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Oxidation of DNA Followed by Conformational Change after OH Radical Attack

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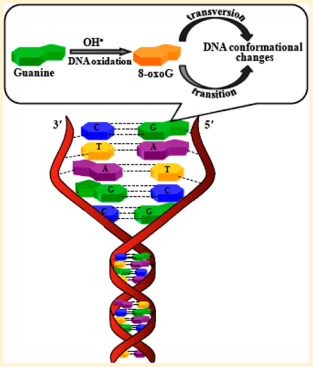
ABSTRACT: Examination of the attack of OH radicals produced in the Fenton way on DNA molecules is important from biological, biochemical, and biosensor points of view. Calf thymus DNA was selected for the investigation, since this natural oligonucleotide is often used in examination of drug−DNA interactions. Particularly useful was the coherent application of five techniques: electrochemical quartz crystal microbalance (EQCM), square wave voltammetry (SWV), circular dichroism (CD), atomic force microscopy (AFM), and UV−vis spectroscopy. These techniques differ in sensitivity to radical concentration and layer thickness of DNA. EQCM appeared to be the most sensitive in monitoring the consequences of OH radical actions; radical activities corresponding to nanomolar concentrations of H_2O_2 could be detected. SWV and AFM detection gave noticeable signal for higher than 1 μM H_2O_2 concentrations. EQCM data led to a conclusion that at higher than 1 μM H_2O_2 concentrations the DNA strands were locally disintegrated. The corresponding DNA loss was ca. 16%. It has been shown that in the presence of α-tocopherol, a strong antioxidant, the damage caused by OH radicals was practically prevented.



In living cells, DNA may be damaged by, e.g., drugs, toxicants, and free radicals. Free radicals are formed in living cells during the metabolic process and by exogenous sources, including carcinogenic compounds and ionizing radiation. It is estimated that 1−5% of oxygen metabolized in living organisms is univalently reduced to yield free oxygen radicals. A human, while resting, consumes ca. 500 L of oxygen per 24 hours and generates approximately 1 mol of free radicals. Free radicals can oxidize all biological macromolecules including proteins, lipids, and DNA.

Despite the fact that both H_2O_2 and $O_2^{\bullet-}$, under physiological conditions, do not react directly with the DNA components, it is accepted that there are two ways involving these molecules that lead to DNA damage. The first way is based on the formation of the OH radical from H_2O_2. Hydrogen peroxide easily penetrates cell membranes, and the Fenton reaction with the Cu^+ and Fe^{2+} ions can take place.[1] There are some limitations to the process between DNA and OH•. First, due to very high reactivity of the hydroxyl radical its ability to diffuse around is limited. Second, the Fenton cascade can be broken in the first reaction of the cascade of Cu(II) reduction to Cu(I) by catechol.[2]

Another explanation of DNA damage, related to the radical attack, involves a series of the metabolic changes in the cell that lead to the activation of nucleases capable of degradation of DNA. It is believed that the occurrence of oxidative stress causes an increase in intracellular free Ca^{2+} and this leads to the activation of Ca^{2+}-dependent endonucleases.[3] Probably, in the cell, the two mentioned mechanisms of DNA damage take place simultaneously. The most reactive form of oxygen in biological systems—the hydroxyl radical—may react with both the deoxyribose molecules and the nitrogen bases.[4] The reaction of OH• with deoxyribose leads to the formation of single and double DNA strands breaks and alkali-metal-sensitive sites.[3,4]

Single and double DNA-strand breaks are responsible for the blocking of the replication process and can cause cell death while the mutation changes are less probable. The latter changes can appear due to the presence of modified nitrogen bases which are formed in the reactions of DNA with free oxygen radicals. OH radical usually binds to pyrimidines at the C5 and C6 positions and to purines at C4, C5, and C8.[5] An alternative of OH• attack, that leads to the strand breaks, is the attack of metal hydroperoxides, such as FeOOH and CuOOH, formed in the reaction of respective metal ions with OH radicals.[6,7] Finally, a variety of modified products appear. Guanine in its free state and as a component of a nucleoside, nucleotide, or polynucleotide (DNA, RNA) is particularly susceptible to oxidation at C8 position and consecutive formation of 8-oxoguanine called 8-oxoG. The hydroxyl radical is the best recognized agent in the conversion of guanine to 8-oxoguanine.

The tools we have chosen for the examination of the attack of OH radicals on DNA allow us to look at the process more selectively, more precisely, and deeply. The aim of this paper is to report examination of the structural transformations of damaged and oxidized double stranded DNA caused by hydroxyl radicals in the presence and absence of an antioxidant. We focused our efforts on finding the way of effective monitoring of this phenomenon for particular OH concentrations and DNA film thickness. The source of free radicals was the Fenton solution which generates just one type of

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radical and the most aggressive to human tissue radicals ($OH^{\bullet}$). Other sources, e.g. UV irradiation, generate a variety of radicals and therefore complicate the investigation. Several analytical techniques, including electrochemical quartz crystal microbalance (EQCM), square wave voltammetry (SWV), circular dichroism (CD), atomic force microscopy (AFM), and UV−vis spectroscopy were applied to strengthen the conclusions.

EXPERIMENTAL SECTION

Materials. Calf thymus (ct) double stranded DNA (dsDNA) was purchased from Fluka; it was sufficiently pure and virtually free of protein. Good criteria for DNA purity are the values of the DNA absorbance ratios: $A_{\lambda=260\ nm}/A_{\lambda=280\ nm}$ in the range 1.7−2.0 and $A_{\lambda=260\ nm}/A_{\lambda=250\ nm}$ in the range 1.4−1.7.[8] The accepted by us ctDNA samples gave an absorbance ratio in the middle of the indicated ranges. ctDNA solutions of 1 mg DNA per 1 mL of phosphate buffer (pH 7.4) were prepared at least 24 h before experiments to get full renaturation. The concentration of ctDNA was determined from the value of the absorbance at λ = 260 nm; ε = 13200 M^{-1} cm^{-1}.[9] Fenton solution (0.025 mM) was always freshly prepared from $(NH_4)_2Fe(SO_4)_2{\cdot}6(H_2O)$ (Merck), EDTA (Merck), 0.01 M acetate buffer (pH 4.7), and H_2O_2 (Merck) solution. The range of the used H_2O_2 concentrations was 0−5 μM. α-Tocopherol, the most biologically active form of vitamin E, was purchased from Fluka. It is an antioxidant that neutralizes the reactive oxygen species.[10] The molar ratio of Fe^{2+}/EDTA was 1:1. Various amounts of H_2O_2 were added to the Fenton solution. Reagent grade NaH_2PO_4, Na_2HPO_4, NaCl, NaOH, and H_2SO_4 were purchased from POCh, Gliwice. All solutions were prepared using Milli-Q water of conductivity 0.056 μS/cm.

Square Wave Voltammetry (SWV) Measurements. Square wave voltammetry was performed using an Autolab, EcoChemie potentiostat, controlled via producer's software, and a special cell with thermal circulation. For each voltammetric measurement the three-electrode system consisting of a glassy carbon electrode, GCE, (ϕ = 3 mm, BAS Instruments) used as the working electrode, a Ag/AgCl/3 M KCl electrode (reference electrode), and a platinum wire used as the auxiliary electrode was employed. Each time before use the working disk electrode was briefly polished with 0.05 and 0.3 μm Al_2O_3 powders on a wet pad. After polishing, to remove alumina oxide completely from the surface, the electrode was rinsed with a direct stream of ultrapure water. The electrode surface was inspected optically with an Olympus, model PME 3, inverted metallurgical microscope. Before measurements the solutions were degassed with pure argon.

Electrochemical Quartz Microbalance (EQCM) Measurements. An electrochemical quartz crystal microbalance, model Autolab-EQCM (Autolab) with 6-MHz Au/TiO_2 quartz crystal resonators, was used in this study. The piezoelectrically active (geometrical) surface area of the working Au electrode was 0.3523 cm^2 and the real surface area, A, was equal to 0.5286 cm^2 (roughness factor: 1.5). The real surface area of the Au−EQCM electrode was determined from the Pb underpotential-deposition voltammetric peak (0.01 M lead(II) perchlorate in 0.1 M perchloric acid). The accepted value for the Pb monolayer on Au is Q_{Pb} = 302 $\mu C/cm^2$ (manufacturer data). The Au−EQCM electrode was electrochemically pretreated by cycling first between 0 and 1.8 V (hold 10 s at 1.8 V) in 0.5 M NaOH with scan rate 50 mV/s, and then by cycling between −0.3 and 1.5 V (vs Ag/AgCl) in 0.1 M H_2SO_4 solution until a stable voltammogram typical for a clean gold electrode was observed.[11] The real surface area was also calculated from the charge of the gold surface oxides formation/reduction in sulfuric acid and was equal to 0.5073 cm^2. This technique was successfully used to monitoring of conformation changes of biomolecules.[12]

Atomic Force Microscopy (AFM) Measurements. Atomic force microscopy experiments were performed with a 5500 AFM instrument (Agilent Technologies, Santa Clara, CA, USA). Magnetic AC mode was used for imaging, and the data were obtained in the Fenton solution. Type VI MAC levers (Agilent Technologies, Santa Clara, CA, USA) were used for imaging. The typical resonance frequency of the cantilevers was 50−60 kHz in air and it was reduced to 18−24 kHz in the solution. The images were acquired at 20 °C. A small gold bead prepared by Clavilier method was utilized as a substrate.[13] The bead was spot-welded to gold foil and atomically flat (111) facets were used for image acquisition. The substrate and the Teflon parts of the liquid cell were cleaned in piranha solution (concentrated H_2SO_4/30% hydrogen peroxide 3:1) and thoroughly rinsed with Milli-Q water. *(CAUTION: piranha solution reacts violently with organics and should be handled with extreme care)*. Gold bead was flame-annealed and quenched in Milli-Q water before each experiment. The root-mean-square (RMS) surface roughness for DNA films was determined for 1 × 1 μm^2 images using Pico Image Basic software provided with 5500 AFM instrument.

Circular Dichroism (CD) and UV−vis Spectroscopy Measurements. A J-815 circular dichroism spectrometer (Jasco), controlled by producer's software, was used for obtaining CD spectra. A quartz cuvette of 1-cm length was used as the optical window. CD parameters were the following: scanning speed 100 nm/min, data pitch 0.5 nm, bandwidth 2 nm, accumulation (number of scans) 10. Absorption spectra were recorded with a PerkinElmer spectrometer, model Lambda 25, at temperature 21 °C. All spectra were obtained for a constant DNA concentration (3 μM base pairs) in the solution and were corrected for the background of the pure Fenton solutions with appropriate addition of H_2O_2. Each spectrum was obtained for a freshly prepared solution.

Five samples were taken for all types of examination and the measurements were repeated three times. The results were well reproducible. The standard deviation was never bigger than 16%.

RESULTS AND DISCUSSION

The oxidative damage of DNA molecules can occur by either the direct reaction of the free radicals with nucleotide located in the DNA helix or the introduction of a damaged nucleotide into DNA during the replication process.[14,15] The reactions of radicals with DNA contribute to the formation of oxidative damage of many molecules, including rupture strands of DNA, damage of single base, and the formation of volume adduct (bulky adducts). The term bulky adducts addresses the cross binding (cross-links) and ring adducts (exocyclic adducts).[16] It is assumed that their presence in DNA can initiate the processes of mutagenesis and carcinogenesis.[16]

In all experiments (except for UV spectroscopy) we worked with DNA accumulated at appropriate substrate (glassy carbon or gold). The accumulation of calf thymus double stranded DNA (ctDNA, negatively charged in pH 7.4)[17] on the substrates was done by adsorption at a constant potential (+0.15 V). To prevent possible adsorption of the chloride ions

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at the gold surface, the accumulation of ctDNA was done in ctDNA solution containing chloride-free phosphate buffer (0.02 M, pH 7.4).

Voltammetric Results. To estimate the extent of DNA damage caused by OH radicals we decided to perform square wave voltammetry with DNA accumulated at the glassy carbon electrode surface. The freshly cleaned electrodes were immersed in a double stranded ctDNA solution (142 μM base pairs, 50 μg/L). The time of immobilization of ctDNA was 30 min. With that electrode it was possible to measure the guanine electrooxidation peak height.[18] The entire electrooxidation of guanine is a two-step process. In the first process we focus on, in a two-electron oxidation 8-oxoguanine is formed; this product is mutagenic. Since 8-oxoguanine can also be formed in the reaction with the radicals, smaller voltammetric peaks will mark the previous oxidation of guanine by radicals. The DNA-modified electrode was first immersed in the Fenton solution containing given concentrations of H_2O_2. After an appropriate time of immersion (e.g., 20 s) the electrode was rinsed with pure water and immersed in 0.02 M PBS buffer (pH 7.4). Then square wave voltammetry was quickly carried out. Next, the electrode was polished, another ctDNA layer was formed on the electrode surface according to the procedure described above, and the freshly prepared electrode was exposed to consecutive Fenton solution with higher concentration of H_2O_2 for 20 s.

The voltammetric curve obtained with the glassy carbon electrode exhibits one peak at 0.68 V, see Figure 1, which

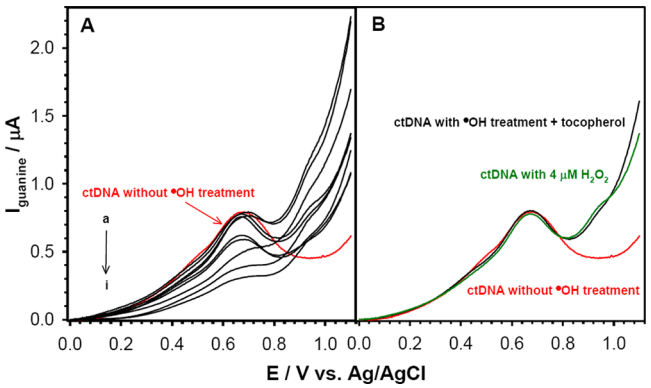

Figure 1. A: Square wave voltammograms of ctDNA obtained after OH radicals treatment in pure PBS buffer. Concentration of H_2O_2: 0.1 (a); 0.2 (b); 0.3 (c); 0.4 (d); 1 (e); 1.5 (f); 2.5 (g); 3 (h); and 4 μM (i). B: Square wave voltammograms of ctDNA obtained before and after OH radicals treatment in pure PBS buffer containing 1 mM α-tocopherol and in the presence of 4 μM H_2O_2. Experimental conditions: diameter of disc glassy carbon electrode 3 mm, 0.02 M phosphate buffer of pH 7.4; concentrations of Fe^{2+} and EDTA in Fenton solution: 0.025 and 0.025 mM, respectively.

corresponds to the oxidation of guanine.[19−21] The decrease in the voltammetric peak height was seen only in the presence of the radicals. The presence of hydrogen peroxide alone (in the same concentration range) and the presence of Fe(II)−EDTA complex did not influence the peak height. The presence of tocopherol (1 mM) apparently deactivated the radicals and finally eliminated the decrease in the voltammetric peak height. Tocopherol reacts faster with the radicals than DNA does and by binding the radicals protects DNA.[1] The applied tocopherol concentration reflects typical concentration of antyoxidants in human body.

As it is seen in Figure 2, the damage of the ctDNA film was displayed by a substantial decrease in the current signal of the

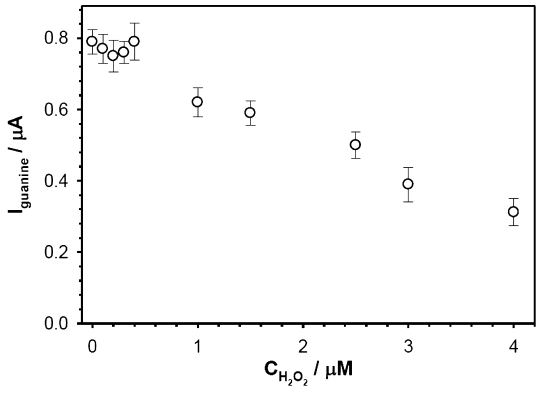

Figure 2. Dependence of oxidation current of guanine in ctDNA versus concentration of H_2O_2. Experimental conditions same as in Figure 1.

electrooxidation of guanine. The extent of DNA damage was measured as the relative decrease (scale 0−1) of the DNA current according to the formula 1:

$$\phi_{DNA} = 1 - \frac{I_{G,H_2O_2}}{I_G} \quad (1)$$

where I_G and I_{G,H_2O_2} are the oxidation currents of guanine without the treatment with Fenton solution and with treatment with different concentrations of H_2O_2 in Fenton solution, respectively.

For H_2O_2 concentrations equal to 0.4, 1.5, 2.5, and 3 μM the relative extent of DNA damage was equal to 0.01 ± 0.1, 0.25 ± 0.09, 0.37 ± 0.11, and 0.51 ± 0.08, respectively (see Figure 2). In the concentration range of H_2O_2 additions 0−1 μM, there is only a slight drop in the oxidation of guanine peak current. At higher concentrations (>1 μM) the guanine peak current rapidly, linearly decreased. The AFM measurements confirmed that the immobilized ctDNA film was tightly covering the electrode surface. Additionally, the ctDNA layer formed in this way consisted of large patches. For smaller than 1 μM OH-radicals concentrations probably the DNA molecules in direct contact with the solution are damaged. Since the voltammetric signal of oxidation of guanine is rather related to the guanine molecules that are in close proximity to the glassy carbon surface, we did not observe significant changes in the electrooxidation guanine peak height. In the case of high concentration of OH radicals ($C_{H_2O_2}$ > 1 μM) the DNA damage size was significant and probably resulted in local removal of DNA fragments from the electrode surface. That is why the guanine residues in the DNA film placed in close proximity to the glassy carbon surface maybe are also oxidized by OH radicals. In consequence, the number of unmodified guanine residues by free radicals dropped what is seen in a decrease of the oxidation peak of guanine. These data are in fact in good agreement with the EQCM and AFM results described below.

EQCM Results. A typical frequency change during DNA immobilization process is shown in the inset in Figure 3. The resonant frequency shift Δf (corresponding to the mass accumulation according to the Sauerbrey equation) was ca. 189 ± 9 Hz. The apparent mass increase related to the observed experimental frequency shift for dsDNA accumulation

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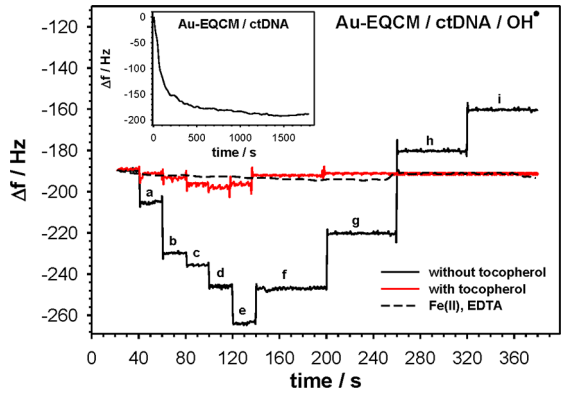

Figure 3. Frequency shifts observed during exposure of Au-EQCM/ctDNA to Fenton solution (Fe^{2+}, EDTA, H_2O_2). Concentration of H_2O_2: 0.1 (a); 0.2 (b); 0.3 (c); 0.4 (d); 1 (e); 1.5 (f); 2.5 (g); 3 (h); and 4 μM (i). Presence of 1 mM α-tocopherol (red line), no H_2O_2 (dashed line). Inset: Frequency shifts observed during accumulation of dsDNA at Au-EQCM electrode surface at 150 mV. Experimental conditions: 0.02 M phosphate buffer of pH 7.4; concentrations of Fe^{2+}, EDTA in Fenton solution: 0.025 and 0.025 mM, respectively.

is: m_{ctDNA} = 4.3·189 = 812.7 ± 39 ng, which corresponds to 1537.4 ± 73 ng/cm^2. To work with the electrodes covered with fixed amount of DNA we kept the adsorption process till the steady state frequency was reached. This amounted to ca. 1600 s.

In the next step the modified electrode (Au-EQCM/ctDNA) was carefully washed with water and exposed to a freshly prepared Fenton solutions with increasing amount of H_2O_2. The time intervals here were so short, since the lifetime of the radicals is very short. A time period of 20 s was satisfactory for the monitoring of the radical action. The reaction of the hydroxyl radicals with DNA can lead to the oxidation of the nucleic bases and the sugar residues; it can even break the phosphodiester bonds connecting deoxynucleotides.[22,23] The oxidation of the nucleic bases in DNA chain can occur in several places. The best known DNA modifications involve the oxidation of guanine in position C8.[23,24] The oxidation of the nucleic bases depends on the type of the bases and can occur in several positions, resulting in the formation of the various reaction products. OH radicals usually binds to pyrimidines at the C5 and C6 positions and to purines at C4, C5, and C8.[5] The position C8 in purines is especially important from the electrochemical point of view. As a result of the reaction of OH radicals with guanine the oxygen atom is bound to the carbon atom at the C8 position, and in consequence of the rupture of the double bond one hydrogen atom is attached to nitrogen N7.[23] The main known product of this process is 8-oxoguanine. The oxidation at this place results in a change of the electrooxidation current of guanine (see Figure 2).

The molecular masses of oxidized DNA elements are significantly higher compared to those that are unoxidized. This fact is supported by the observed decrease in the resonant frequency. The process of the oxidation of DNA (drop of the frequency), according to Figure 3, was observed till the addition of H_2O_2 reached the value of 1 μM. This means that the DNA weight, due to the oxidation process, increased by 42%. This significant increase cannot reflect just the oxidation process and rather mirrors the additional hydration of the oxidized nucleotide. For the higher than 1 μM additions of H_2O_2 an increase in the frequency appeared. This increase suggests that the double helix DNA chain was locally cut and a part of it moved away to the solution. To support this conclusion the UV spectra of this solution were obtained. They are presented in Figure 4. They show that for concentrations of H_2O_2 higher

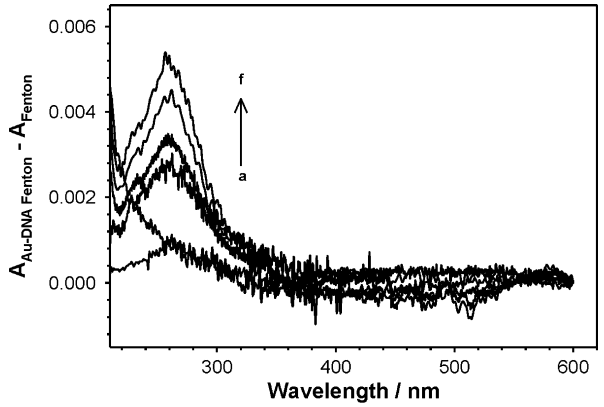

Figure 4. UV−vis spectra of the solution after 15-min exposure of modified Au−EQCM electrode (Au−EQCM/ctDNA) to Fenton solutions with different concentrations of H_2O_2. All spectra were corrected by subtracting the appropriate signal of Fenton solution without DNA. Concentration of H_2O_2: 0.5 (a); 1 (b); 1.5 (c); 2.3 (d); 3 (e); and 4 μM (f).

than 1 μM, DNA species were detected in the solution. The amount of accumulated ctDNA at the gold surface was determined from the absorbance values (obtained for the solution before and after ctDNA immobilization)[25] at λ = 260 nm; ε = 13 200 M^{-1} cm^{-1} and was equal to 8.4 ± 0.9 nmoles of base pairs. After adding H_2O_2 to the Fenton solution to the level 4 μM, ca. 1.5 nmoles of base pairs (18%) of ctDNA was removed from the electrode surface. These results are in a good agreement with the EQCM data. The obtained DNA surface coverage based on the EQCM data before OH radicals treatment was 1.54 ± 0.2 μg/cm^2. After increasing H_2O_2 concentration to 4 μM the DNA surface coverage reached the value 1.3 ± 0.13 μg/cm^2. The corresponding DNA loss was ca. 16%. However, these numbers are just approximations because of the involvement of solvent and electrolyte in the surface film and the viscoelastic frequency shift due to the change in the degree of hydration.[26]

The increase in the content of oxidized nitrogen bases in the DNA chain contributes to the emergence of the point mutations. It is estimated that the oxidized adenine (8-oxoA) is at least an order of magnitude less mutagenic than 8-oxoG.[27] It was shown that in vivo pairing of oxidatively modified guanine (8-oxoG) during DNA replication leads to a transversion 8-oxoG → T and to the formation of oxidation products of other nitrogenous bases. The latter leads to either the transition G → A or C → T.[28−30] Too big a number of transitions and/or transversions in DNA chain lead to the conformational changes of double stranded DNA, which was confirmed by CD measurements.

In the presence of tocopherol, a strong antioxidant, no changes in the resonant frequency shifts were observed, see the red line in Figure 3. This means that the oxidation process of DNA chains did not take place. Simply tocopherol binds the radicals and in this way deactivates them.[1]

AFM Results. AFM imaging was performed to evaluate the DNA layer structure in the absence and the presence of H_2O_2. Figure 5 shows a typical image of freshly prepared gold substrate modified with a ctDNA layer. The immobilization of

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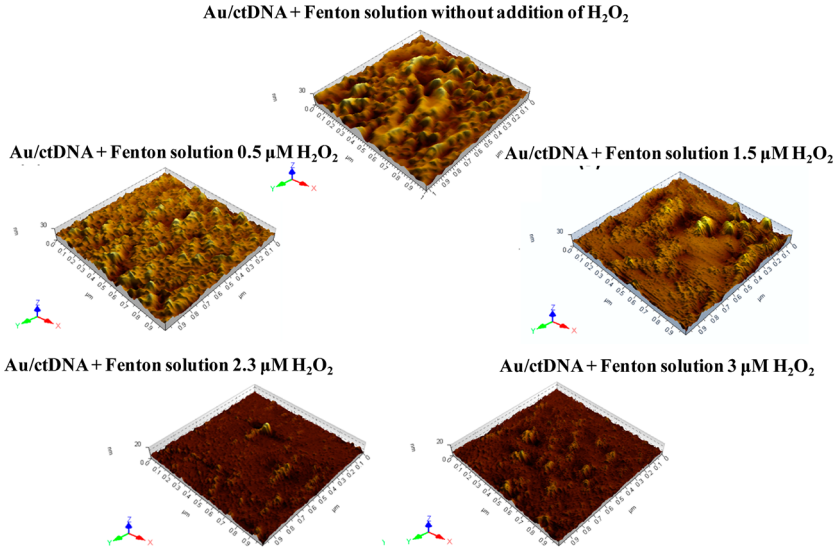

Figure 5. AFM image of the Au(111) substrate modified with ctDNA film recorded in Fenton solution without addition of H_2O_2 and with increasing concentration of H_2O_2. Concentrations of Fe^{2+} and EDTA in Fenton solution: 0.025 and 0.025 mM, respectively.

ctDNA by electrostatic interactions resulted in formation of large patches with the size ranging from 20 to 100 nm. Such behavior is consistent with previously reported results for amorphous DNA layers on hard and flat surfaces where the aggregated DNA formed lumps with the average diameter of 50−60 nm.[31,32] Formation of amorphous DNA layers is commonly observed for the films with high molecular density and for long DNA molecules.[33] The changes in the morphology of the film upon increasing amount of H_2O_2 are also shown in Figure 5. As the concentration of H_2O_2 increases the aggregates become smaller and the topography of the film changes significantly: the film flattens. This may indicate that the hydroxyl radicals induce dispersion of the aggregates to fine-grained structures. It is also possible that some part of the previously immobilized material is detached from the substrate. To describe these changes more quantitatively, we have analyzed the RMS surface roughness (S_q) of the DNA films. The standard deviation of the height distribution was taken as a measure of RMS defined according to the ISO 4287 and ASME B46.1 standards. It was calculated using formula 2:

$$S_q = \sqrt{\frac{1}{NM}\sum_{x=0}^{N-1}\sum_{y=0}^{M-1} z_{x,y}^2} \qquad (2)$$

where N is the number of points per scan line, M is a number of scan lines, and $z_{x,y}$ is the amplitude at point (x, y). Figure 6 presents the changes of the surface roughness with increasing amount of H_2O_2. Within the concentration range 0−1.5 μM, there is only a slight change in surface roughness reflecting break-up of the aggregates to fine grains. At higher concentrations (>1.5 μM) the surface roughness drops to significantly lower values, which indicates that the film is flattened. This behavior suggests that the aggregates forming the film are dispersed and subsequently removed from the substrate. Such explanation of the AFM data is supported by the results of EQCM measurements where the loss of the material from the electrode surface was observed for concentration of H_2O_2 higher than 1 μM. Similarly as in the EQCM and SWV cases the presence of tocopherol did not lead to any morphological changes.

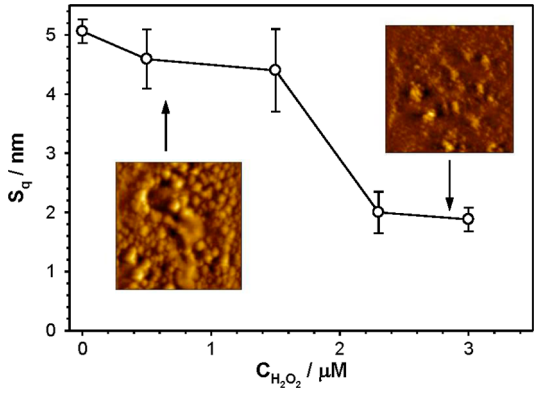

Figure 6. Change in surface roughness as a function of hydrogen peroxide concentration. Insets show representative AFM images (taken from 1 μm^2 of the sample) obtained in the absence of H_2O_2 (left) and at highest concentration of H_2O_2 (right).

CD Results. The measurement of ellipticity (θ) is widely used to monitor the conformational changes of dsDNA caused by various factors.[34,35] The changes in the DNA CD spectra are caused by the interplay of three kinds of the interactions: the hydrogen bonding between the complementary bases (i), the vertical (stacking) interactions (ii), and the electrostatic repulsion of the negatively charged phosphate groups (iii). After treatment of DNA with OH radicals, 8-oxoguanine appears to be a typical oxidative base lesion. Other DNA components can be attacked too. The presence of 8-oxoguanine in the double stranded helix causes some disorder compared to the undamaged double helix and disturbs the circular hydrogen bonding.[36] Also, the extent of hydration may be increased around the polar oxygen atom at C8. Interestingly, the increased concentration of radicals (increased concentration of H_2O_2) does not cause a continuous change in the CD spectrum. The obtained changes in ellipticity in function of wavelength are shown in Figure 7. All CD spectra were corrected by subtracting the signals obtained in the Fenton solution without DNA. For small H_2O_2 concentrations (<1 μM) the main positive and negative bands are only slightly affected; the plot for undamaged dsDNA (B-DNA) is

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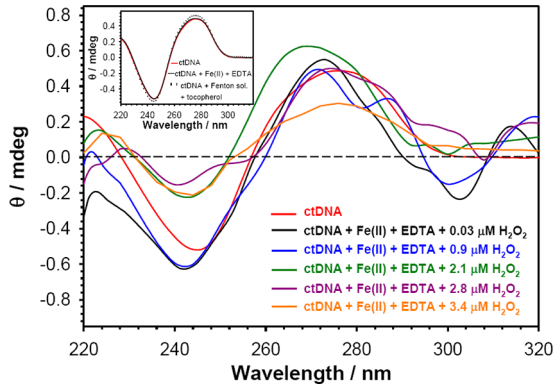

Figure 7. CD spectra of ctDNA present in Fenton solutions with different concentrations of H_2O_2. All spectra were corrected by subtracting the appropriate signals of Fenton solution without DNA. Deactivation of OH radicals by tocopherol is shown in inset. Experimental conditions same as in Figure 3.

presented in Figure 7. However, the negative band intensity was increased and, at ca. 300 nm, a new small negative band appeared. Since such band and the shift of the main positive band toward shorter waves is characteristic for Z-DNA, it may mean that the DNA strands were locally converted into C- and Z-DNA forms.[37] An increase in H_2O_2 concentration to 2.1 μM (see in Figure 7) led to a very different situation. The negative new band disappeared, the magnitude of the main negative band strongly decreased, and the position of the main positive band moved to ca. 270 nm, which may mean that locally the conformation of the strands was turned into A-type. Further increase in H_2O_2 concentration (up to 3.4 μM) led to the restoration of the initial (untreated DNA) positions of both main bands, however, the intensities of both bands dropped severely. This may mean that the chains were simply cut by the radicals.

CONCLUSIONS

The results presented in this paper show that the action of OH radicals present at a very small concentration can be monitored by the EQCM technique. The changes in the frequency of Au–EQCM electrode occurred instantaneously after the addition of hydrogen peroxide at submicromolar level. The fast stabilization of the frequency means that the lifetime of the radicals is very short; it is in the range of several nanoseconds.[2] The precise data cannot be given since the determination of the concentration of the radicals is not possible. The oxidation of guanine and the disintegration of the DNA strands can be measured quantitatively. EQCM supported by SW voltammetry, CD measurements, UV–vis spectroscopy, and AFM micrographs allowed us to quantitatively characterize the action of radicals. Our approach gave a possibility to distinguish between simple oxidation of guanine and local cutting of DNA. We could conclude that at concentrations of H_2O_2 in the Fenton solution lower than 1 μM (weak efficiency of action of radicals) dsDNA is oxidized at nucleic-base centers. After the oxidation the biomolecule undergoes a local transformation from B- to A-, C-, and Z-DNA. Finally, at higher $OH^{\bullet}$ concentrations (>3 μM), a local destruction/disintegration of DNA strands by the radicals takes place. As expected, in the presence of tocopherol, which neutralizes the free radicals by reacting with them, no conformation changes of DNA and a negligible oxidative damage were observed. The characterization of structural changes and damages of DNA caused by free radicals may help in the study of DNA biosensors and, e.g. guanine-rich telomeres or CpG islands in promoter regions of enzymes in cancer cells.

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Notes

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