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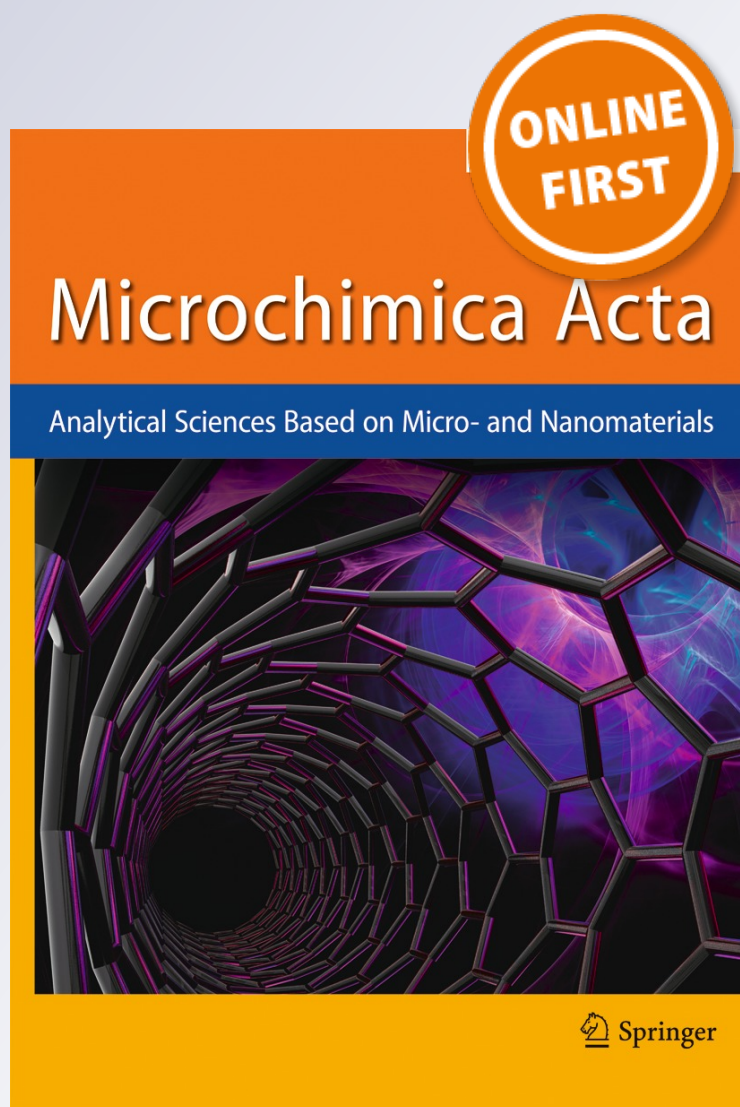
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An electrochemical DNA-based biosensor to study the effects of CdTe quantum dots on UV-induced damage of DNA

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Abstract A DNA-based biosensor is presented that can be applied to the detection of DNA damage caused by UV-C radiation (254 nm) in the presence of CdTe quantum dots (QDs). The sensor is composed of a glassy carbon electrode whose surface was modified with a layer of dsDNA and another layer of CdTe QDs. The response of this sensor is based on (a) the intrinsic anodic signal of the guanine moiety in the DNA that is measured by square-wave voltammetry, and (b) the cyclic voltammetric response of the redox indicator system hexacyanoferrate(III/II). Depending on the size of the QDs, they exert a significant effect on the rate of the degradation of dsDNA by UV-C light, and even by visible light. Time-dependent structural changes of DNA include opening of the double helix (as indicated by an increase in the redox response of the guanine moiety due to easy electron exchange with the electrode when compared to the original helix state and by an increase in the voltammetric peak current of the hexacyanoferrate(III/II) anion after degradation of the negatively charged DNA backbone on the electrode). The effects of QDs were verified for salmon sperm DNA and calf thymus DNA, and further corroborated by experiments in which DNA solutions were irradiated in the presence of QDs.

Introduction

Quantum dots (QDs) represent semiconductor nanocrystals that consist of a metalloid crystalline core, an encapsulating shell, and an organic coating. They exhibit unique optical, electrical properties, and bright and stable fluorescence that can be applied in biomedical imaging and electronic industries [1]. QDs are highly popular in numerous research areas [2, 3]. Cadmium telluride (CdTe) represents the second most utilized solar cell material in the world after silicon. The most important advantage of QDs is that the emission wavelength can be continuously tuned by altering the size of the particle. The band gap in a quantum dot which determines the frequency range of emitted light is inversely related to its size. At the same time, properties such as size, shape, concentration, charge, redox activity, surface coatings and mechanical stability need to be considered for toxicological assessment. The toxicity of nanomaterials including QDs can be broadly classified into chemically induced and photoinduced one [4]. Generally, mechanical damage of biological tissues can be also caused by nanosized materials. Because of their ability of being energy donors, QDs could transfer energy to proximate oxygen molecules, inducing the generation of reactive oxygen species (ROS) [5]. Nucleic acid bases are one of the most affected targets, whose damage can cause changes leading to disorders and diseases development [6–8]. Elevated levels of ROS induce double-strand breakages and

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nucleobase damages in DNA leading to cell damage or death.

The capacity of QDs to induce oxidative damage to DNA has been investigated mostly at semiconductor nanomaterials from II-VI group of the chemical element table. The toxic effect of QDs seems to be a complex challenge where not only coverage (protective shell) of the QD nanoparticle, but also the synthesis process is of importance [9]. Yin showed that the toxic effect of PbSe QDs under UV irradiation is caused by release of Pb^{2+} and generated ROS, inducing DNA damage [10]. Green and Howman reported damage to supercoiled DNA by the CdSe QDs modified with a ZnS shell which inhibited a release of Cd^{2+} ions. This study showed an effect of the incubation under UV irradiation or in dark on damage to DNA, showing importance of both, photoactivation and surface covering at the generation of ROS. They suggest that the damaging mechanism for DNA incubated with quantum dots is not a simple photo-induced free radical process [11]. Ipe et al. investigated a production of free radicals by CdS, CdSe, and CdSe/ZnS QDs stabilized by mercaptoacetic acid (MPA) under the UV radiation. They found that the type and quantity of the free radicals depends on the QDs material, e.g. the CdS QDs generated superoxide and hydroxyl radicals, the CdSe QDs generated hydroxyl radicals while the CdSe/ZnS did not produce free radicals under the irradiation [12]. Liang et al. also analysed DNA damage by CdSe-MPA QDs after the UV exposure using a ruthenium complex light switch as a probe. No significant DNA damage was observed in the dark; however, approximately 70 % of the DNA was damaged under irradiation. They suppose that DNA damage was caused by ROS and not by photo-induced liberation of cadmium ions [13]. With respect to toxicity, simple cadmium based QDs seem to be of rather limited applications. In the case of CdTe, it has different qualities than cadmium and tellurium taken separately and is less toxic than elemental cadmium and its the nephrotoxicity could be minimized, for instance, by silica coating [14]. Nevertheless, QDs of other chemical composition like InP/ZnS [15] and CuInS/ZnS [16] or even metal-free QDs become to be of interest [17].

There is no doubt that investigation of factors leading to nucleic acids damage can be a key how to diagnose a disease or to evaluate the effectiveness of the treatment. Therefore, the study of toxic features of new advanced materials like QDs towards DNA damage is of increasing interest. With respect to electroactivity of the DNA bases, different solid electrodes have been used to study oxidation processes of the bases and damage to DNA [18–21]. Electrochemical DNA biosensors with immobilized DNA layers on the electrode surface are known as effective tools at the investigation of DNA interactions and damage [22–27].

We present here the study of the DNA damage induced by UV-C (λ=254 nm) and visible light in the presence of QDs. For the detection of damage, the DNA-based biosensors represented by glassy carbon electrode (GCE) modified with calf thymus (ct) or salmon sperm (ss) dsDNA and an additional layer of the CdTe QDs (green or red—depending on the size) were prepared. The DNA molecules with multiple HOMO/LUMO (Highest Occupied Molecular Orbital/Lowest Unoccupied Molecular Orbital) energy levels and QDs have to be well aligned to provide efficient conduction pathway [28]. We have chosen a large CdTe QDS (red) and a small CdTe QDs (green) because of their good fluorescent properties. We considered that this difference in size allows us to estimate the effect of the QDs size on the damage of DNA caused by UV irradiation and to investigate if a desired energetic alignment of the nanobioelectronic material QDs-DNA was obtained. Cyclic voltammetry (CV) and square wave voltammetry (SWV) were employed to measure signals of the DNA redox indicator $[Fe(CN)_6]^{3-/4-}$ present in solution phase and the anodic guanine moiety response. To verify the results, DNA was irradiated by UV-C in solution in the presence of QDs followed by its immobilization at the GCE and CV and SWV investigation.

Materials and methods

Reagents

Salmon sperm dsDNA with low molecular weight was obtained from Sigma–Aldrich, Germany (www.sigmaaldrich.com). Calf thymus dsDNA was purchased from Calbiochem USA/Canada (www.merckmillipore.com). Their stock solutions (0.1 mg mL^{-1}) were prepared in 0.1 mol L^{-1} phosphate buffer (PB) of pH 7.4 and stored at 4 °C. CdTe QDs of two different sizes (app. 3 nm—green emitting and app. 6 nm—red emitting) stabilized by mercaptosuccinicacid (MSA) were obtained according to the published protocol [29].

TAE (Tris-acetate-EDTA) buffer and ethidium bromide were purchased from AppliChem Germany (www.applichem.com). Agarose, 1 kb DNA ladder and Loading dye were obtained from Cleaver Scientific Ltd (www.cleaverscientific.com). 1 kb DNA ladder was used as the DNA lenght marker. Other chemicals, obtained from Mikrochem, Slovakia (www.mikrochem.com) or Lachema, Brno (www.erbalachema.com/cz), were of analytical reagent grade purity and used as received. During the measurements, nanopure water (18 MΩ cm) was used.

Apparatus

Voltammetric measurements were performed using the potentiostat Autolab M101 and the software Nova version 1.10.3 (MetrohmAutolab, Netherlands, www.metrohm-autolab.com). A three-electrode system consisting of working glassy carbon electrode (GCE) with a diameter

of 3 mm active area, silver/silver chloride reference electrode and Pt wire as counter electrode. All measurements were performed at ambient temperature in 3 and 10 mL glass voltammetric cell. UV lamp (Gajdoš, Slovakia) with working wavelength 254 nm was used for the UV radiation.

The average size of the nanoparticles and the size distribution were determined by quasielastic laser light scattering with a Malvern Zetasizer (NANO-ZS, Malvern Instruments Ltd., Worcestershire, U.K, www.malvern.com). 1.5 milliliter of water solution of nanoparticle (1 mg mL^{-1}) was put into a polystyrene latex cell and measured at a detector angle of 173°, a wavelength of 633 nm, a refractive index of 0.30, a real refractive index of 1.59, and a temperature of 25 °C. Spectrometric measurements were performed using a multifunctional microplate reader Tecan Infinite 200 PRO (Tecan group Ltd., Männedorf, Switzerland, www.tecan. com). The absorbance scans were recorded in the range of 200–800 nm each 5 nm. Emission wavelengths were measured at different excitation wavelengths with 2 μL of the sample placed on a NanoQuant plate, 16 well, with a quartz optical lens. The concentration of samples was 2 mmol L^{-1} and the same gain (70) was set for measurements.

Preparation of biosensor

The surface of the working GCE electrode was first mechanically polished with 0.3 μm alumina slurry and then rinsed with ethanol and nanopure water. Subsequently, the electrode was cleaned by performing cyclic voltammetric scans within the potential range of −1.0 V to +1.0 V in 1 mmol L^{-1} [Fe(CN)$_6$]$^{3-/4-}$ solution in PB pH 7.4 till reversible behaviour of the redox couple was achieved. Before the immobilization of DNA, the working electrode was treated at the potential +1.6 V vs. Ag/AgCl for 120 s in PB pH 7.4. Salmon sperm (ss) DNA was adsorbed under potential stimulation from 0.1 mg mL^{-1} solution in PB pH 7.4 by applying potential of +0.5 V for 300 s. In the case of calf thymus (ct) DNA, the immobilization was performed by dropping 10 μL of 0.1 mg mL^{-1} DNA solution and drying for 3 h in an oven at 35 °C. The newly prepared DNA/GCE biosensor was washed by immersion in PB pH 7.4 for 2 min under stirring at open circuit condition. Stability of the adsorbed DNA layer was confirmed by repeated CV scans in 1 mmol L^{-1} [Fe(CN)$_6$]$^{3-/4-}$ solution.

Procedures

Damage to DNA by UV-C

The layer of DNA/GCE biosensor was exposed to radiation for various times from 15 cm light source distance. After the exposure, the biosensor was rinsed with nanopure water. Subsequent measurements were carried out under conditions as follows: (i) CV was carried out in 1.10^{-3} mol L^{-1} [Fe(CN)$_6$]$^{3-/4-}$ in PB of pH 7.4 from −250 to 600 mV using the scan rate 100 mV s^{-1} and the step potential 2 mV; (ii) SWV was performed in PB pH 7.4 using the pulse amplitude 40 mV, the frequency 100 Hz, the scan rate 1500 mV s^{-1} and the step potential 5 mV. The guanine moiety SWV peak current was evaluated against the baseline and corrected to a blank.

Damage to DNA by UV-C in the presence of QDs

Fifteen microliter of QDs solution (stock solution diluted 1:10 with PB pH 7.4) were dropped onto the surface of the DNA/GCE biosensor and immediately exposed to UV-C (or under exposure to visible light) for a given time. Then, the biosensor surface was rinsed with nanopure water. Subsequent measurements were carried out under the conditions as mentioned above.

Damage to DNA by UV-C in the presence of QDs in solution phase

Three millilitre of 0.1 mg mL^{-1} ss dsDNA solution in PB without and with addition of red- or green- emitting CdTe QDs in a ratio of 1:10 was exposed to UV-C radiation for 60 and 300 s. Subsequently, DNA was adsorbed under potential stimulation onto the polished surface of the GCE electrode by applying +0.5 V for 300 s. Further measurements were carried out under conditions as in the previous cases.

Gel electrophoresis

Salmon sperm dsDNA was treated and irradiated as listed before. Positive control DNA damage experiment was done at 40 min of UV-C irradiation. At negative control experiment, DNA was exposed to visible light or dark for 40 min. Consequently, the DNA samples were subjected to 2 % agarose gel electrophoresis at 40 V for 30 min and visualized with ethidium bromide with the BioSpectrum 415 Imaging system.

Data treatment

At the CV measurements, portion of survived DNA after the UV-C radiation was expressed as normalized biosensor response using the equation:

$$\Delta I_{rel} = \frac{I_{surv\ DNA} - I_{GCE}}{I_{DNA} - I_{GCE}} \times 100\% \tag{1}$$

where $I_{surv\ DNA}$ and I_{DNA} are the anodic current values for the 1.10^{-3} mol L^{-1} [Fe(CN)$_6$]$^{3-/4-}$ redox system obtained at the biosensor after and before the irradiation, respectively, and measured at the potential of the [Fe(CN)$_6$]$^{3-/4-}$ anodic peak

current at bare GCE (0.250 V), and I_{GCE} is the $[Fe(CN)_6]^{3-/4-}$ anodic peak current at the bare GCE.

At the SVW measurements, the damage to DNA is expressed as normalized anodic current of guanine moiety at 0.980 V:

$$\Delta I_{g(rel)} = \frac{I_g}{I_{g0}} \times 100\% \qquad (2)$$

where I_g and I_{g0} are intrinsic guanine moiety responses at the biosensor after and before the damage to DNA, respectively.

The relative standard deviations (RSD) of the data were obtained for three repeated DNA damage experiments (the error bars were constructed for the significance level $\alpha = 0.05$). The RSD was about 2 % for the I value at bare GCE, about 2.3 % for I and 1.5 % for $I_{g,0}$ values at the ss dsDNA/GCE, and about 4.2 % for I and 1.9 % for $I_{g,0}$ values at ct dsDNA/GCE, respectively. This was the reason why the normalized values of the biosensor responses were used.

Results and discussion

Characterization of QDs

CdTe QDs were prepared from stock solution of cadmium acetate, mercaptosuccinic acid and sodium tellurite by microwave heating [21]. The process was optimized to obtain different QDs. The green QDs were formed using temperature of 60 °C whereas 120 °C was applied for the preparation of red QDs. Colour of the QDs was checked under UV transilluminator. Further, the absorbance and the emission spectra of CdTe QDs were measured (Fig. 1a). The highest fluorescence intensity was found for the red CdTe QDs (28,000 AU) at the emission wavelength of 640 nm. The green CdTe QDs show the maximum fluorescence at 530 nm with the intensity of 13,000 AU.

The size of the QDs was measured using zeta sizer. Dynamic Light Scattering (DLS) was used to determine the sizes of CdTe QDs. The results are shown in Fig. 1b and c. The size of green CdTe QDs was found to be 3 ± 2 nm and that of red ones 6 ± 2 nm.

Effect of QDs present on the surface of the dsDNA/GCE biosensor

First, CV of the negatively charged redox indicator $[Fe(CN)_6]^{3-/4-}$ present in the solution and SWV of guanine moiety were employed for the detection of degradation of both, the salmon sperm dsDNA and calf thymus dsDNA layers, caused by UV-C radiation in the absence of QDs. The $[Fe(CN)_6]^{3-/4-}$ indicator works on a principle of repulsion with the negatively charged DNA backbone which leads to a bad developed CV curve recorded at the DNA/GCE biosensor. The gradual deep degradation of the DNA layer leads to an enhancement of the reversibility of the electrochemical reaction of the indicator; the CV curve becomes better developed and reaches gradually the shape obtained at the bare GCE. Hence, the DNA

Fig. 1 Absorbance and the emission spectra of CdTe QDs (**a**), determination of QDs size using zeta sizer (**b**, **c**)
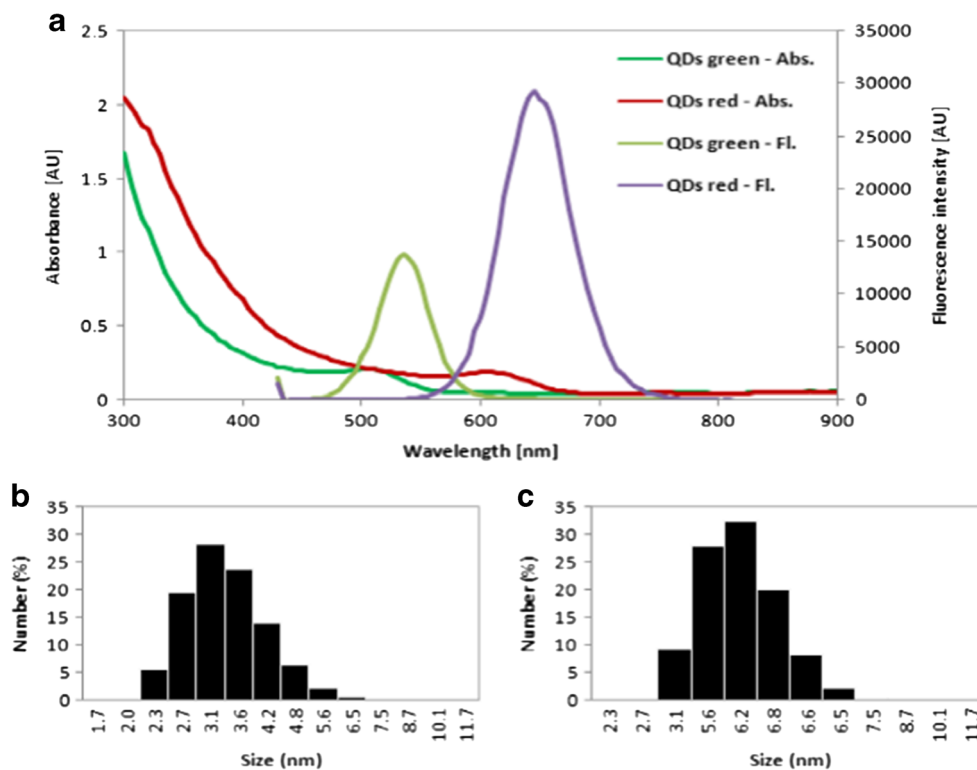
degradation can be evaluated by measuring an increase in the voltammetric current as well as decrease in the anodic to cathodic peak potential separation [25]. The stability of the quasireversibility/reversibility of the indicator signal was checked by repeated CV records at each individual time of DNA damage.

The guanine moiety is known to be electrochemically active possessing an intrinsic response at the anodic polarization of the electrode which can be measured by sensitive technique like SWV. This response becomes higher at the opening of the DNA helix structure when the electron exchange between the guanine moiety and the electrode is easy and latter gradually decreases with the deep DNA degradation and loss the base from the electrode surface [26].

Figure 2a, b represents voltammograms recorded after treatment of the salmon sperm dsDNA/GCE biosensor without QDs by the UV-C radiation for various time. It is seen that the degradation of the DNA layer becomes deeper with increasing time of exposure to UV-C and for 300 s irradiation reaches about (30.2± 3.0) % for CV and (57.1±3.9) % for SWV normalized signals due to a release of DNA fragments from the electrode surface [23]. The second SWV peak at about 1.27 V which belongs to the anodic oxidation of adenine moiety was not evaluated due to more complex behaviour. The structure of calf thymus dsDNA was found to be more stable under these conditions than that of salmon sperm DNA (Table 1).

In Fig. 3, there is a comparison of the CV and SWV normalized data obtained at DNA/GCE biosensor with the salmon sperm and calf thymus DNA. The behavior of simple biosensor without QDs and the biosensors covered with the layer of red-emitting CdTe QDs or green-emitting CdTe QDs after 60 and 300 s application of UV-C radiation to the biosensor surface is depicted. In difference to simple DNA layer exhibiting damage by opening the helix structure (an increase of the SWV guanine moiety anodic signal above 100 %), the presence of

Table 1 Portion of survived salmon sperm (ss) DNA and calf thymus (ct) DNA (expressed by the normalized CV and SWV responses) after the treatment of the dsDNA/GCE biosensor by UV-C ($\lambda = 254$ nm) for given time

Irradiation time, s	ss DNA		ct DNA	
	ΔI_{rel}, %	$\Delta I_{p,g(rel)}$, %	ΔI_{rel}, %	$\Delta I_{p,g(rel)}$, %
0	100.0±0.5	100.0±0.3	100.0±0.4	100.0±0.5
20	86.7±3.3	104.5±1.4	–	–
40	65.5±3.9	135.0±3.1	–	–
60	63.4±2.8	120.7±4.1	83.7±1.4	103.3±1.4
120	56.1±0.9	110.9±0.2	–	–
180	50.2±2.8	104.5±0.4	–	–
240	33.5±3.9	81.2±1.1	–	–
300	30.2±3.0	57.1±3.9	69.5±1.0	72.7±0.7

both red-emitting QDs and green-emitting QDs leads immediately to strand breaks and releasing the DNA fragments from the electrode surface. The determining factors of quantum dots toxicity are the size, charge and concentration [30]. Our observation is in agreement with results of the similar investigation reported previously [31]. Red-emitting QDs were more effective than green ones up to 21 %. Again, the layer of calf thymus dsDNA was less damaged when compared to the layer of salmon sperm dsDNA.

An exposure of the biosensors with QDs under visible light was also tested. While the CV and SWV signals of simple DNA/GCE exposed to visible light are unchanged, an evident DNA degradation is observed at the biosensors with the QDs layers, but in significantly lower degree than that at the UV-C irradiation. The CV data indicate leaching of the DNA fragments from the electrode and the SWV data show relatively small change in the guanine response which is larger for red-

Fig. 2 CV (**a**) and baseline-corrected SWV (**b**) curves recorded before and after exposure of the DNA/GCE biosensor with salmon sperm dsDNA to UV-C radiation ($\lambda = 254$ nm) for given time
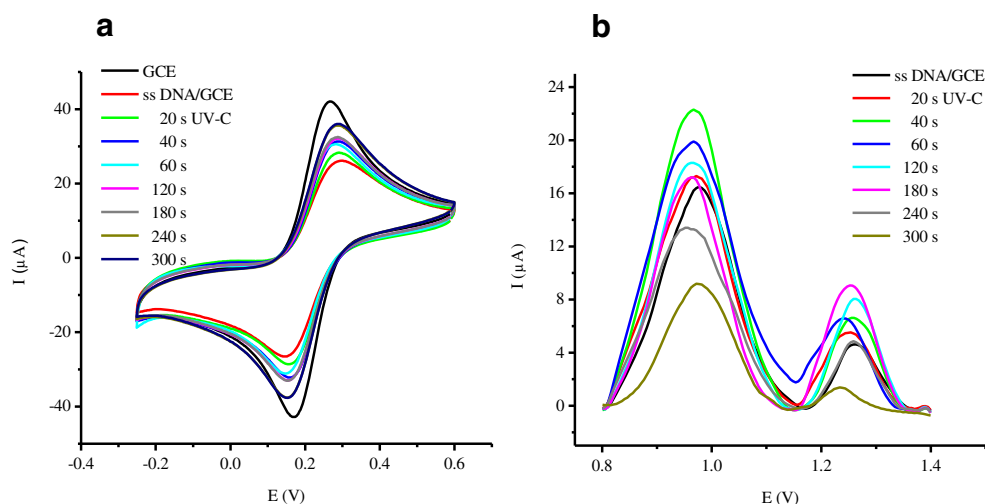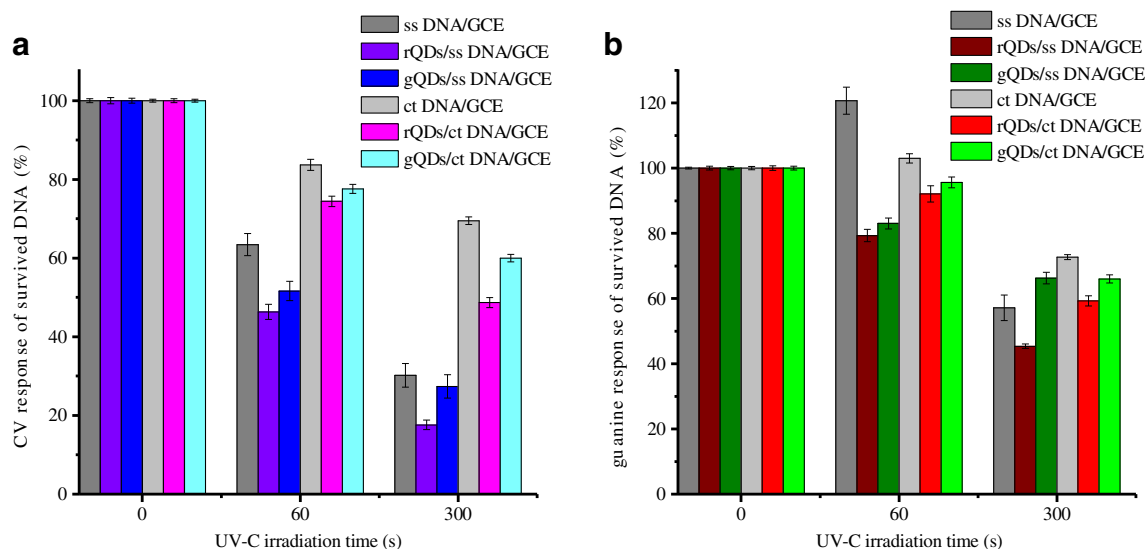
Fig. 3 Comparison of the normalized CV (**a**) and SWV (**b**) responses of the biosensor with the salmon sperm (ss) and calf thymus (ct) DNA as well as red or green CdTe QDs after the UV-C irradiation (λ = 254 nm) for given time

emitting QDs than for green-emitting QDs. These data are summarized in Tables 2 and 3. In difference to this observation, no DNA degradation was found by voltammetric measurements performed after keeping the QDs/DNA/GCE biosensors for corresponding time in dark. Our experiments have been performed under aerobic conditions. In literature, reports can be found, for instance, that oxygen is required to mediate a photodamage by UV radiation [32, 33].

Effect of QDs on dsDNA in solution phase

To verify the effect of both types of QDs, the UV-C irradiation of salmon sperm dsDNA in solution in the presence of QDs was also carried out followed by the potential stimulated adsorption of degraded DNA from solution onto the bare GCE surface and the voltammetric measurements. A portion of survived DNA was evaluated as at the experiments with irradiation of the DNA biosensors described above.

Figure 4 shows a comparison of the data obtained at the irradiation of simple dsDNA and dsDNA in the mixture with

QDs. The DNA degradation reached lower degree than that at the DNA/GCE biosensor evidently due to other concentrations of DNA and QDs in solution and other amount of the DNA fragments immobilized at the bare GCE after the irradiation. Again, deeper DNA degradation was found in the presence of red-emitting QDs than the green ones. By these experiments, the same mechanism of the QDs effect on dsDNA attached to the electrode surface and present in the solution phase was confirmed.

Gel electrophoresis

Agarose gel electrophoresis was used as an independent analytical method for the confirmation of DNA damage in the presence of semiconductor nanocrystallites. Typical electropherogram obtained is depicted in Fig. 5. The electrophoretic experiments show that both, red- and green-emitting colloidal QDs enhance the salmon sperm dsDNA fragmentation under the UV-C irradiation after 40 min of exposure (lane B5 and C5). In contrast, at the absence of QDs, dsDNA degrades to

Table 2 Portion of survived DNA (expressed by the normalized CV and SWV responses) after the treatment of the dsDNA/GCE biosensor with layers of the salmon sperm (ss) DNA and red or green CdTe QDs by daily light for given time

Irradiation time, s	ss DNA and rQDs		ss DNA and gQDs	
	ΔI_{rel}, %	$\Delta I_{p,g(rel)}$, %	ΔI_{rel}, %	$\Delta I_{p,g(rel)}$, %
0	100.0±0.5	100.0±0.5	100.0±0.5	100.0±0.6
60	54.0±2.5	94.1±1.9	70.8±4.0	95.6±0.5
300	56.7±1.5	95.7±1.6	80.3±4.2	95.6±1.7

Table 3 Portion of survived DNA (expressed by the normalized CV and SWV responses) after the treatment of the dsDNA/GCE biosensor with layers of the calf thymus (ct) DNA and red or green CdTe QDs by daily light for given time

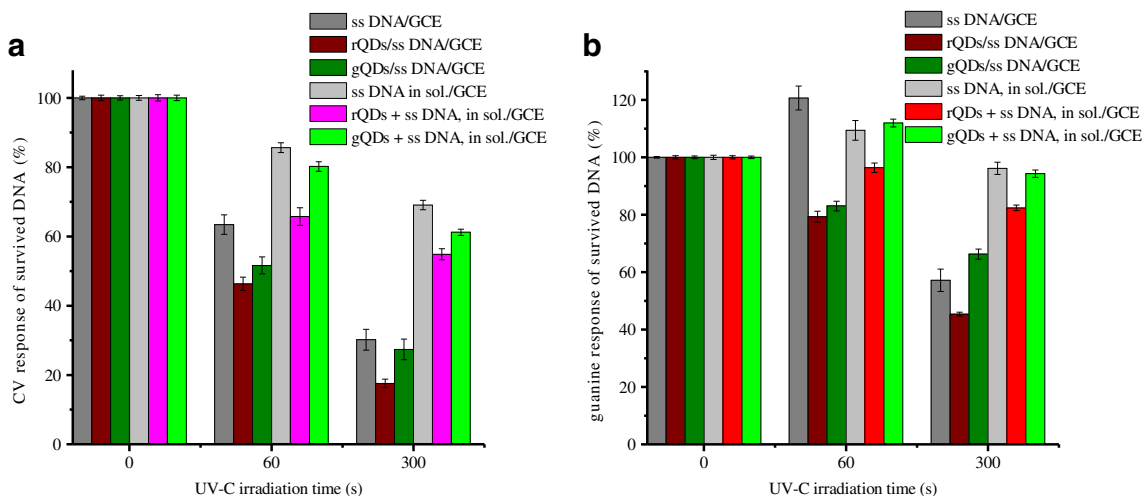| Irradiation time, s	ct DNA and rQDs		ct DNA and gQDs	
	ΔI_{rel}, %	$\Delta I_{p,g(rel)}$, %	ΔI_{rel}, %	$\Delta I_{p,g(rel)}$, %
0	100.0±0.7	100.0±0.6	100.0±0.5	100.0±0.4
60	82.4±2.3	96.8±1.3	79.8±2.2	98.2±1.9
300	84.6±1.7	96.5±2.1	77.9±2.7	97.6±0.8

Fig. 4 Comparison of the normalized CV (**a**) and SWV (**b**) responses of the biosensor with the salmon sperm DNA (ss DNA/GCE) and the GCE electrode modified by salmon sperm DNA survived after the UV-C irradiation (λ = 254 nm) of its solution both after the irradiation in the presence of red or green CdTe QDs

larger fragments which are visible as a smear in the bottom part of the lane A5. There was no degradation of salmon sperm DNA under the exposure to dark (A1) and visible light (A2) even in the presence of red-emitting QDs (B1—dark, B2—visible light) and green-emitting QDs (C1—dark, C2—visible light). Exposure of DNA to only UV-C for 60 and 300 s revealed no detectable breakage of DNA (A3 and A4). Also, the presence of red-emitting QDs (B3 and B4) and green-emitting QDs (C3 and C4) did not revealed DNA degradation after 60 and 300 s of the UV-C irradiation, resp. These experiments confirmed the degradation of salmon sperm dsDNA under the long term UV-C irradiation in the presence of CdTe QDs. However, sensitivity of the electrophoretic method under given conditions is lower than that of the DNA biosensor.

Conclusion

Using dsDNA preparative from two animal species (salmon sperm and calf thymus), two independent electrochemical methods of investigation (CV and SWV) and two arrangements of experiment (DNA at the biosensor and solution phase), a stimulation effect of CdTe quantum dots on the dsDNA degradation by UV-C radiation has been proved. Larger (red–emitting) QDs were more effective than the smaller (green-emitting) ones. Coupling the DNA molecule with different size QDs, an energetic bridge was realised that provides efficient ways for transporting different energy electrons and holes. Changing the size of nanoparticles it was possible to alter the band gap of nano-scaled semiconductor. Probably, the valence band (VB) of green-emitting QDs is lower in energy compared with the HOMO level of the DNA molecule leading to a barrier for energetic carriers. On the other hand, red-emitting QDs show a better alignment and an efficient transport in the nanobioelectronic material (QDs-DNA) and an energetic overlap of the CdTe QDs with HOMO/LUMO levels of DNA (nitrogenous bases). This leads to a conclusion that the degradation of dsDNA structure, i.e. toxic effect of the nanoparticles, is evidently size dependent.

Generally, the approach described here may represent a simple and inexpensive scheme for the detection and

Fig. 5 Agarose gel electropherogram of salmon sperm dsDNA nontreated (**a**) and treated with red QDs (**b**) or green QDs (**c**) under exposure conditions as follows: dark (1), visible light (2), UV-C for 60 s (3), UV-C for 300 s (4) and UV-C for 40 min (5). Line M represents 1 kb DNA Ladder
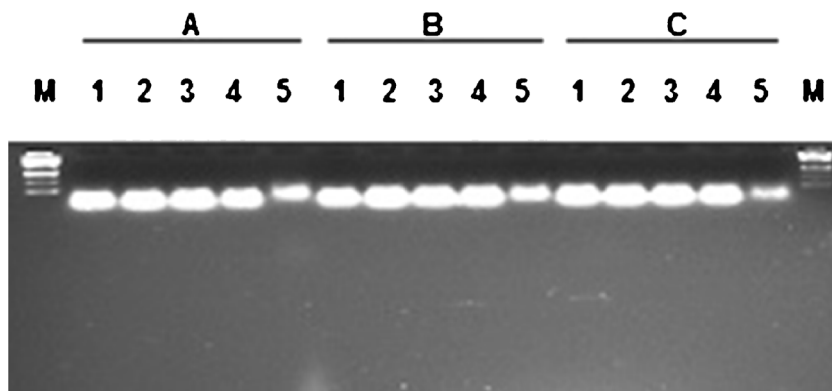
evaluation of damage to DNA by various (physical) agents and various types of nanoparticles. At the same time, the prepared and used DNA-based biosensors represent simple and effective tools for such assay.

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