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First paraben substituted cyclotetraphosphazene compounds and DNA interaction analysis with a new automated biosensor

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ABSTRACT

Cancer, as one of the leading causes of death in the world, is caused by malignant cell division and growth that depends on rapid DNA replication. To develop anti-cancer drugs this feature of cancer could be exploited by utilizing DNA-damaging molecules. To achieve this, the paraben substituted cyclotetraphosphazene compounds have been synthesized for the first time and their effect on DNA (genotoxicity) has been investigated. The conventional genotoxicity testing methods are laborious, take time and are expensive. Biosensor based assays provide an alternative to investigate this drug/compound DNA interactions. Here for the first time, a new, easy and rapid screening method has been used to investigate the DNA damage, which is based on an automated biosensor device that relies on the real-time electrochemical profiling (REP™) technology. Using both the biosensor based screening method and the *in vitro* biological assay, the compounds **9** and **11** (propyl and benzyl substituted cyclotetraphosphazene compounds, respectively), have resulted in higher DNA damage than the others with 65% and 80% activity reduction, respectively.

1. Introduction

The cancer, as one of the leading cause of death in the world, is caused by the persistent cell division that requires DNA replication. To develop anti-cancer drugs this feature of cancer could be used and DNA damaging molecules can be utilized that may kill rapidly dividing cells. Although a number of compounds exhibit anti-cancer properties and are currently used for treatment, the search for new drugs with improved efficiency and minimum side effects still continues. One of the studied compounds for anti-cancer activity are cyclophosphazenes (Akbas et al., 2013; Bovin et al., 1978; Ciftci et al., 2013; Siwy et al., 2006; Yildirim et al., 2012; Elmas et al., 2014) that are an important family of inorganic heterocyclic rings with a large variation in ring size. The cyclophosphazene core can exhibit different physical and chemical characteristics depending on the side groups replacing the chlorine atoms. One of the cyclophosphazene compounds that have attracted attention as a potential anti-cancer agent is octachlorocyclotetraphosphazene ($N_4P_4Cl_8$ (**1**). This compound has a robust heterocyclic ring with eight pendant chlorine atoms that can be substituted with special groups. To enhance the anti-cancer properties, a variety of compounds may be appended to the octachlorocyclotetraphosphazenes. For this purpose, in this study parabens have been considered as a side group to enhance the anti-cancer properties of the octachlorocyclotetraphosphazenes. Parabens are considered ideal preservatives because they have a wide spectrum of anti-microbial activity, and are highly stable in regard to variation in pH, relatively safe to use and their production costs are low (Bledzka et al., 2014; Soni et al., 2005). In addition to anti-microbial activity, Nes and Eklund (1983), found out that parabens also inhibit the DNA and RNA synthesis in *Escherichia coli* and *Bacillus subtilis*. In another study, Park et al. (2012) have studied the effects of butyl paraben on DNA methylation using rat epididymal spermatozoa. These studies suggest that parabens may exhibit DNA damaging properties, and hence can be appended to cyclotetraphosphazenes to afford new compounds

Abbreviations: m.p., melting point; THF, tetrahydrofuran; TLC, thin layer chromatography; DMSO, dimethylsulfoxide; TMS, tetramethylsilane; HRP, horseradish peroxidase; TMB, 3,3′,5,5′-Tetramethylbenzidine; Tris–HCl, ethylenediaminetetraacetic acid; NHS, N-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; NA, NeutrAvidin

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Table 1
Oligonucleotide sequences.

HPLC purified oligonucleotides	SET 1	Molecular weight (g/mol)	SET 2	Molecular weight (g/mol)
Surface probe	5′-Biotin-CAA TAT TTG GCG T GA ATG GGT CGG AAA ACA-3′	9813.1	5′-T GCT GTT TCT AGT GAT GTT AAT TAT CTC CAT TTC-3′-Biotin	14581.4
Target probe	5′- AGA CGT TAA TAC ATT GAA CCT GTT TTC CGA CCC ATT C -3′	11911.6	5′-TAA TTA ACA TCA CTA GAA ACA GCA AGA TGA CAA TAT AAT GTC TAA GT-3′	14576.4
Detecton probe	5′-GGT TCA ATG TAT TAA CGT CTA TGG AAA-Biotin-3′	8780.3	Biotin- 5′-T CAC TAC TTA GAC ATT ATA TTG- 3′	9057.6

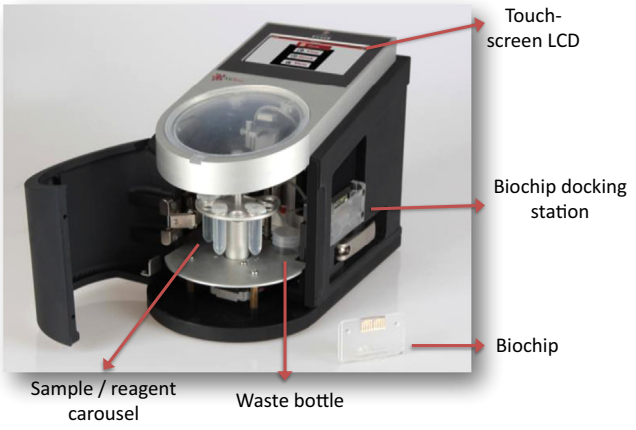

Fig. 1. MiSens biosensor and the biochip.

with potential genotoxic and anti-cancer properties.

To assess the effects of a potentially genotoxic compound to DNA, *in vivo* or *in vitro* tests need to be performed using a variety of techniques. The conventional DNA interaction analysis methods include comet assay and cytogenetic assays using different mammalian cells. During the comet assay, live cells are treated with a compound and later the cells are lysed to release their DNA for analysis by gel electrophoresis. In a study, Fox and colleagues have screened 4000 compounds using a cell-based quantitative high-throughput ATAD5-luciferase assay to detect genotoxic compounds (Fox et al., 2012). Other alternative methods for the recognition of DNA interaction and DNA-binding properties have been developed based on chemical assays (Garas et al., 2009) or surface-enhanced Raman scattering spectroscopy (Masetti et al., 2015). These types of tests are laborious, take time and are expensive. Biosensor based assays provide an alternative to investigate the drug/compound DNA interactions (Chiba et al., 2014; So et al., 2008). For example in a review paper published by Palecek et al. (1998), electrochemical biosensors was described as a tool to investigate the toxicity of compounds by looking into DNA damage and DNA hybridization, and since than many researchers implemented biosensors for genotoxicity tests (Chiba et al., 2014; Simkova et al., 2009; So et al., 2008; Stobiecka et al., 2010).

Electroanalytical biosensors represent highly sensitive tools for the investigation of anti-carcinogen compounds or mutagens and their toxic effects on DNA. For example, Hajkova et al. has investigated the interactions of genotoxic environmental pollutant 2-aminofluoren-9-one with DNA using glassy carbon electrodes (Hajkova et al., 2015). In another study, Kuralay et al. have measured DNA and anti-cancer drug (mitomycin) interactions using polymer coated disposable pencil graphite electrodes (Kuralay et al., 2014). Although electroanalytical techniques/biosensors have been proven to be a useful tool for genotoxicity tests as shown above, lack of automated and fast devices prevented its use as the routine toxicology analysis tools. In a review paper published by Rusling et al. in (2007), it had been stated that if the sensing devices had been inexpensive and better developed, they had potential to be used for toxicity screening for drug development. To answer the need, here the use of a new automated biosensor device (MiSens) has been described, where the assay has been performed using a microfluidic channel integrated biochip with the sequential injections of the assay reagents. MiSens biosensor device relies on the Real-time Electrochemical Profiling (REP™) technology and can be utilized to investigate the genotoxicity by measuring the DNA hybridization efficiency on the biochip surface (Olcer et al., 2015; Uludag et al., 2014a). The DNA hybridization (that is the formation of the DNA duplex by annealing two complementary single strands) is achieved by implementing a surface immobilized capture probe and a hybridized target and detection probes.

In the study, the reactions of cyclotetraphosphazene, ($N_4P_4Cl_8$, **1**) with sodium derivatives of parabens [methyl 4-hydroxybenzoate (**MP**) (**2**), ethyl 4-hydroxybenzoate (**EP**) (**3**), propyl 4-hydroxybenzoate (**PP**) (**4**), butyl 4-hydroxybenzoate (**BP**) (**5**) and benzyl 4-hydroxybenzoate (**BzP**) (**6**) in a THF solution gave full substituted cyclotetraphosphazene compounds. All paraben-substituted cyclotetraphosphazene compounds (7–11) were fully characterized by elemental analysis, FT-IR, MALDI mass spectrometry, ^{1}H and ^{31}P NMR spectroscopies. The molecular structures of compounds (**7** and **8**) were also determined by X-ray crystallography. The crystal structures of these compounds were solved by direct methods and refined by full-matrix least squares. Later, for the first time a new screening method has been used to investigate the DNA damage, which is based on an automated biosensor device that relies on the real-time electrochemical profiling (REP™) technology and the *in vitro* biological assay.

2. Materials and methods

2.1. Materials

Octachlorocyclotetraphosphazene (Otsuka Chemical Co., Ltd) was purified by fractional crystallization from hexane. Sodium hydride, (60% dispersion in mineral oil, Merck; prior to use the oil was removed by washing with dry heptane followed by decantation). Methyl 4-Hydroxybenzoate (99.0%), Ethyl 4-Hydroxybenzoate (99.0%), n-Propyl 4-Hydroxybenzoate (99.0%), n-Butyl 4-Hydroxybenzoate (99.0%) were obtained from Alfa Aesar and Benzyl 4-hydroxybenzoate (99.0%) was obtained from Aldirich. Tetrahydrofuran ($\geq$99.0%), dichloromethane ($\geq$99.0%), n-hexane (95.0%), Ethyl Acetate ($\geq$99.0%), were obtained from Merck. THF was distilled over a sodium–potassium alloy under an atmosphere of dry argon. All reactions were performed under a dry argon atmosphere. Silica gel 60 (230–400 mesh) for column chromatography was obtained from Merck. CDCl3 for NMR spectroscopy are obtained from Goss Scientific. Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) tablets, mercaptoundecanoic acid, ethanolamine, horseradish peroxidase (HRP), 3,3′,5,5′-Tetramethylbenzidine (TMB) ready to use reagent, hydrochloric acid (HCl), Tris–HCl, ethylenediaminetetraacetic acid, N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (Poole, UK). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and NeutrAvidin (NA) was purchased from Thermo Scientific. Au nanoparticles (40 nm) were obtained from BBI

International (Cardiff, UK). The oligonucleotide sequences were obtained from TIB Molbiol (Berlin, Germany) (Table 1), details about the complementarity of the DNA sequences is given at the Supplementary Information.

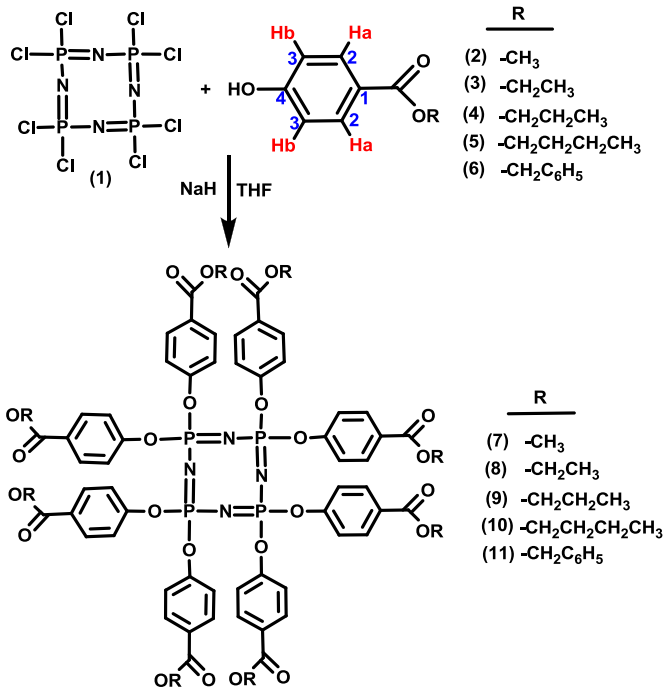

Fig. 2. Synthesis route of paraben substituted cyclotetraphosphazene derivatives.

2.2. Chemical analysis methods

Elemental analyses were obtained using a Thermo Finnigan Flash 1112 Instrument. Positive ion and linear mode MALDI-MS of compounds were obtained in 2,5-dihydroxybenzoic acid as MALDI matrix using nitrogen laser accumulating 50 laser shots using Bruker Microflex LT MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight) mass spectrometer. All reactions were monitored using thin-layer chromatography (TLC) on Merck silica gel plates (Merck, Kieselgel 60, 0.25 mm thickness) with F_{254} indicator. Column chromatography was performed on silica gel (Merck, Kieselgel 60, 230–400 mesh; for 3 g crude mixture, 100 g silica gel was used in a column of 3 cm in diameter and 60 cm in length). All reactions were carried out under an argon atmosphere. Melting points were measured on a Gallenkamp apparatus using a capillary tube. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded in $CDCl_3$ solutions on a Varian INOVA 500 MHz spectrometer using TMS as an internal reference for ^{1}H NMR and 85% H_3PO_4 as an external reference for ^{31}P NMR. FT-IR spectra were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometer. DNA interaction analysis tests were performed using an automated MiSens biosensor device and its sensor chips (BILGEM-TUBITAK, Kocaeli, Turkey).

2.3. X-ray crystallography

Chemical synthesis procedures are described in detail at the Supplementary Information. Intensity data were recorded on a Bruker APEX II QUAZAR diffractometer. Absorption correction by multi-scan has been applied (Bruker, 2005) and space groups were determined using XPREP implemented in APEX2 (Bruker, 2008). Structures were determined using the direct methods procedure in SHELXS-97 and refined by full-matrix least squares on F^2 using SHELXL-97 (Sheldrick, SHELXL-97). All non-hydrogen atoms were refined with anisotropic displacement factors and C–H hydrogen atoms were placed in calculated positions and allowed to ride on the parent atom. The final geometrical calculations and the molecular drawings were carried out with PLATON (Spek, 2003), MERCURY (Macrae et al., 2006), and DIAMOND (Version 3.1) (Brandenburg, 2006) programs. Structure determinations have been deposited with the Cambridge Crystallographic Data

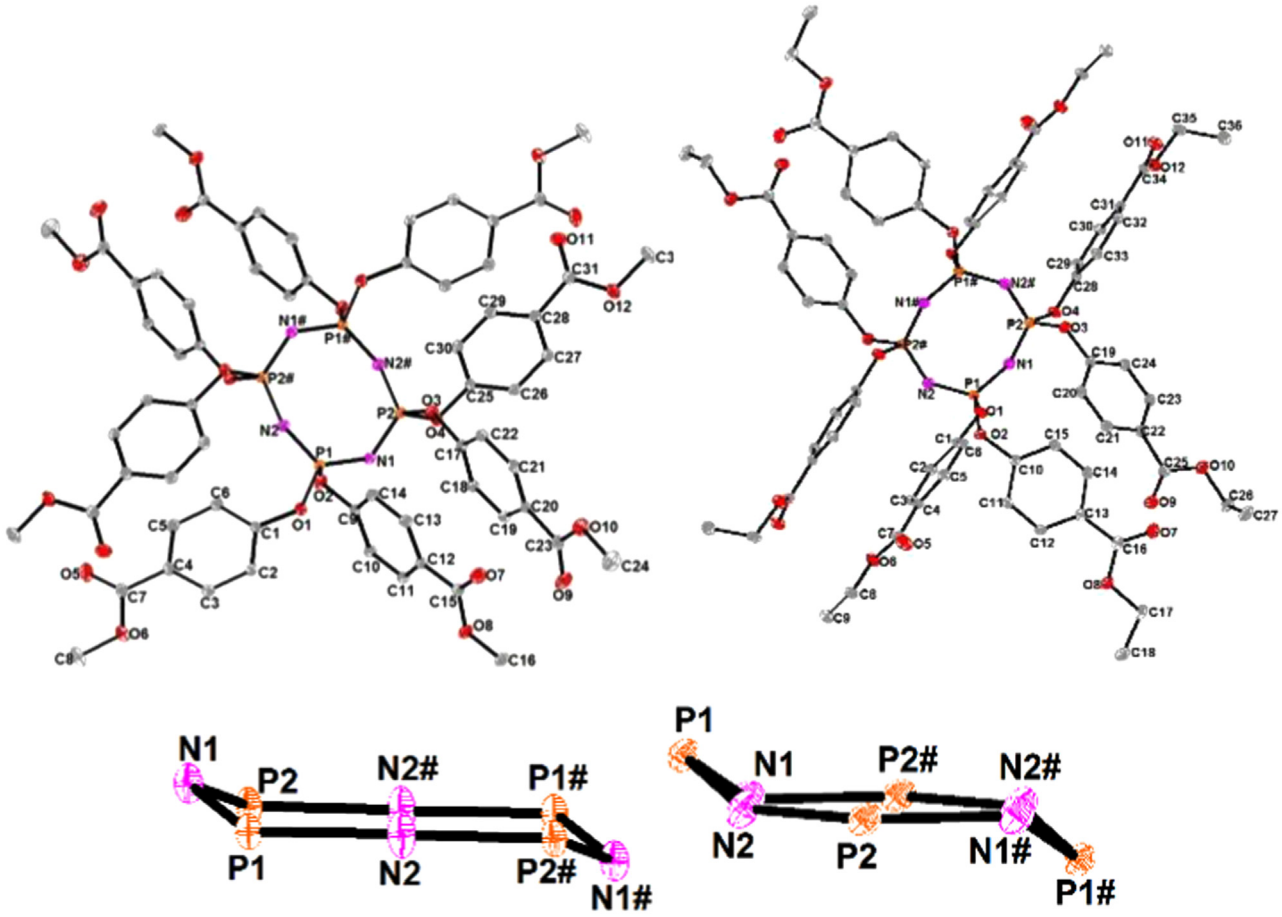

Fig. 3. Crystal structures of compound **7** (left) and **8** (right) with the atom-numbering scheme. Displacement ellipsoids are drawn at the 30% probability level. The hydrogen atoms have been omitted for clarity. For comp. **7** [Symmetry (#): −x, −y+1, −z+1], for comp. **8** [Symmetry (#): −x, −y, −z+1].

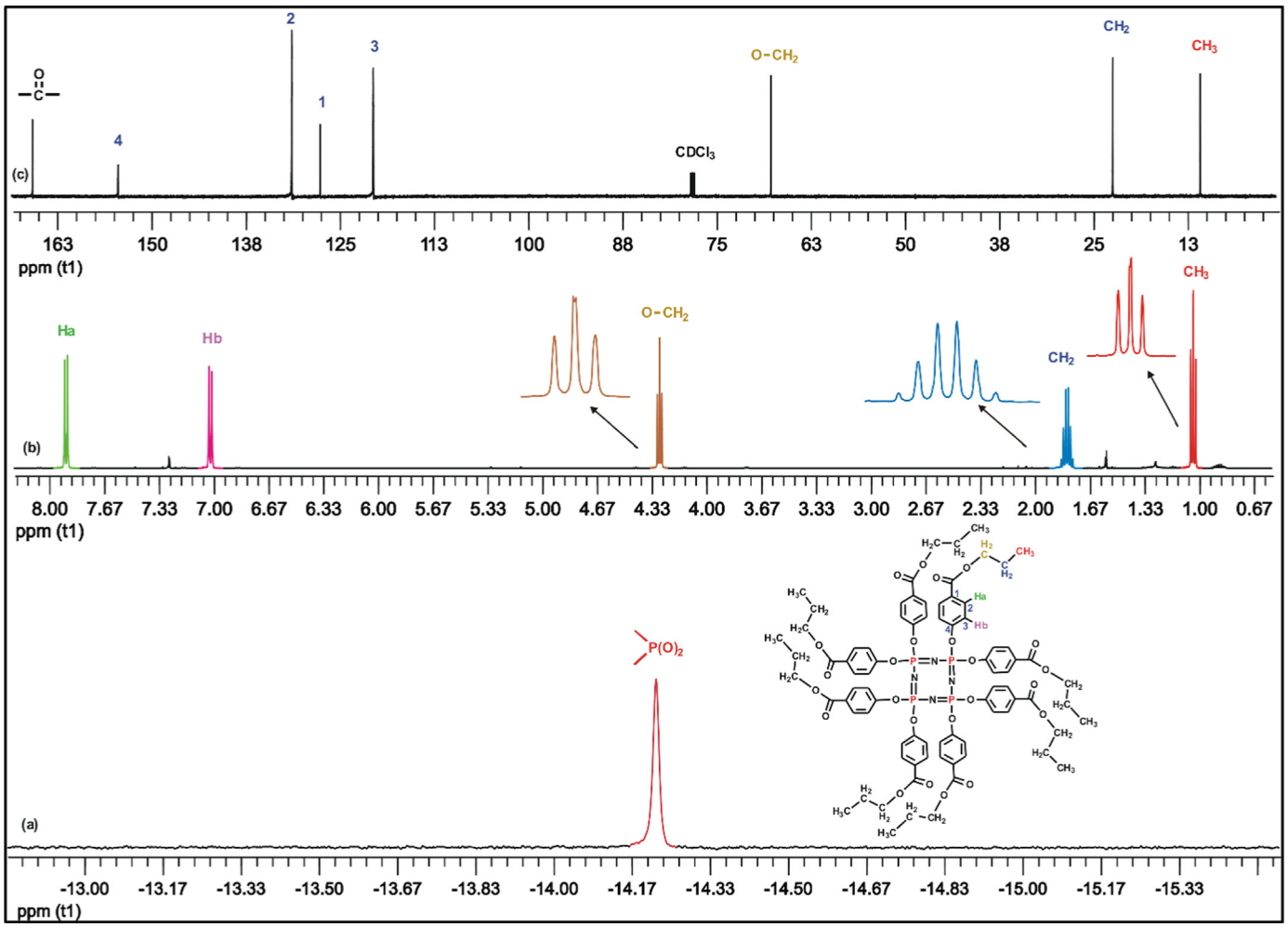

Fig. 4. (a) The proton decoupled ^{31}P NMR spectrum of the compound **9** in $CDCl_3$ solution, (b) the ^{1}H NMR spectrum, (c) the ^{13}C NMR spectrum.

Centre with references CCDC 1040472 and 1040473 for structures **7** and **8**, respectively.

2.4. DNA interaction tests using plasmid DNA and electrophoresis

Interactions between pUC18 plasmid DNA and cyclotetraphosphazene compounds have been examined with agarose gel electrophoresis technique. All the compounds were first dissolved in THF (final concentration 0.1%) and later diluted with TE (Tris-EDTA) buffer. The test solutions were prepared in several doses and immediately mixed with 0.03 μg/μl pUC18 plasmid DNA (Thermo Scientific) and 2 μl loading buffer (0.1% bromophenol blue and 0.1% xylene cyanol) in 10 μl total volume. Samples were incubated in the incubator at 37 °C for 24 h. Later, the compound samples (0.8–0.4 mg/ml) and 0.05 μg/μl marker DNA were loaded onto the 1% agarose gel. Electrophoresis was carried out for 1 h at 100 V with 1 × TBE (Tris-Boric acid-EDTA) buffer. Untreated and 0.1% THF treated plasmid DNA were used as control. After running the gel, it was stained with 0.5 μg/L ethidium bromide. Gel was viewed using imaging system (Vilber Lourmat Fusion FX5).

2.5. DNA interaction tests using MiSens biosensor

DNA interaction analyses have been performed using an automated MiSens biosensor device and its biochips (Fig. 1) developed by the Bioelectronic Devices and System Development Group of BILGEM Research Center (BILGEM-TUBITAK, Kocaeli, Turkey). MiSens biosensor relies on the Real-time Electrochemical Profiling (REP™) technology (Olcer et al., 2014; Uludag, 2014b). A biochip that has two sets of Au electrode arrays, each consist of shared reference/counter electrodes and 3 working electrodes has been fabricated and used for the assays (Uludag et al., 2015). The biochip has been integrated to a microfluidics system and all the steps of the assay have been performed during fluid flow.

The biochip surface has been functionalised with a self assembled monolayer prior to the immobilization of neutravidin (NA) that has been used to capture the biotinylated surface probe. Varying concentrations of the compounds (0, 0.8 and 1.6 mg/mL) have been initially diluted in dimethyl sulfoxide or dichloro methane, and 50 μL of this mixture has been added to the 200 μL of hybridized target (1×10^{-9} M) and detection probe (2×10^{-9} M) solution. This mixture has been incubated for 15 min, prior to the injection on to the capture probe immobilised sensor surface for hybridisation. This was followed with the injection of the neutravidin (NA) and enzyme (horseradish peroxidase) modified Au nanoparticles. The subsequent injection of the enzyme substrate (3,3′,5,5′-Tetramethylbenzidine) and simultaneous amperometric measurements (against −0.1 V potential) during the flow, resulted in a real-time amperometric reading due to the Reaction (1).

$$TMB_{red} + H_2O_2 \xrightarrow{\text{Horseradish peroxidase}} TMB_{ox} + 2\,H_2O \qquad \text{Rxn. 1}$$

3. Results and discussion

3.1. Compound synthesis

In the current study, new paraben substituted compounds (7–11) have been synthesized for the first time (Fig. 2) and their effects on DNA have been investigated first time using an automated biosensor device based screening method.

Full substituted parabens of cyclotetraphosphazene compounds (7–11) were synthesized and characterized by elemental analysis, mass spectrometry, FT-IR, ^{1}H, ^{13}C and ^{31}P NMR spectroscopy (see Experimental section, ESI†). The crystal structures of the compounds **7** and **8** have been established from single crystal X-ray diffraction and their molecular structures are presented in Fig. 3. The crystallographic data are summarized in Tables S1 and S2, ESI†.

Table 2
A list of genotoxicity screening tests that are performed using a variety of techniques.

Technique	Aim of the test	Chemicals	DNA-chemical incubation time	Assay time	References
Real-time Electrochemical Profiling (assay using fully automated device)	Genotoxicity screening of synthesised chemicals	Paraben substituted cyclotetraphosphazene compounds	15 min	25 min	Current study
UV/vis spectrophotometry	Screening compounds for interaction with DNA	Antracycline drug family	1 h	2–3 h	Garas et al. (2009)
Cell-based quantitative high-throughput ATAD5-luciferase assay	Assay identifies antioxidants as inducers of DNA damage response	Antioxidants including resveratrol, genistein, baicalein	16 h	21 h+30 min	Fox et al. (2012)
Surface-enhanced Raman scattering spectroscopy	DNA interactions with exogenous agents	Cisplatin, methyleneblue, cytotoxic metal ion Hg	2 h	2 h+20 min	Masetti et al. (2015)
Quartz crystal microbalance pyrolytic graphite disks	Screening toxic reactive metabolites of arylamines	Arylamines	5 min	40 min	So et al. (2008)
Electrochemical	Estimation of damaged DNA duplexes	Photo-damaged	13 h	270 min	Chiba et al. (2014)
Electrochemical quartz crystal nanobalance	Evaluation of DNA damage by toxic agents	Herbicides pesticides	10 min	65 min	Stobiecka et al. (2010)
Electrochemical – Voltammetry on a glassy carbon electrode	Genotoxicity of environmental pollutants	2-Aminofluoren-9-one	180 s	35 s	Hajkova et al. (2015)
Electrochemical – Chitosan modified disposable pencil graphite electrodes	Search for anticancer drug	Mitomycin	20 min	1 h+180 s	Kuralay et al. (2014)
Electrochemical impedance spectroscopy	Detection of antioxidative effects	Tea extracts, phenolic acids	15 min	~10 min	Simkova et al. (2009)
Impedimetric DNA-biosensor	Genotoxicity analysis	Catecholics	2 h	60 min+360 min	Ensafi et al. (2014)
Arrays based on electrochemiluminescence	New biosensor technology is compared to other emerging methods for toxicity	Review	x	x	Rusling et al. (2007)

The ^{1}H NMR data also confirmed the structures of **7**–**11**. The aromatic and aliphatic protons for all the compounds were observed between 8.07–6.99 and 5.31–1.03 ppm, respectively. In aromatic and aliphatic groups for compound **9**Ha, Hb, OCH_2, CH_2 and CH_3 protons were resonated at 7.89, 7.05, 4.28, 1.80 and 1.03 ppm in which those have three bond-coupling constants, average of *ca.* ^{3}J=8.71, 6.70, 7.73 Hz, respectively (Fig. 4b). The chemical shifts of aromatic and aliphatic protons and coupling constants were shown in experimental sections. The carbons for all the compounds were observed between 165 and 15 ppm and the ^{13}C NMR spectrum of compound **9** was shown as an example in Fig. 4c.

3.2. Genotoxicity tests

To assess the effects of a potentially genotoxic compound to DNA, *in vivo* or *in vitro* tests need to be performed using a variety of techniques (Table 2). Electroanalytical biosensors represent highly sensitive tools for the investigation of anti-carcinogen compounds and their toxic effects on DNA. For example, Kuralay et al. (2014) have measured DNA and anti-cancer drug (mitomycin) interactions using polymer coated disposable pencil graphite electrodes. Palecek et al. (1998) and Ensafi et al. (2014) have assessed the DNA damage caused by various agents by investigating the level of DNA oxidation on electrode surfaces using electrochemical biosensors. Stobiecka and colleagues have utilized a different approach (Stobiecka et al., 2010). Rather than testing the DNA biorecognition properties, they have investigated the sensitivity of the intercalator-probe uptake to the conformational alterations of the DNA molecule.

As could be seen from the studies mentioned above and in Table 2, electrochemical biosensors have been proven to be a useful tool for genotoxicity tests with respect to the conventional expensive and time consuming *in vivo* and *in vitro* tests. However, the used electrochemical biosensor systems mentioned in the literature mostly relies on homemade set ups consist of separate parts such as a potentiostat, a connector and a sensor electrode. These systems results in manual and cumbersome assay procedures that does not allow the test of several compounds in a short period of time. As stated by Rusling et al. (2007), if the sensing devices had been inexpensive and better developed, they had potential to be used for toxicity screening for drug development. To answer the need, here the use of a new automated biosensor device (MiSens) has been described, where the assay has been performed using a microfluidic channel integrated biochip with the sequential injections of the assay reagents. As the MiSens device allows automated and fast electrochemical analysis, it is possible to test a number of compounds easily in a short period of time. And hence, to reduce the number of compounds that undergo the conventional genotoxicity analysis, we propose **a pre-screening procedure** using MiSens where DNA interaction analysis can be performed on the surface of the biosensor chip. MiSens device results in quantitative results, therefore, allows easier and comparable comparison between the compounds that undergo pre-screening. Small molecule/drug and DNA interaction can occur in the forms of intercalation, groove binding or DNA cleavage. The driving force for these interactions could result from hydrophobic, ionic, hydrogen bonding and van der Waals interactions. After the screening of the compounds and selecting the possible anti-cancer molecules out of many using the MiSens assay, comprehensive biological assays can be performed to assess the mechanism of the anti-cancer properties of these selected molecules.

MiSens biosensor device relies on the Real-time Electrochemical Profiling (REP™) technology and can be utilized to investigate the genotoxicity by measuring the DNA hybridization

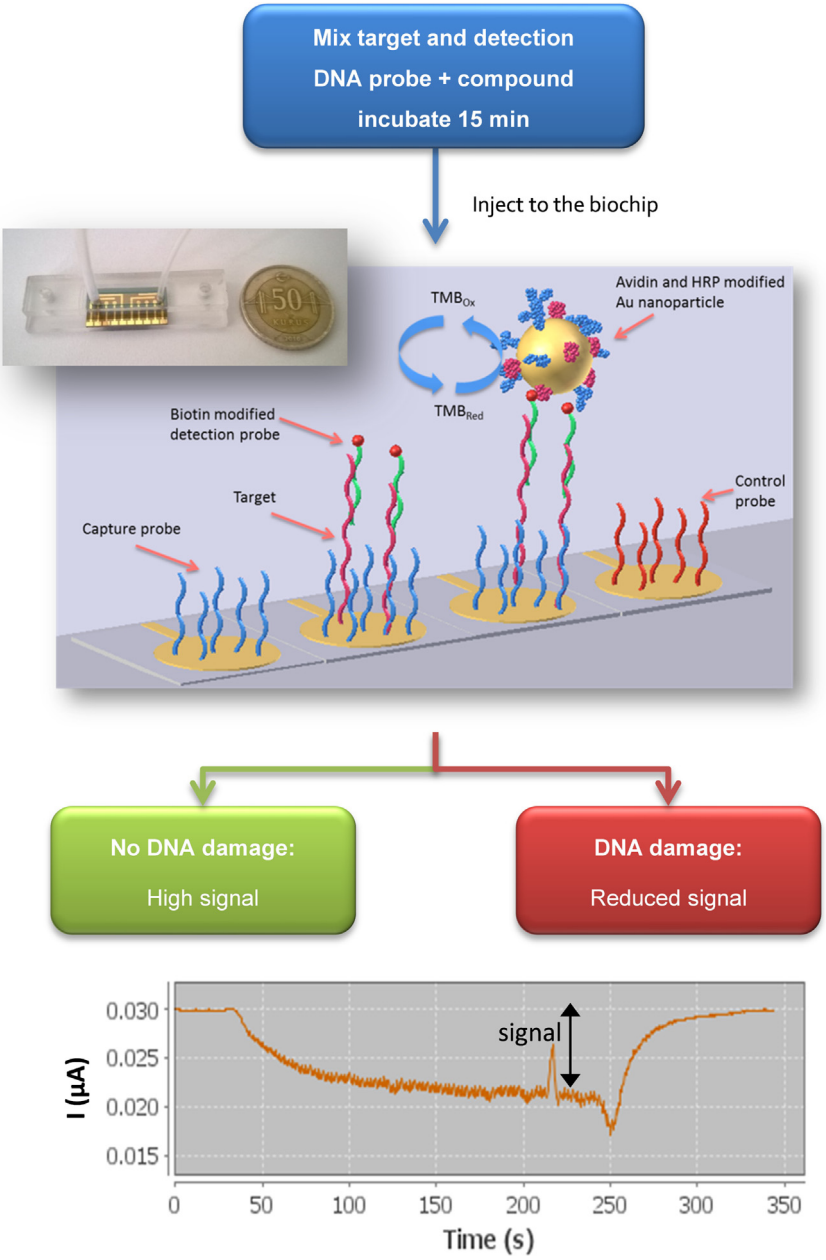

Fig. 5. Schematics of the DNA damage test using MiSens device and the biochip.

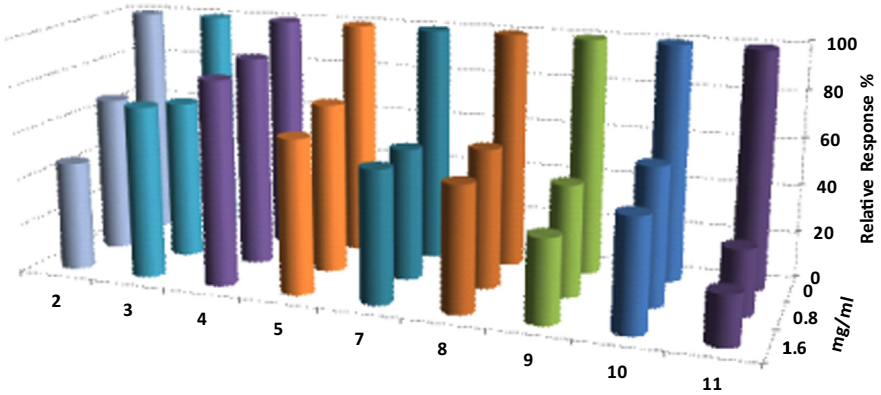

Fig. 6. The amperometric responses obtained from the REP assay have been shown in the figure as percent relative responses. Varying concentrations of the compounds (0, 0.8 and 1.6 mg/mL) have been incubated with target and detection DNA probes prior to the injection on to the biochip.

efficiency on the biochip surface. The DNA hybridization (that is the formation of the DNA duplex by annealing two complementary single strands) is achieved by implementing a surface immobilized capture probe and a hybridized target and detection probes. In the study, the hybridized target and detection probe solution have been incubated in a solution containing varying concentrations of parabens, and later this mixture has been sent to the capture probe immobilised surface to assess the hybridisation of the target to its complementary surface probe (Fig. 5). The compounds **1** and **6** did not dissolve in DMSO, therefore they have not been used for the screening tests. Both the individual reactives (2–5) and the synthesized products (7–11) have been tested and the results are shown Fig. 4 as percent DNA hybridization activity. When the hybridization activity of the reactive **2** has been compared to its synthesized compound **7**, it has been found that the synthesis has not resulted in a further DNA damage with respect to its reactive. However, the synthesized compounds **8** and **10**, resulted in more than 20% activity loss with respect to their reactive **3** and **5**, respectively. Compound **9**, resulted in more than 50% activity loss with respect to its reactive **4**. When all the synthesized compounds have been compared (7–11), it has been found that compounds **9** and **11**, resulted in the highest DNA damage than the others with 65% and 80% hybridization activity reduction, respectively (Fig. 6). These results indicate that compounds **9** and **11** (propyl and benzyl substituted cyclotetraphosphazene compounds, respectively) are potential candidate as anti-cancer agents.

As a further study, the effect of the compounds to the plasmid DNA structure has been analyzed by comparing the existence of the supercoil form I, open circular form II and linear form III plasmid on the electrophoresis gel, after the incubation of the plasmid DNA with the compounds. Supercoiled DNA (form I) is the native plasmid confirmation found *in vivo*. Open circular (form II) or linearized DNA (form III) occurs when the DNA helix strands are cut or damaged either due to an enzymatic reaction or affect of a chemical compound. These 3 different plasmid confirmations can be observed as different lines on the electrophoretograms. In order to assess the effect of the synthesized compounds to the plasmid DNA, in the electrophoretograms, in all the cases one lane was was run with THF (solvent treated DNA as control) while the other lanes contained plasmid DNA interacted with the compounds (0.8–0.4 mg/ml) (Supplementary Information, Figures 1–2). The analysis of the electrophoretogram revealed some reduction in form I, hence an increase in the form II and III for the compound **11** (benzyl substituted cyclotetraphosphazene compound) at the tested highest concentration (0.8 mg/ml) (Supplementary Information, Fig. 2). This result indicates that compound **11** have an effect on the plasmid DNA and complies with the biosensor assay, where the compound **11** (0.8 mg/ml) has caused 72% hybridization activity reduction. The biosensor assay results are quantitative; hence it is much easier to judge and compare the level of DNA damage caused by different compounds.

4. Conclusion

In the current study, for the first time paraben substituted cyclotetraphosphazene compounds have been synthesized and their effect on DNA has been screened using an automated biosensor device. The results of the screening tests showed that compounds **9** and **11** (propyl and benzyl substituted cyclotetraphosphazene compounds, respectively) resulted in higher DNA damage (65% and 80% activity reduction) with respect to the other compounds. Further studies with a conventional assay using plasmid DNA and electrophoresis have showed that compound **11** induce a change in plasmid confirmation hence affecting the DNA. It is believed that the data obtained from the biosensor assays help researchers to choose the most probable drug candidates out of many, as this method directly and quantitatively shows the effect of the compounds to the DNA strand. Hence, this pre-screening method can be applied prior to the conventional *in vitro* and *in vivo* genotoxicity tests. As the findings of this work revealed the anti-cancer properties of specific cyclotetraphosphazene derivatives that may be used for the targeted cancer therapy, an investigation of these materials will continue in our laboratories.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.01.061.

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