the diffusion of the electrochemical probe $[Fe(CN)_6]^{3-/4-}$ to the electrode surface and, thus, changes in the relative charge-tranfer resistance were observed. These changes are in good agreement with the amount of lipid peroxidation products determined by the peroxide and TBARS values.

Keywords: DNA biosensor, lipid peroxidation, screen-printed carbon electrode, chitosan, carbon nanotubes, edible oils.

Introduction

As a consequence of oxidation of oils, especially their components – the polyunsaturated fatty acids, hydroperoxides in the primary stage of oxidation are formed. These compounds can decompose to the low-molecular-weight breakdown products or condense to polymers in the presence of metal ions or specific enzymes or at high temperature. The decomposition of hydroperoxides generates breakdown products such as aldehydes, ketones, alcohols, short fatty acids, esters, hydrocarbons, furans, and lactones, named as secondary products (Burcham 1998). Some of them are rather long-living and can drift far from membranes, damaging a wide variety of proteins, lipids and nucleic acids (Spiteller 2003, Cadenas 1989).

Studies of the interaction of hydroperoxides with DNA show that these compounds are reactive and cause cleavage of double-stranded DNA (dsDNA) or formation of hydroperoxide-induced DNA adduct (Termini 2000, Blair 2001, Williams et al. 2006). The secondary products, mainly unsaturated aldehydes, ketones and epoxides, cause DNA damage via the formation of base adducts. Depending upon structure, a DNA adduct may block or significantly slow replication, leading to arrested cell division or chromosomal aberrations, direct misincorporation of bases during replication, leading to mutations or undergo hydrolysis, leading to abasic sites and increasing the probability of strand scission (Kanner 2007, Uchida 2007). Malondialdehyde (MDA) is unusual among lipid peroxidation products. It can act as a nucleophile as well as an electrophile and is able to form a pyrimidopurinone adduct with deoxyguanosine (M1dG) (Seto et al. 1985). It also reacts with deoxyadenosine and deoxycytosine to form M1dA and M1dC, resp. M1dG can be made by the reaction of the corresponding bases with base propenals, which are generated at oxidative damage of deoxyribose. Adducts of DNA with this compounds (acrylaldehyde, crotonaldehyde, MDA, 4-hydroxy-2-nonenal) are considered as biomarkers of certain diseases, therefore they are widely studied (Marnett et al. 2003, Yang et al. 2003).

The DNA biosensors based on screen-printed electrochemical sensors are of great interest for their simplicity, mass production and detection possibilities. They can be used to study specific interactions of DNA with compounds of low molecular mass, DNA hybridization as well as damage to DNA. Sensitivity of the DNA biosensors can be enhanced via a way of the DNA immobilization by using new supporting matrices such as nanohybrids, for example polymer - carbon nanotubes (CNT) composites. In recent years, polysaccharide chitosan (CHIT) is of interest for this purposes (Galandova et al. 2008, Li et al. 2005). It has

been revealed that chitosan is efficiently protective against the protonation of single-walled carbon nanotubes (Takahashi et al. 2005). Due to its charge CHIT is more efficient dispersing agent in comparison to other media because of allowing quicker dispersion of the nanotubes. Moreover, CHIT also offers higher stability of dispersion (Tkac et al. 2006). It was also used in a combination with "non-carbonic" nanoparticles such as CHIT-metal composite with Ag, Au, Pt and Pd for glucose sensing (Huang et al. 2004, Okitsu et al. 2007, Du et al. 2007) and with ceramic materials (sol-gel) together with multi-walled carbon nanotubes (MWNT) for cholesteroloxidase immobilization at amperometric biosensor (Tan et al. 2005). CHIT as a polysaccharide containing free amino- and hydroxyl- groups, and having good film-forming ability, high water permeability, good adhesion, is perspective for biosensing (Zhang et al. 2004). It is biocompatible, biodegradable and non-toxic cationic polymer which forms polyelectrolyte complexes with negatively charged molecules including DNA (Kara et al. 2002).

In this article we report the preparation and electrochemical characterization of the electrochemical sensors CHIT-CNT/SPCE and DNA/CHIT-CNT/SPCE. Multi-walled carbon nanotubes (MWNT), single-walled carbon nanotubes (SWNT) and carboxylated single-walled carbon nanotubes (SWNT-COOH) were tested for this purpose. The DNA biosensors were used for testing the interaction of DNA with lipid peroxidation products.

Materials and Methods

Apparatus

Voltammetric measurements were performed using the potentiostat Autolab and the software GPES version 4.9.005 (Eco Chemie, Netherland). EIS measurements were carried out on the Autolab using FRA module, version 4.9.006. Screen-printed carbon electrode assembly (SPCE) consisting of working carbon electrode (21 mm^2 geometric surface area), a silver/silver chloride reference electrode Ag/AgCl/SPCE (potential of 0.284 V vs conventional Ag/AgCl/saturated KCl electrode) and the same counter electrode was obtained from Food Research Institute, Biocentrum, Modra, Slovakia. Spectrophotometric measurements were realized with Spekol 11 (Carl-Zeiss, Jena, Germany).

Chemicals

Carbon nanotubes – MWNT (o.d. 40–60 nm, i.d. 5–10 nm, length 0.5–500 μm), SWNT (o.d. 0.7–1.2 nm, length 2–20 μm) and SWNT-COOH (o.d. 4–5 nm, length 500–1500

nm) obtained from Sigma-Aldrich Chemie (Germany) were used as received. Sodium dodecyl sulphate (SDS) used for the preparation of MWNT and SWNT dispersions was from Sigma-Aldrich Chemie (Germany). The dispersion of SWNT-COOH was prepared in deionized water. CHIT (M = 600 000, degree of deacetylation 85 %) was obtained from Fluka. Its 0.5 % (w/w) solution was prepared in 1 % (v/v) acetic acid (Lachema, Czech Republic) and filtered through a simple paper strip. The final CHIT solution was of pH 5.0 (Galandova et al. 2008). Calf thymus dsDNA was obtained from Merck. Its stock solution (5 mg mL^{-1}) was prepared in 1.10^{-2} mol dm^{-3} Tris–HCl and 1.10^{-3} mol dm^{-3} EDTA (pH = 8.0).

Malondialdehyde (MDA) was prepared by the hydrolysis of tetraethoxypropane in 0.1 mol dm^{-3} HCl at 40 °C for 40 min and subsequent neutralization. Edible oils (soybean oil, sunflower oil, rapeseed oil, sunflower oil fortified with oleic acid and olive oil) were purchased from a local store. Samples of oxidized oils were prepared as follows: oil was resuspended in 0.1 mol dm^{-3} phosphate buffer (PBS, pH = 7.0) and oxidized at 85 °C during 24 h. The degree of oxidation of oil was determined by peroxide value and TBARS value (the amount of reactive species interacting with 2-thiobarbituric acid).

A mixture of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ with concentration of 1.10^{-2} mol dm^{-3} was used as electrochemical DNA indicator. PBS (0.1 mol dm^{-3}) of pH 7.0 was used as the supporting electrolyte. Other chemicals used were of analytical grade purity.

Isolation of DNA samples after its interaction with MDA and oxidized oils

DNA solution (0.1 mg mL^{-1}) was incubated with MDA of different concentration (1.10^{-3} mol dm^{-3}, 1.10^{-4} mol dm^{-3}, 1.10^{-5} mol dm^{-3} and 1.10^{-6} mol dm^{-3}) in 0.1 mol dm^{-3} PBS (pH = 7.0) at 37 °C during 72 h. After incubation, DNA was isolated using precipitation with ethanol. Briefly, ethanol was added to sample of DNA (2.5 times of sample volume) after the addition of 3 mol dm^{-3} sodium acetate (pH = 5.5, tenth of sample volume). The mixture was left at –18 °C for 2 h and subsequently washed with 70 % ethanol. DNA extracted was dried at room temperature, resolved in ultra-pure water and stored at –18 °C.

DNA (0.1 mg mL^{-1}) was incubated with oxidized oil (0.01 g mL^{-1}) during 24 h at 37 °C. DNA was isolated from reaction mixture by using the precipitation with ethanol (the procedure described above).

Preparation of modified electrodes

The electrode modifier was prepared by mixing of the CNT dispersion with CHIT solution in the 1 : 1 ratio (v/v) followed by the sonification. 5 μL of resulted modifier solution

was put onto the electrode surface and let to evaporate to dryness. Then 5 μL of DNA sample was added and let to evaporate to dryness.

Cyclic voltammetry (CV) of $[Fe(CN)_6]^{3-/4-}$

Before the first measurement, each newly prepared DNA modified electrode was preconditioned by immersing in 1.10^{-3} mol dm^{-3} PBS (pH = 7.0) for 5 min under stirring to remove unattached modifier. The cyclic voltammograms of 1.10^{-3} mol dm^{-3} $[Fe(CN)_6]^{3-/4-}$ in 0.1 mol dm^{-3} PBS were recorded from −650 mV to 700 mV using a scan rate of 50 mV s^{-1} and evaluated agaist the CV record obtained in blank PBS.

Electrochemical impedance spectroscopy (EIS)

Electrochemical impedance spectroscopic measurements were carried out in presence of 10^{-3} mol dm^{-3} $[Fe(CN)_6]^{3-/4-}$ as the redox probe in 0.1 mol dm^{-3} PBS (pH = 7.0) at ambient temperature and at the potential of 0 V, within the frequency range of 12–10^4 Hz and the amplitude of 10 mV.

Peroxide value determination

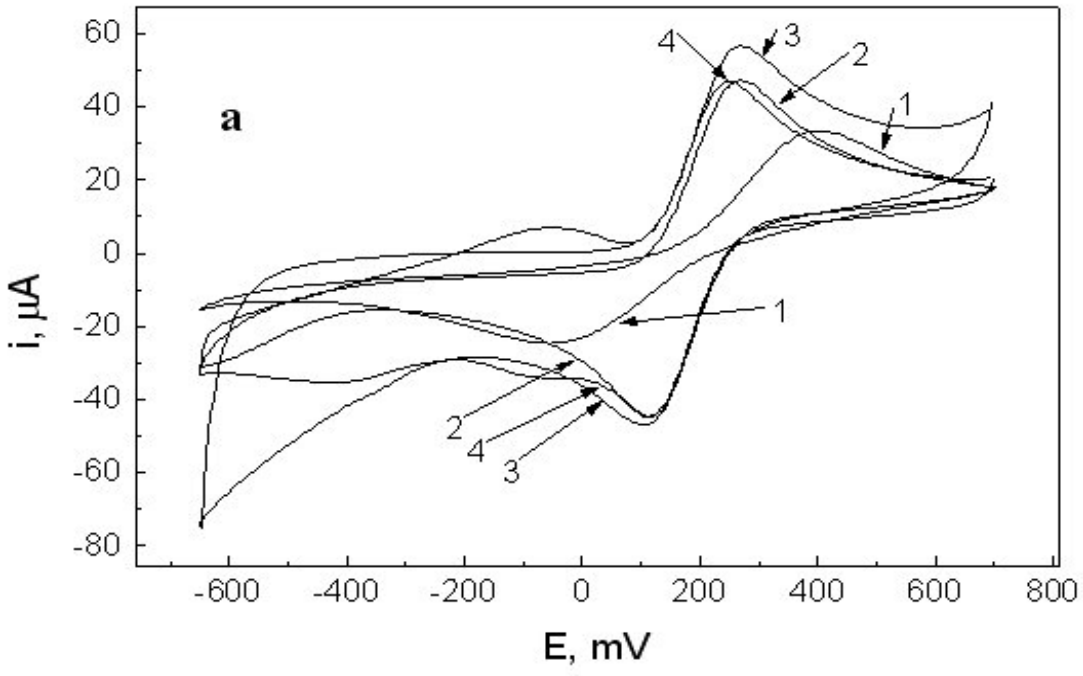
Peroxide value was determined by method of Shantha (Shantha et al. 1994) with slight modification in the preparation of sample. Briefly, oxidized oil solution (5 mL) was extracted with hexane (1 mL). Hexane layer was mixed in test tube with a mixture of chloroform : methanol 7 : 3 (v/v). Then, NH_4SCN and subsequently Fe(II) solutions were added to the fatty acid solution. After 5 min incubation of the mixture in the dark absorbance was measured spectrophotometrically at 500 nm against blank solution. Peroxide value was expressed as miliequivalents of peroxides per kg of oil.

TBARS value determination

1 mL of suspension of oxidized oil and 2 mL of each 1 % of 2-thiobarbituric acid (TBA) and 20 % of ice cold acetic acid and 1 mL of deionized water were mixed and then heated in a water bath to 100 °C for 1 h. After cooling, 5 mL of chloroform were added to the mixture and entire mixture was centrifuged at cetrifugal force 2000 g for 10 min. The absorbance of supernatant was measured spectrophotometrically at 532 nm. The value of TBARS was expressed as μmol of MDA equivalent per g of fatty acid.

Results and Disccusion

Fig. 1a shows the comparison of cyclic voltammograms of $[Fe(CN)_6]^{3-/4-}$ obtained at unmodified SPCE and SPCE modified with different types of carbon nanotubes. It is possible to see that the presence of CNT caused an increase in the current signals almost two times. The peak potential separations were reduced from value 416 mV at SPCE to 140 mV, 149 mV and 128 mV at SWNT/SPCE, MWNT/SPCE and SWNT-COOH/SPCE, resp. This current enhancement is caused by an enlargement of the electrode surface area and improved electrochemical properties of the electrode. Electrochemical impedance spectroscopy showed no significant differences in the results obtained for individual CNT modified electrodes. The impedance spectra obtained are represented by Nyquist plots (Fig. 1b). The Nyquist plot of unmodified SPCE shows semicircle which is typical for the faradayic electron transfer at high frequences. The straight line at low frequences is typical for diffusion limited electron transfer. In contrast to this, spectra obtained for CNT modified SPCE does not show the semicircle or its diameter is very high. It indicates that the modification of the electrode surface with CNT caused an enhancement of the electric properties of SPCE resulted to fast electron transfer.



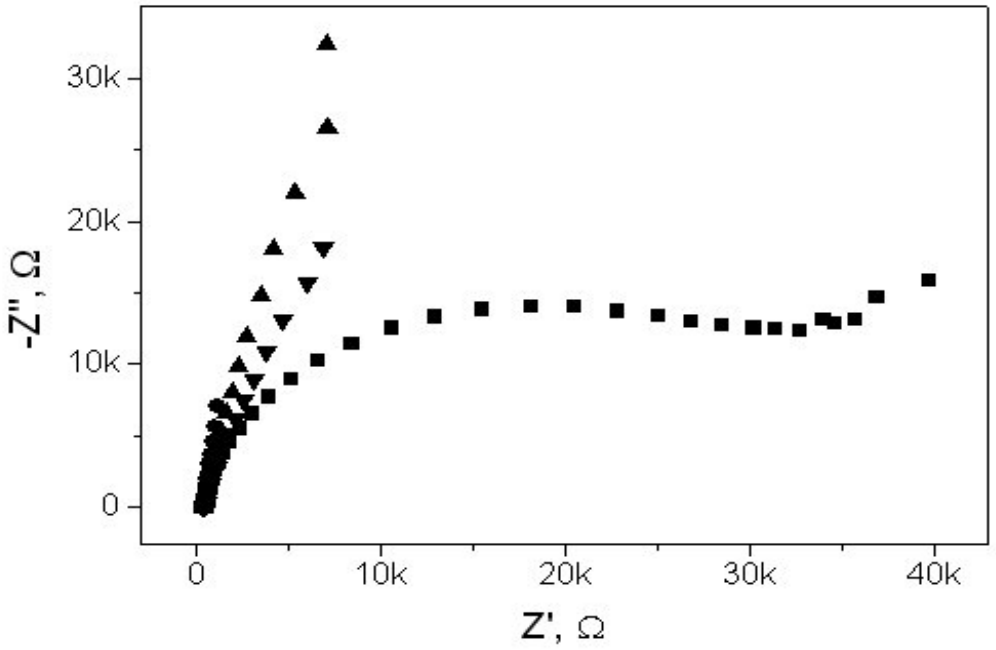


Fig. 1. Comparison of cyclic voltammograms of $[Fe(CN)_6]^{3-/4-}$ (a) and electrochemical impedance spectra (b) obtained at SPCE (1), MWNT/SPCE (2), SWNT/SPCE (3) and SWNT-COOH/SPCE (4). Conditions: 1.10^{-3} mol dm^{-3} $[Fe(CN)_6]^{3-/4-}$, 0.1 mol dm^{-3} PBS, (a) scan rate 50 mV s^{-1} ; (b) amplitude 10 mV.

In the next part of experiments we used chitosan to improve properties of the DNA biosensors. Chitosan is the polycation and DNA and other anions can be effectively immobilized on the surface modified with the chitosan film through the electrostatic attractions. SPCE were modified with the suspension of CHIT-CNT and covered with DNA. Fig. 2a–d shows CV of $[Fe(CN)_6]^{3-/4-}$ obtained at all prepared sensors and biosensors. Each Fig. contains three CV: those obtained at unmodified SPCE, CHIT-CNT/SPCE and DNA/CHIT-CNT/SPCE. For comparison Fig. 2a shows CV scans obtained at electrodes without CNT.

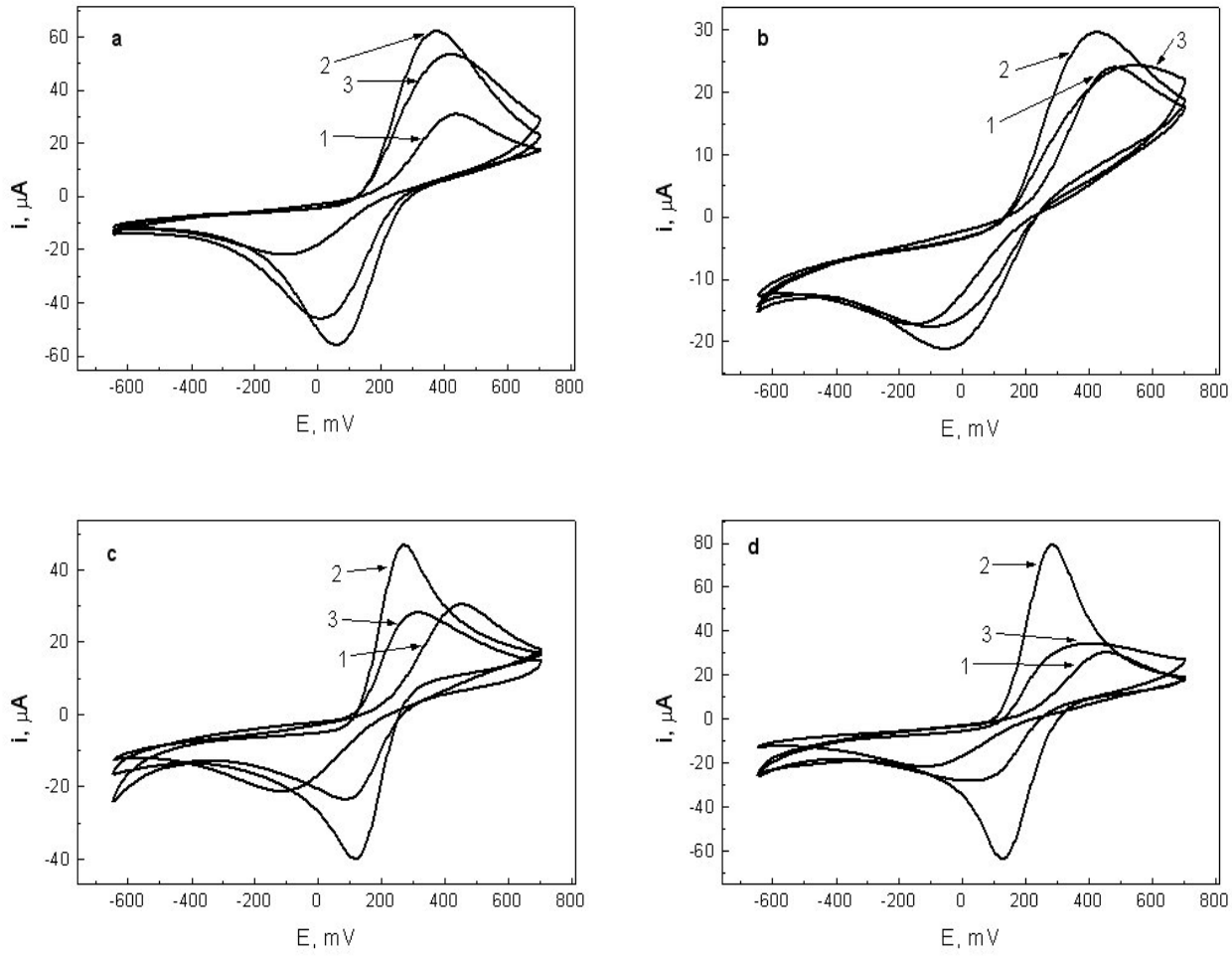


Fig. 2. Comparison of cyclic voltammograms of $[Fe(CN)_6]^{3-/4-}$ obtained at SPCE (1), CHIT-CNT/SPCE (2) and DNA/CHIT-CNT/SPCE (3) ((a) without CNT, (b) SWNT, (c) MWNT, (d) SWNT-COOH). Conditions: 1.10^{-3} mol dm^{-3} $[Fe(CN)_6]^{3-/4-}$, 0.1 mol dm^{-3} PBS, scan rate 50 mV s^{-1}.

In all cases, the modification of SPCE with the CHIT-CNT suspension improves its electrochemical properties resulted to higher peak currents and lower peak potential separations. The best results were observed in the case of CHIT-SWNT-COOH/SPCE where the peak height was increased 3.5-times and peak potential separation was reduced from 577 mV to 152 mV with respect to SPCE (Table 1). After introducing the DNA layer to CHIT-CNT/SPCE peak currents were decreased and the peak potential separations were increased. It indicates that the DNA layer acts as an insulator of the electrode surface. Again, the best results were obtained with DNA/CHIT-SWNT-COOH/SPCE, i. e. the peak currents were decreased almost 3-times and peak potential separation was increased from 152 mV to 388 mV with respect to CHIT-SWNT-COOH/SPCE (Table 1). These differences are suitable enough for the detection of damage to DNA.

Table 1. CV peak currents and peak potential separations of 1.10^{-3} mol dm^{-3} $[Fe(CN)_6]^{3-/4-}$ obtained at prepared sensors and biosensors. Conditions: 0.1 mol dm^{-3} PBS, scan rate 50 mV s^{-1}

Electrode	I_{pa}, μA	I_{pc}, μA	ΔE_p, mV
SPCE	21.3	−18.2	498
CHIT/SPCE	52.1	−56.4	296
DNA/CHIT/SPCE	38.2	−44.6	409
SPCE	19.3	−16.8	536
CHIT-SWNT/SPCE	28.8	−26.3	355
DNA/CHIT-SWNT/SPCE	11.3	−14.4	613
SPCE	24.3	−23.4	573
CHIT-MWNT/SPCE	43.9	−41.9	145
DNA/CHIT-MWNT/SPCE	17.6	−21.7	197
SPCE	19.8	−17.4	577
CHIT-SWNT-COOH/SPCE	73.7	−62.3	152
DNA/CHIT-SWNT-COOH/SPCE	24.6	−26.1	388

Two equivalent circuits (Fig. 3) were used to fit the impedance spectroscopic data obtained at sensors and biosensors mentioned above. Simple Randles circuit (Fig. 3a) contained R_S (solution resistance), R_{CT} (charge-transfer resistance) and C (constant phase element) was used to fit impedance spectra with well defined semi-circles. Second circuit (Fig. 3b) contained W (Warburg impedance) was used to fit impedance spectra where the semi-circle was diminished indicating that diffusion limited electron transfer took a part.

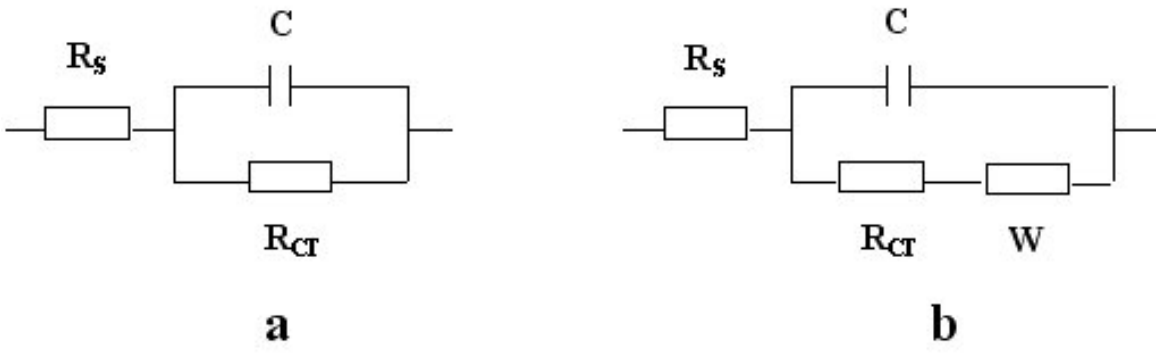


Fig. 3. Equivalent circuits used for fitting experimental EIS data.

Table 2 shows R_{CT} values obtained from the fitting the impedance spectra obtained at unmodified SPCE, CNT-CHIT/SPCE and DNA/CNT-CHIT/SPCE. It is possible to see that modification of SPCE with the mixture of CHIT-CNT caused the decrease in the charge-transfer resistance indicating the enhancement of electrochemical properties of the sensor. Adding the DNA layer caused an increase of the R_{CT} value because of insulating properties of the DNA. The best results were obtained in case of SPCE modified with CHIT-SWNT-COOH, which is in agreement with CV results.

Table 2. Parameters obtained from fitting the Nyquist plots

Electrode	R_{CT}, kΩ
SPCE	5.87
CHIT/SPCE	2.85
DNA/CHIT/SPCE	3.35
CHIT-SWNT/SPCE	0.86
DNA/CHIT-SWNT/SPCE	3.93
CHIT-MWNT/SPCE	0.48
DNA/CHIT-MWNT/SPCE	1.76
CHIT-SWNT-COOH/SPCE	0.17
DNA/CHIT-SWNT-COOH/SPCE	2.64

Tests of the lipid peroxidation products

With respect to the results obtained above, CHIT-SWNT-COOH/SPCE was chosen to test the quality of the DNA after its interaction with MDA and oxidized edible oils. CHIT-SWNT-COOH/SPCE were modified with DNA samples prepared as described in the Experimental section. Electrochemical impedance spectroscopy was used to test the quality of the DNA layer. In all cases impedance spectra showed significant diffusion component therefore we used the equivalent circuit depicted in Fig. 3b. Significant differences in charge-transfer resistance R_{CT} were obtained. Results were evaluated using relative value of R_{CT} ($R_{CTrelat}$) calculated as follows:

$$R_{CTrelat} = (R_{CT}/R_{CT,0})^{-1} \quad (1)$$

where $R_{CT,0}$ is the charge-transfer resistance of DNA/CHIT-SWNT-COOH/SPCE modified with undamaged DNA and R_{CT} is the charge-transfer resistance of DNA/CHIT-SWNT-COOH/SPCE modified with DNA pretreated with MDA of different concentrations or with oxidized edible oils.

Fig. 4 shows the dependence of the relative $R_{CTrelat}$ on MDA concentration. MDA reacts with DNA bases to form adducts. These adducts probably inhibit the diffusion of the redox probe which leads to an increase of R_{CT}.

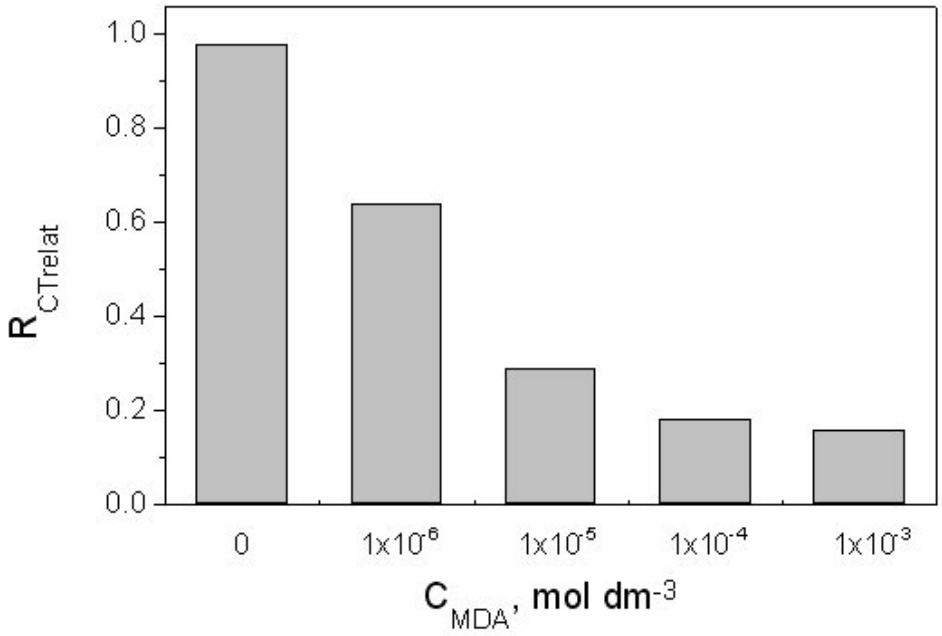


Fig. 4. Dependence of $R_{CTrelat}$ on MDA concentration.

During the peroxidation of oils primary oxidative products are formed and their decomposition generates structuraly various secondary products. Their presence can be determined using degree of peroxidation of oils in laboratory tests, such as peroxide value (amount of primary products - hydroperoxides) and TBARS value (amount of secondary products - substances able to react with TBA). The results of these tests are shown on Fig. 5.

Fig. 6 shows the dependence of the relative R_{CT} on type of oxidized oil. During DNA pretreatment with oxidized oils many different adducts can be formed with lipid peroxidation products which inhibit the diffusion of electrochemical marker. Therefore more or less increasing in R_{CT} value was observed. These results are in good agreement with those on Fig. 5 indicating that changes of R_{CT} are connected with the amount of lipid peroxidation products.

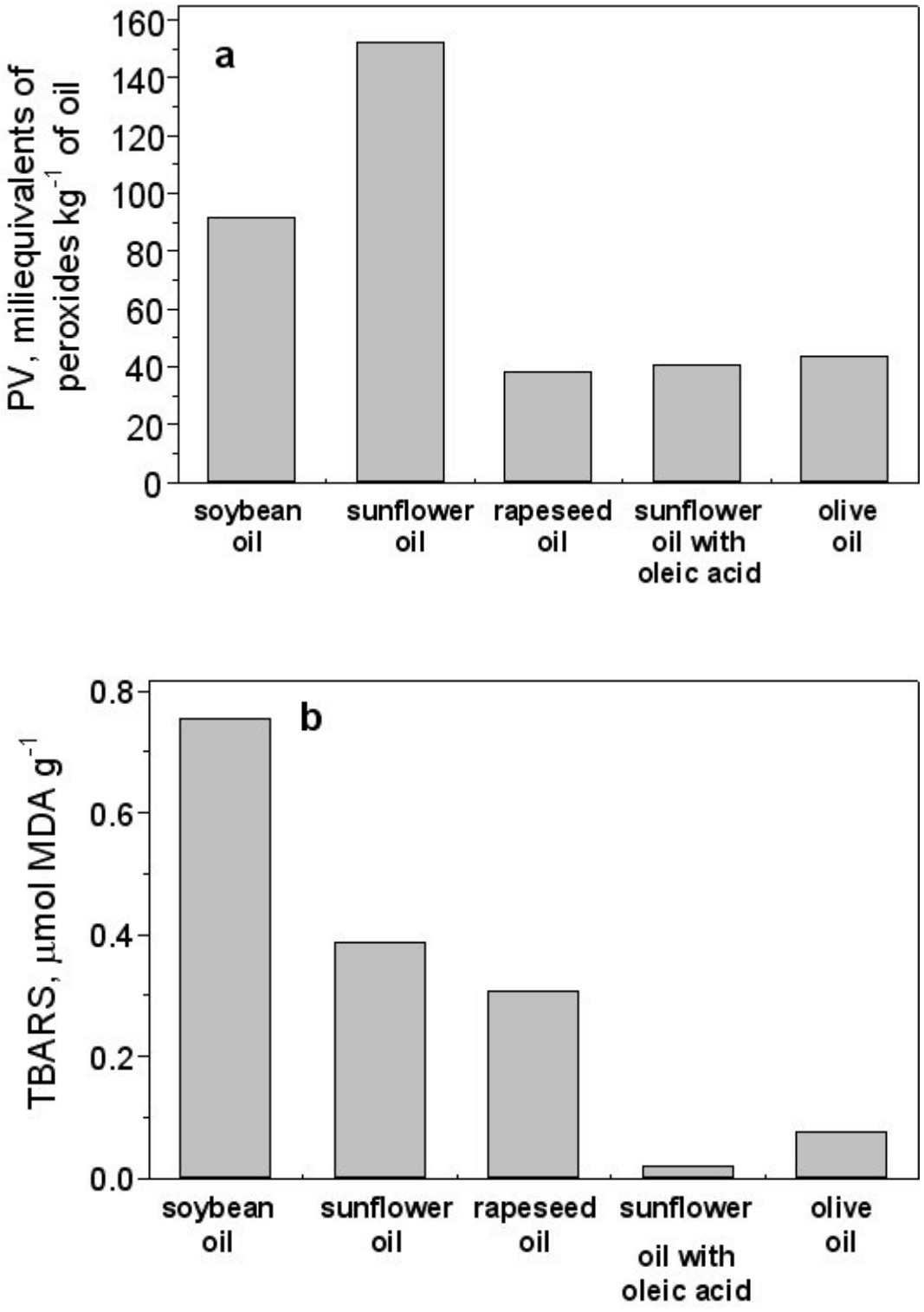


Fig. 5. Peroxide value of oils (0.01 g mL^{-1}) after oxidation at 85 °C during 24 h in 0.1 mol dm^{-3} PBS (pH = 7.0) (a) and TBARS value of oils (0.01 g mL^{-1}) after oxidation at 85 °C during 24 h in 0.1 mol dm^{-3} PBS (pH = 7.0) (b).

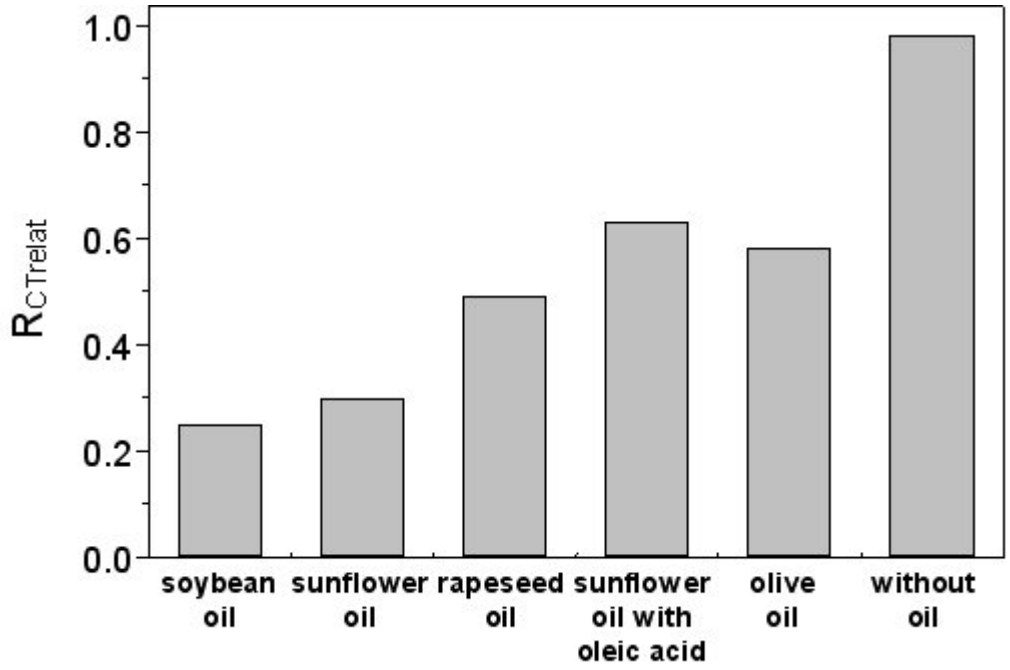


Fig. 6. Dependence of $R_{CTrelat}$ on edible oil.

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