

# 1 **Quantitative fluorescent determination of DNA – Ochratoxin A interactions supported** 2 **by nitrogen-vacancy rich nanodiamonds**

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4 **Wioleta Białobrzaska<sup>1</sup>, Maciej J. Głowacki<sup>2</sup>, Monika Janik<sup>2,3</sup>, Mateusz Ficek<sup>2</sup>, Krzysztof Pyrchla<sup>2</sup>,**  
5 **Mirosław Sawczak<sup>4</sup>, Robert Bogdanowicz<sup>2</sup>, Natalia Malinowska<sup>1</sup>, Sabina Żołędowska<sup>1</sup> and Dawid**  
6 **Nidzworski<sup>1</sup>**

7 <sup>1</sup> *Institute of Biotechnology and Molecular Medicine, 3 Trzy Lipy St., 80-172 Gdańsk, Poland*

8 <sup>2</sup> *Department of Metrology and Optoelectronics, Faculty of Electronics, Telecommunications and*  
9 *Informatics, Gdańsk University of Technology, 11/12 Gabriela Narutowicza St., 80-233 Gdańsk, Poland*

10 <sup>3</sup> *Institute of Microelectronics and Optoelectronics, Warsaw University of Technology, 75 Koszykowa*  
11 *St., 00-662 Warsaw, Poland*

12 <sup>4</sup> *The Centre for Plasma and Laser Engineering, The Szewalski Institute of Fluid-Flow Machinery,*  
13 *Polish Academy of Sciences, 14 Fiszerza St., 80-231 Gdańsk, Poland*

14

## 15 **ABSTRACT**

16 Ochratoxin A (OTA) is a hazardous contaminant of a large variety of plant and animal originated  
17 food. Herein, we report an interaction of OTA with calf thymus DNA (ct DNA) on the nanodiamond  
18 surface. We employed multispectroscopic techniques to elucidate the binding mechanism of OTA with  
19 ct DNA. The fluorescence and UV-Vis spectroscopy results show that OTA binds to ds ct DNA and  
20 forms complexes.

21 We obtained the binding constants of OTA and ct DNA using fluorescence quenching and UV-Vis  
22 spectroscopy. The binding constant ( $K_b$ ) for the interaction of OTA with ct DNA was determined using  
23 spectroscopic methods and was determined as  $3.27 \times 10^5 \text{ M}^{-1}$  (UV-Vis) and  $8.12 \times 10^5 \text{ M}^{-1}$   
24 (fluorescence) for nanodiamond in green tea beverage OTA. Performed analyses directly indicate that  
25 OTA can interact with calf thymus DNA in a groove-binding mode as proved by the hyperchromic effect  
26 of the absorption spectra. This study of OTA–ct DNA interaction may provide novel insights into the  
27 toxicological effect of the mycotoxins.

28

29 **KEYWORDS:** fluorescence; nitrogen-vacancy nanodiamonds; ochratoxin A, calf thymus DNA; UV-  
30 Vis spectroscopy;

31

32

## 34 1. Introduction

35 Mycotoxins are naturally occurring toxins produced by certain mold species. The main fungal genera  
36 producing the mycotoxins include *Aspergillus*, *Fusarium*, and *Penicillium*. The most common  
37 mycotoxins are aflatoxins, OTA, fumonisins, deoxynivalenol, T-2 toxin, and zearalenone <sup>1</sup>. The  
38 mycotoxins are present at all stages of the food chain and can be found in products such as meat, fruits,  
39 wine, beer, coffee, tea, milk, nuts, dried fruits, cereals, and many more. Additionally, these compounds  
40 exhibit high chemical stability. Their crystallization state and high resistance to high temperatures or  
41 even gamma radiation <sup>2</sup> make them insensitive to conventional sterilization methods. On the other hand,  
42 chemical products that are able to neutralize their toxic effect are often causing other health problems.

43 Among the mycotoxins listed above, OTA is recognized as one of the most toxic and has been classified  
44 as a group I carcinogen. Although the toxicity of OTA has been studied for many years, its complex  
45 mechanism of action has not been elucidated in detail yet. However, based on *in vitro* and animal studies,  
46 this toxin is 'this time was as carcinogenic, teratogenic, hepatotoxic, neurotoxic, and immunotoxic.

47 OTA is also known to generate covalent DNA adducts and to promote oxidative DNA damage based on  
48 the production of reactive oxygen species <sup>3</sup>. DNA is of key importance in the molecular processes of all  
49 living organisms. Therefore, exploring the OTA–DNA interaction mechanisms is of great importance—  
50 first, to understand the exact DNA damage mechanism, and second, to develop the therapeutic drugs in  
51 the future. Throughout the years, DNA has been reported as a target molecule of various drugs and toxic  
52 compounds, which can lead to a change in DNA structures and genetic mutations. Molecules can interact  
53 with DNA in three different ways. First, through the intercalation between the DNA base pairs, which is  
54 the most effective and thus destructive one; second, through the groove binding; and the last one, through  
55 the electrostatic interactions.

56 To study the interaction mechanisms of small molecules with DNA, several techniques have been  
57 utilized and reported, i.a. fluorescence spectroscopy <sup>4</sup>, UV-spectrophotometry <sup>5</sup>, circular dichroism <sup>6</sup>,  
58 mass spectrometry <sup>7</sup>, molecular docking <sup>5</sup>, and electrochemical methods <sup>6</sup>. Moreover, techniques like  
59 competitive displacement, iodide quenching effect, viscosity measurements, and DNA melting assays  
60 have been used for further identification of the binding mode.

61 According to the literature, the mycotoxins have already been grafted on a few different surfaces. <sup>8</sup>. In  
62 our experiment, we used a nanodiamond surface with COOH functional groups, previously studied by  
63 Tian *et al.* <sup>9</sup>. The surface coverage, according to *ab-initio* calculations for COOH presented in <sup>9</sup>, is  
64 approximately 6%. The carboxylated nanodiamonds (NDs) are efficient fluorescent agents enabling  
65 grafting various surface species <sup>10</sup> or interacting with biomatter <sup>11</sup>. The fields of physics and biology  
66 have discovered nanodiamond as an ideal material for at least two very different scientific applications.  
67 Biologists, on the one hand, have found that diamond nanoparticles show promise as exceptionally  
68 robust fluorescent dyes, with many potential applications in biolabeling <sup>12,13</sup>. On the other hand,

69 physicists have made tremendous progress in understanding and controlling the electronic properties of  
70 vacancy<sup>12,14</sup>. Before introducing the nitrogen-vacancy (NV) center in nanodiamond and its use for  
71 nanoscale sensing, we note that several excellent reviews have appeared highlighting different aspects  
72 of diamond nanoparticles and NV centers. Jelezko & Wrachtrup<sup>15</sup> have given an early overview of NV  
73 centers in diamond. More detailed descriptions of photoelectric detection and quantum readout of the  
74 NV center have been given by Bourgeois et al.<sup>16</sup>. Chi-Cheng Fu et al.<sup>17</sup> have provided an overview of  
75 the nanodiamonds as fluorescent cellular biomarkers.

76 NV centers in the nanodiamonds are atomic-level defects which display a remarkable range of  
77 properties, including sustained fluorescence, long quantum coherence times under ambient conditions,  
78 and demonstrated biocompatibility<sup>18-20</sup>. The nanodiamonds containing negatively charged NV centers  
79 have proven to be a promising tool in bio labeling, biomarking<sup>21</sup>. Recently, an important element of  
80 nanodiamonds has been surface functionalization<sup>22</sup>.

81 In this work, we have explored interactions between calf thymus DNA (ct DNA) and OTA grafted at the  
82 fluorescent nanodiamond's surface. To date, fluorescent nanodiamonds have been used only as a sorbent  
83 / carrier of the mycotoxins to facilitate their removal from an organism and to neutralize their toxic effect  
84<sup>23,24</sup>. Thanks to NDs tailored chemistry, OTA has been immobilized stably on the NDs' surface during  
85 experiment conduction. The OTA immobilization density and stability were also revealed by *ab-initio*  
86 simulations at the diamond [111] surface covered with 6% of carboxyl linkers.

87 Furthermore, the immobilization of OTA has been performed in a few, widely available drinks,  
88 beverages, and liquids to assess the influence of the environment on OTA toxicity. What is more,  
89 complex of the OTA and NDs enables the investigations of the DNA-OTA interactions using both  
90 fluorescence and UV-Vis spectroscopy.

91

## 92 **2. Experimental**

### 93 *2.1. Materials*

94 Carboxylated, fluorescent nanodiamonds with an average size of 750 nm, purchased from Adámas  
95 Nanotechnologies, USA have been chosen as substrates for immobilization of OTA. The Ochratoxin A  
96 from *Aspergillus ochraceus* has been received from Sigma-Aldrich, Poland. The stock solution of ct  
97 DNA has been obtained from Sigma Aldrich, Poland

98 Both OTA and ct DNA have been stored in phosphate buffer saline (PBS; pH=7.4) containing 140 mM  
99 of sodium chloride (NaCl), 2.7 mM of potassium chloride (KCl), 0.1 mM of sodium phosphate dibasic  
100 (Na<sub>2</sub>HPO<sub>4</sub>), and 1.8 mM of potassium phosphate monobasic (KH<sub>2</sub>PO<sub>4</sub>), all of which have been supplied  
101 by Sigma-Aldrich, Poland. OTA was suspended in PBS at 1 mg/mL concentration and stored as aliquots  
102 at -20°C. The ct DNA has been suspended in 0.01 M PBS, and its concentration was determined using  
103 UV-Vis spectroscopy based on the UV absorbance measurement at 260 nm using a molar extinction  
104 coefficient of 6600 M<sup>-1</sup>cm<sup>-1</sup>.

105 For immobilization of OTA on the nanodiamonds' surfaces, the following chemicals have been selected:  
106 4-aminophenylacetic acid, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-  
107 carbodiimidehydrochloride (EDC), potassium ferricyanide ( $K_3[Fe(CN)_6]$ ), potassium ferrocyanide  
108 ( $K_4[Fe(CN)_6]$ ), dimethylsulfoxide (DMSO), sulfuric acid ( $H_2SO_4$ ) (98%), and ethanol (98%). All of the  
109 chemicals listed above have been supplied by Sigma-Aldrich, Poland.

110 For the assessment of an impact of a liquid environment on OTA's interaction with ct DNA, the  
111 following comestibles have been selected: 10% spirit vinegar, 100% lemon juice, 100% apple juice, and  
112 green tea. Deionized water has been used as a reference. Detailed information on pH, and the origins of  
113 the comestible liquids is summarized in **Table 1**.

114

115 **Table 1.** Origins, and values of pH of the comestible liquids used in the experiment.

Comestible liquid	pH	Manufacturer
Spirit vinegar, 10%	2.38	OCTIM Sp. z o.o., Poland
Lemon juice, 100%	2.55	Polenghi LAS s.r.l., Italy
Apple juice, 100%	3.51	Tymbark - MWS Sp. z o.o. Sp.k., Poland
Green tea	7.10	Unilever Polska Sp. z o.o., Poland

116

## 117 2.2. Experimental Procedure

118 The nanodiamonds were separately suspended in every one of the 4 comestible liquids, and in deionized  
119 water. A concentration of the diamond in the suspensions was 0.1% (w/w). To ensure a repeatability of  
120 the samples, the following procedure for the tea brewing has been established: one sachet bag of the  
121 green tea was poured with 250 mL of filtered, boiling water, and removed after exactly 5 minutes. Before  
122 OTA was immobilized on the nanodiamonds' surfaces, the suspensions were being homogenized in an  
123 ultrasonic bath (Polsonic Sonic-3, 40 kHz) for 30 min.

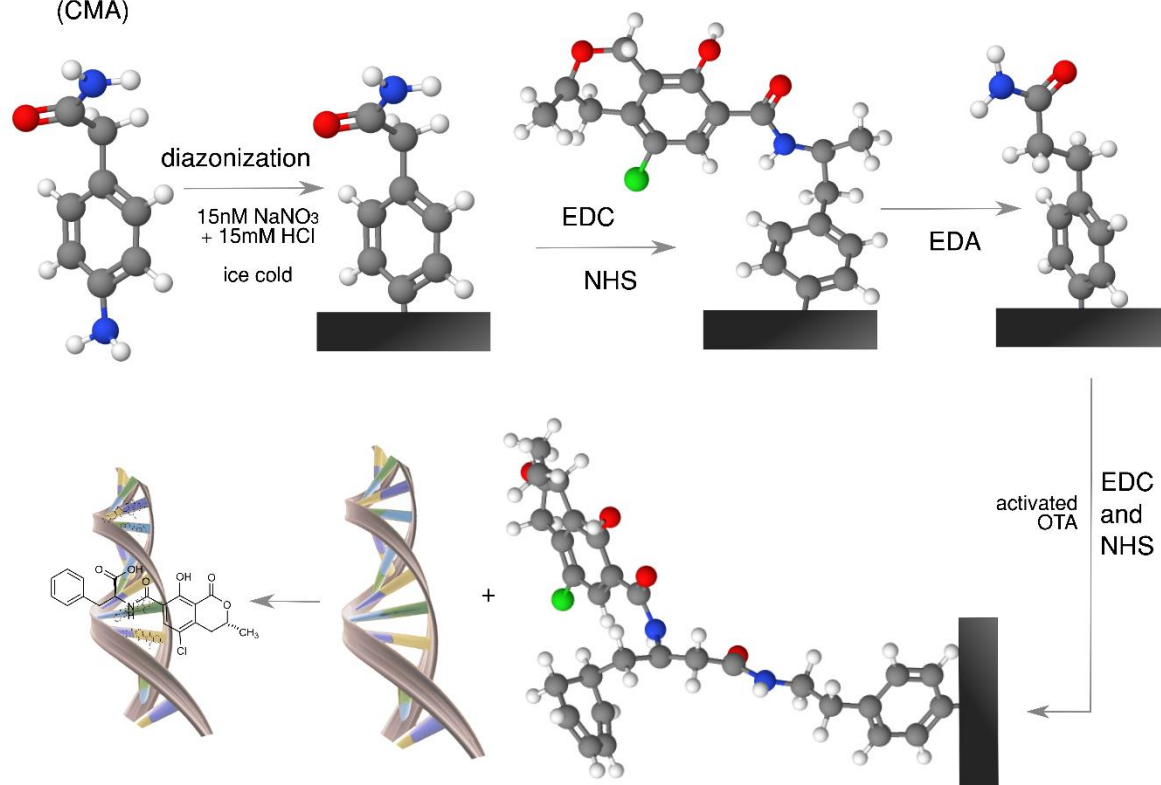
124 In the present study, OTA was directly immobilized to 4-carboxymethylaniline (CMA). The proposed  
125 reaction scheme (**Figure 1**) consists of an activation of carboxyl groups on OTA with EDC/NHS, and a  
126 crosslinking of the activated carboxyl groups to amine groups generated on CMA-coated  
127 nanodiamonds' surfaces. First, 10 mg of 4-carboxymethylaniline (CMA) were activated in the presence  
128 of 20.5 mg of EDC, and 11.5 mg of NHS in 1 mL of PBS for 30 min with stirring. After the activation,  
129 carboxylic acid terminal moieties of CMA were converted to amine groups during incubation with 1 M  
130 ethylenediamine (EDA), pH 8.5 for 7 min. Free carboxylic groups were blocked with 1 M ethanolamine  
131 (EOA), pH 8.5, during incubation lasting 2 min.

132 After OTA was attached to the surfaces of the nanodiamonds, its interaction with ct DNA was  
133 investigated by means of UV-Vis spectroscopy, fluorescence spectroscopy, and analysis of zeta  
134 potential.

135



4-carboxymethyl aniline  
(CMA)



136

137 **Figure 1.** Scheme of nanodiamond modification by OTA and graphical representation of DNA-OTA  
138 interactions.

### 139 2.3. Apparatus

140 A high-precision quartz glass cuvette with outer dimensions of 45 × 12.5 × 12.5 mm and an optical path  
141 length of 10 mm has been used to analyze the UV-Vis absorption, and the fluorescence of the samples.  
142 All the absorption spectra were recorded in 250–650-nm range with the Perkin Elmer Lambda 35 UV–  
143 Vis spectrometer equipped with the integrating sphere module for reflectance measurements.

144 The fluorescence spectra were measured using a custom-made setup with 532 nm CW Nd:YAG SHG  
145 laser (Millenia, Spectra Physics) as an excitation source. The spectra were analyzed using 0.3 m  
146 monochromator (SR303i, Andor) equipped with 600 groves/mm grating and recorded with ICCD  
147 detector (DH740, Andor). The samples were excited by the laser from the front at 45° angle. The  
148 fluorescence signal was collected using the quartz lens end focused on the entrance of optical fiber. In  
149 the detection path the band-pass filter (OG570, Schott) was used to block the laser radiation.

150 The electrical properties (conductivity and zeta potential) of the samples were measured using the  
151 Zetasizer Nano ZS (Malvern Panalytical, UK) particle analyzer equipped with a high concentration cell.

152

### 153 2.4. Measurement Procedures

154 In the analysis of both the UV-Vis absorption, and the fluorescence, an empty cuvette was measured  
155 first to register a background signal, which would later be subtracted from the spectra of the samples.

156 Next, 2 mL of the suspension of OTA-immobilized nanodiamonds were placed in the cuvette and  
157 analyzed. Then, the suspension of ct DNA was added portion-wise to the suspension present in the  
158 cuvette. The following volumes of ct DNA suspension in total were added to the cuvette: 20  $\mu$ L, 40  $\mu$ L,  
159 60  $\mu$ L, 80  $\mu$ L, 100  $\mu$ L, 150  $\mu$ L, 200  $\mu$ L, 500  $\mu$ L. After each portion of the ct DNA suspension was added,  
160 the sample was vortexed (VELP Scientifica TX4) for 10 sec at 400 rpm, and then left for 5 minutes,  
161 after which the absorption and fluorescence measurements were carried out.

162 The conductivity and the zeta potential of the samples were examined at 25°C at three stages of  
163 modification of the nanodiamonds: after the nonmodified nanodiamonds were dispersed in the liquids  
164 in 0.1% concentration; after OTA was immobilized on their surfaces; and finally after the addition of  
165 500  $\mu$ L of the solvent containing ds ct DNA to 2 mL of the suspensions of the OTA-modified  
166 nanodiamonds.

167

### 168 *2.5 Ab-initio and Molecular Dynamics Simulation of ND-OTA Complexes*

169 The *ab-initio* simulations were conducted on the [111] oriented diamond slab containing 4950 carbon  
170 atoms (9 layers, each containing 550 atoms). Model was created using the Atomistix Tool Kit  
171 Quantumwise<sup>25</sup> (ATK, Synopsys, USA). The diamond slab was terminated by COOH groups with  
172 covalently grafted OTA. The thickness of the slab was 19.6 Å. The model is assumed to be an infinite  
173 plane; on the borders of supercell the periodic boundary conditions were applied. To leave sufficiently  
174 large space for the adsorbing molecules and analyze interaction with images, the 6.8 nm vacuum buffer  
175 was added above the surface. Periodic boundary conditions were applied in the unit cell, while the ratios  
176 between atom types within the cell were kept similar for the entire surface.

177 The [111] diamond surface consists of 225 surficial carbon atoms, where each of the carbon atoms forms  
178 three bonds with other carbons. Priorly, hydrogen atoms are bonded to each carbon atom resulting in  
179 the 100% hydrogen termination. If the single hydrogen atom is removed, the surface gets radical site  
180 (per unit cell). Radical site could be saturated by the COOH (or OTA), then the percental coverage  
181 changes to  $1/225 \approx 0.44\%$ . Surface coverage is usually estimated in reference to all possible radical sites  
182 on the given surface, regardless the occupation. Such a convention was already used in the papers  
183 describing surface chemistry simulations<sup>9</sup>. Large molecules such as OTA could cover up the diamond  
184 surface which is already covered by relatively small atoms such as H enabling the intersection of the  
185 covered areas. This phenomenon does not impact the percentage coverage, which is independent of the  
186 molecule geometrical size. Models used in Force-Field and density-functional theory (DFT) calculations  
187 have different cell sizes but similar percentage coverages of COOH and OTA. During all Force-Field  
188 calculations on the diamond surface, 13 COOH species were present corresponding to 5.77% coverage.

189 The applied ochratoxin structure was downloaded from the PubChem<sup>26</sup> databases (CID 442530). The  
190 binding to the diamond surface was conducted to reproduce the mechanism presented in **Figure 1**. Six





191 separated slabs were designed containing perpetually increased number of OTA molecules bounded to  
192 diamond surface, from one to six per chosen area (see **Figure 2**). The binding energy  $E_{bind}$  of the model  
193 system can be calculated using the formula (1).

$$194 \quad E_{bind} = \frac{1}{n}(E_{slab+OTA} - E_{slab} - nE_{OTA}) \quad (1)$$

195 Where  $E_{slab+OTA}$  is the total energy of the system with OTA and all terminating species,  $E_{slab}$  is the  
196 energy of the system without OTA (the radical sites created by removing OTA are left passivated), and  
197  $E_{OTA}$  is the energy of one isolated OTA species. Small  $n$  denotes the number of OTA molecules in the  
198 supercell.

199 To describe the energy of the system, the ATK Force-Field has been used. This module describes the  
200 interatomic integration using the classical potentials set. This implementation is described elsewhere<sup>27</sup>.  
201 To calculate the operating forces, the reactive force field ReaxFF\_CHONSMgPNaCuCl\_2018\_08 was  
202 used<sup>28</sup>. Before the total energy calculation, the atomic positions and the cell size were optimized based  
203 on the Broyden-Fletcher-Goldfarb-Shanno (LBFGS) algorithm. The maximal force in the structure after  
204 optimization did not exceed 0.05 eV/Å.

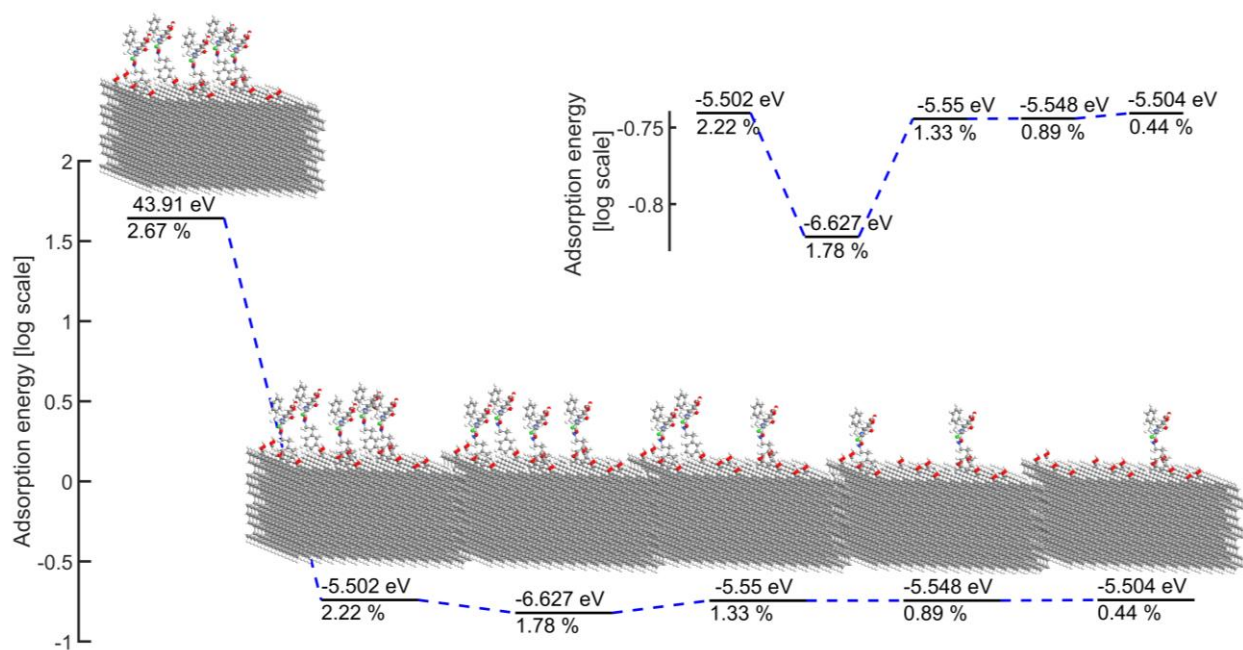
205 The diamond slab containing single OTA was applied for *ab-initio* band structure simulations by means  
206 of DFT. The Quantum ATK toolbox enables conduction of such calculations based on a couple of  
207 variants. The Linear Combination of Atomic Orbitals<sup>29</sup> (LCAO) was used to calculate electron density  
208 functionals. To solve the matrix equations of the LCAO method, self-consistent field (SCF) iterations  
209 were performed. The density mesh cut-off energy was set to 125 Hartree and k-point sampling utilizing  
210 MonkhorstPack scheme was 3x2x1. During DFT calculation, the exchange-correlation of electrons was  
211 included using GGA method with PBE (Perdew, Burke and Ernzerhof) functionals. The PseudoDojo<sup>30</sup>  
212 pseudopotentials with medium basis set were utilized to calculate the electron wave functions. The  
213 detailed description of the mentioned basis set can be found in the paper of Smidstrup *et al.* (appendix)  
214<sup>31</sup>.

215

### 216 **3. Results and Discussion**

#### 217 *3.1. Simulations of the Adsorption Energy Dependence on the Surface Coverage*

218 The series of numerical studies were carried out to estimate the dependence of OTA adsorption energy  
219 on the ND surface. The key aspect of this calculation was to estimate energetically favorable OTA  
220 coverage density. The OTA coverage density and adsorption energies are plotted in **Figure 2**.



221

222 **Figure 2.** The binding energy for a series of [111] diamond models with different OTA concentration  
 223 on the surface.

224 According to Force-Field calculation, the energetically favorable OTA surface coverage is equal to  
 225 1.78%, exhibiting the lowest energy. The adsorption energy at this coverage is -6.627 eV per adsorbed  
 226 ochratoxin molecule. The adsorption energy per each molecule for low concentration decreases  
 227 monotonically with increasing percentage of coverage. This phenomenon could be attributed to the  
 228 stabilization of the long-range interactions between the adsorbed OTA molecules<sup>32</sup>. The well-known  
 229 fact is that the significantly different energies are associated with various conformation of the organic  
 230 molecules<sup>33</sup>. Once the percental surface coverage exceeds the limiting value, the distance between  
 231 molecules is as low as the cross-linking and bond breaking processes starts to occur<sup>9</sup>. The initialization  
 232 of the breakdown leads to the massive surface recombination which is associated with the desorption of  
 233 OTA molecules. Such a process is indicated by the rapid increase of the adsorption energy. The  
 234 competitive influence of mentioned processes will push the system to preserve the OTA concentration  
 235 at the level close to optimal energetically stable.

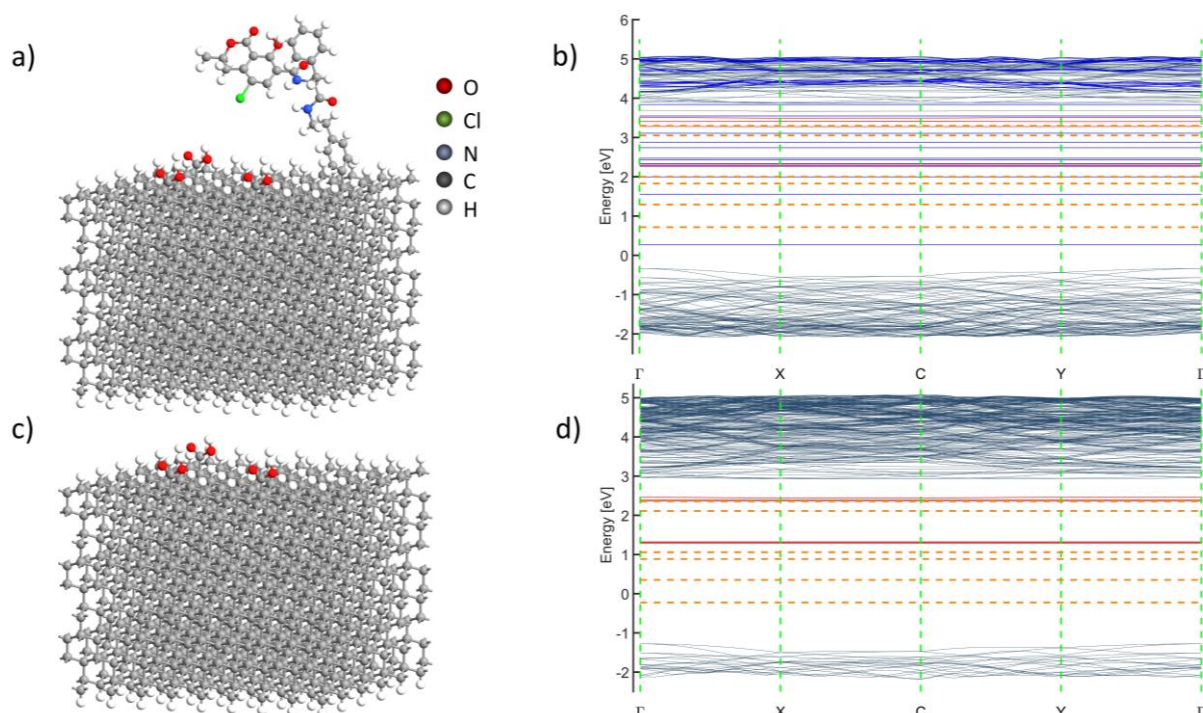
236

### 237 3.2. *Ab-initio* Simulations of ND-OTA Complex

238 The diamond slab containing single OTA molecule was utilized to simulate *ab-initio* band structure of  
 239 the studied ND-OTA complex by means of DFT. The horizontal size of the slab was aligned to set the  
 240 OTA coverage to 1.78%. The coverage of COOH was kept at 6.66%. The slab is displayed in **Figure**  
 241 **3a**. Additionally, the reference, H-terminated diamond slab, covered only by COOH (see **Figure 3c**),  
 242 has been created. **Figures 3b,d** display projected band structure based on the *ab-initio* simulations. This



243 type of the band structure includes the projection of a state on a specific group of atoms, in this case  
244 OTA and COOH.



245  
246 **Figure 3.** a) The model of diamond [111] surfaces with adsorbed ochratoxin; b) Projected band structure  
247 of the ND-OTA complex; c) Reference model of the slab terminated by the mixture of COOH and H;  
248 d) Projected band structure of the slab presented in c, red states were introduced by adsorbed molecules.  
249 (Attn. The blue states were introduced by OTA, red by COOH, orange dashed lines indicate position of  
250 states of NV<sup>-</sup> defects as calculated previously<sup>34</sup> while black states are associated with the diamond slab).

251 In this work, the interaction of the adsorbed OTA molecules with NV<sup>-</sup> color centers is particularly  
252 investigated. The energy states of NV<sup>-</sup> center were extensively studied up to now<sup>34-37</sup>. Here, the available  
253 literature data<sup>34-37</sup> has been applied instead of calculating the position of the NV states. The band gap  
254 of diamond as shown in **Figures 3b,d** (orange dashed lines).

255 The presented projected band structure data clearly shows that adsorption of both COOH and OTA  
256 introduces the electron states in the band gap of the diamond. In the case of COOH functionalization  
257 new energy band is introduced below the lower conduction band. As an effect, the band gap shrinks by  
258 the 40% relative to the hydrogen-terminated surface bandgap. A similar effect, however at higher  
259 concentration of carboxyl, was predicted on the ground of ab-initio simulation of nanodiamond cluster  
260 electronic properties<sup>38</sup>. Introduction of the OTA functionalization results in further shrinking of the  
261 fundamental band gap, divided both by introduction of OTA energy levels into fundamental band gap  
262 of diamond and upshift of the valence band. As an effect of combined processes, the bandgap width  
263 shrinks by 84% in comparison to the hydrogen-terminated [111] diamond surface.

264 The introduction of the surface states by the adsorbent species is well-known, but in these particular  
265 conditions the most important is the location of these states in the energy-momentum space. The states  
266 residues in the region close to the states introduced by the NV<sup>-</sup> centers. The overlapping of surface  
267 termination states is particularly evident in **Figure 3b**, which presents the band structure of [111]  
268 diamond surfaces covered by both COOH and OTA. Several states are positioned within the NV<sup>-</sup>  
269 bandgap, implying the strong interactions between ND-NV and OTA, which could even deactivate the  
270 NV fluorescence<sup>34,39</sup>.

271 To get qualitative measure of influence of OTA functionalization effect on the electronic properties, the  
272 work function (WF) calculations were conducted. Both band structure and WF were simulated using  
273 similar model as shown in **Figure 3**. The electron WF was calculated for different cases of surface  
274 functionalization. The fully hydrogenated surface results in WF of 4.83 eV being in agreement with  
275 prior experimental data<sup>40,41</sup> for [111] diamond. The partially hydrogen-terminated diamond  
276 functionalized with 6.66% of COOH groups (see **Figure 3c**) manifests WF equal to 6.21 eV. Finally,  
277 the adsorption of 1.78% OTA results in WF of 6.49 eV (see **Figure 3a**). Observed changes are in  
278 agreement with approach described in<sup>42</sup> where WF values could be recalculated to the electron affinities.

279 The hydrogen termination induces the formation of the positively-charged surface, causing band  
280 bending close to the diamond surface<sup>43</sup>, thus NV centers will be favored to convert in the neutrally  
281 charged state. According to conducted DFT simulation, OTA-coated diamond [111] surface will exhibit  
282 positive electron affinity. This suggests that the surface will be negatively-charged.

283 Despite the relatively low concentrations of both COOH and OTA, the band structure of the ND-OTA  
284 complex was significantly distorted by the covalent adsorption of OTA. Qualitative analysis of the  
285 projected band structure and quantitative studies on the chemical potentials lead to the conclusion that  
286 binding of OTA to the nanodiamond surface should stabilize concentrations of the NV<sup>-</sup> centers.

287 Spectroscopic experiments revealed the decay in the fluorescence emission of the ND-OTA complexes  
288 in function of increased the ct DNA concentrations (**Figure 6**). The simulation results suggest that the  
289 light scattering and absorption or fluorescence quenching in the DNA-rich buffer are additional  
290 processes responsible for the observed decay effect.

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298 *3.3. Spectroscopic studies on the ND-OTA interactions with DNA*

299 Phosphate chromophores and aromatic bases are the cause of the characteristic DNA absorption peak  
300 positioned at *ca.* 260 nm. The chromophore groups, namely purines—adenine and guanine—and  
301 pyrimidines—cytosine and thymine, are the cause of the given maximum absorption peak<sup>44</sup>. Thanks to  
302 this characteristic, one can utilize UV-Vis absorption measurement for the detection of various complex  
303 formations. This technique, taking into account its simplicity and effectiveness, enables the detection of  
304 various DNA–molecule interactions and the formation of new complexes. As the result of these spatial  
305 changes, the absorbance intensity in the UV-Vis range as well as the band position are altered.  
306 Bathochromism is observed during intercalation if the  $\pi^*$  orbital of the intercalating molecule couples  
307 with the  $\pi$  orbital of the DNA base pairs, resulting in the decreased  $\pi^*$  transition energy. However, the  
308 coupling  $\pi$  orbital is not fully saturated with electrons. To detect an interaction between DNA and a  
309 chosen molecule, one can observe the shift in the maximum position of this band before and after the  
310 addition of the ligand to the tested DNA solution.

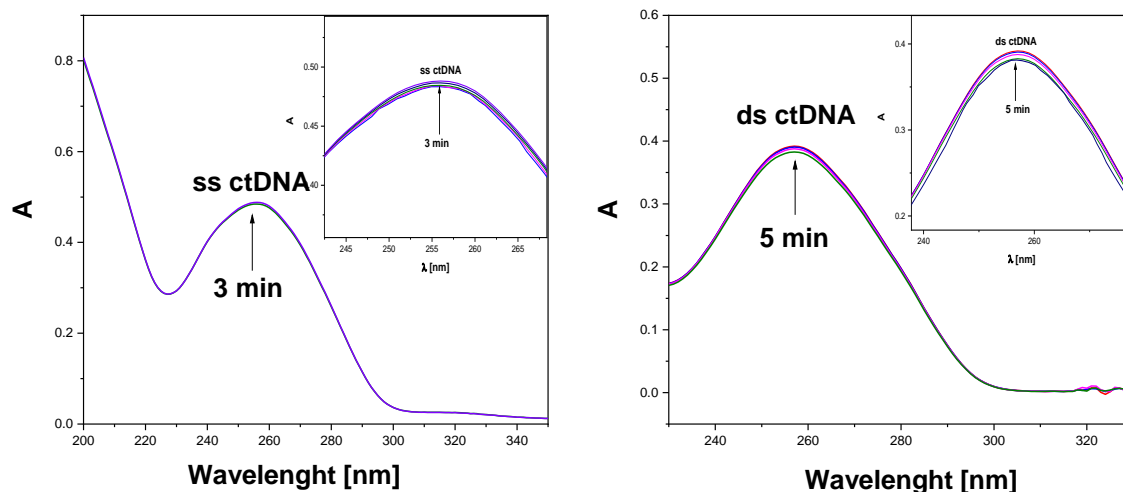
311 UV–Vis is usually used as a standard technique to quantify the amount of DNA in a sample. DNA bases  
312 absorb strongly at 260 nm in an aqueous buffered solution at pH 7.4<sup>6</sup>. The absorbance values obtained  
313 for this wavelength are used for the calculation of the DNA concentration and the stability analysis.  
314 OTA was dissolved in PBS, pH 7.4. In the present experiments, the addition of 10  $\mu$ L of calf thymus  
315 DNA (ct DNA) to OTA strongly influenced the UV spectra. The absorption band intensity regularly  
316 decreased over time, which was also observed in many other studies<sup>45,46</sup>. Usually, the time needed to  
317 establish the reaction equilibria was around 3–5 min<sup>47</sup>. In the present study, the overall time for the  
318 determination of equilibria was 3 min for ss ct DNA and 5 min for ds ct DNA (**Figure 4**). This time was  
319 accepted as optimal for the intercalation, to study the effects of OTA interactions with ct DNA<sup>46</sup>.

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323  
 324 **Figure 4.** The influence of the incubation time on the band intensity of OTA ( $2 \times 10^{-4}$  M) after addition of ct DNA,  
 325 10  $\mu$ L (ss ct DNA and ds ct DNA) in aqueous phosphate buffer solution, pH 7.4.

326 The UV spectra of the ND-OTA suspensions with different concentrations of ct DNA were obtained.  
 327 As shown in **Figure 5**, with increasing concentrations of ct DNA, the intrinsic binding constant  $K_b$  of  
 328 OTA with ct DNA can be determined according to Benesi–Hildebrand equation:

$$329 \quad \frac{A_0}{A - A_0} = \frac{\varepsilon_G}{\varepsilon_{H-G} - \varepsilon_G} + \frac{\varepsilon_G}{\varepsilon_{H-G} - \varepsilon_G} \times \frac{1}{K_b [DNA]}$$

330  
 331 where  $K_b$  is the binding constant,  $A_0$  and  $A$  are the absorbance of the OTA and its complex with ct DNA,  
 332 and  $\varepsilon_{H-G}$  are the absorption coefficients of the OTA

333 **Figure 5** shows the absorption spectra of OTA in the presence of the increasing amounts of ct DNA to  
 334 decrease in the peak intensities. Intercalating binding commonly results in the hypochromic and  
 335 bathochromic effect of the intercalated chromophore transition. The spectral changes observed in the  
 336 form of ‘hypochromism’ effect during the process, reflect the change in conformation of DNA and  
 337 structure of DNA. It has been reported in literature that hypochromism is due to the intercalative mode  
 338 involving a strong stacking interaction between an aromatic chromophore of a molecules and base pairs  
 339 of DNA. Generally, the external groove binding causes a slight spectral shift, a hyperchromic effect. In  
 340 all the spectra obtained, we observed the band with the maximum absorption in 270 nm, but for 10%  
 341 spirit vinegar we observed the maximum at 230 nm.

342 Furthermore, comparing with the  $K_b$  value of other DNA groove mycotoxin binders, the binding mode  
 343 between OTA and the ct DNA was deduced as groove binding. The association constant can be obtained  
 344 from the intercept-to-slope ratios of  $A_0/(A - A_0)$  vs.  $1/[DNA]$  plots. Based on the intersection-to-slope  
 345 ratio, the greatest  $K_b$  value was calculated as  $3.27 \times 10^5 \text{ M}^{-1}$  for the diamond suspended in the green tea

346 solution. The  $K_b$  binding constant values reported herein are higher than those typically found for the  
347 well-known toxins (**Table 2**) such as Aflatoxin B1 ( $K_b = 7.60 \times 10^4 \text{ M}^{-1}$ ) and Wortmannin ( $K_b = 2.20 \times 10^4$   
348  $\text{M}^{-1}$ ).

349

350 **Table 2.** Comparison of the binding parameters for various toxins–ct DNA interactions obtained using UV-Vis  
351 spectroscopy.

Compound	Binding constant [ $\text{M}^{-1}$ ]	References
Isoxazolcurcumin	$1.15 \times 10^4$	45
Wortmannin	$2.20 \times 10^4$	48
Farrerol	$11.80 \times 10^5$	49
Aflatoxin B1	$7.60 \times 10^4$	50

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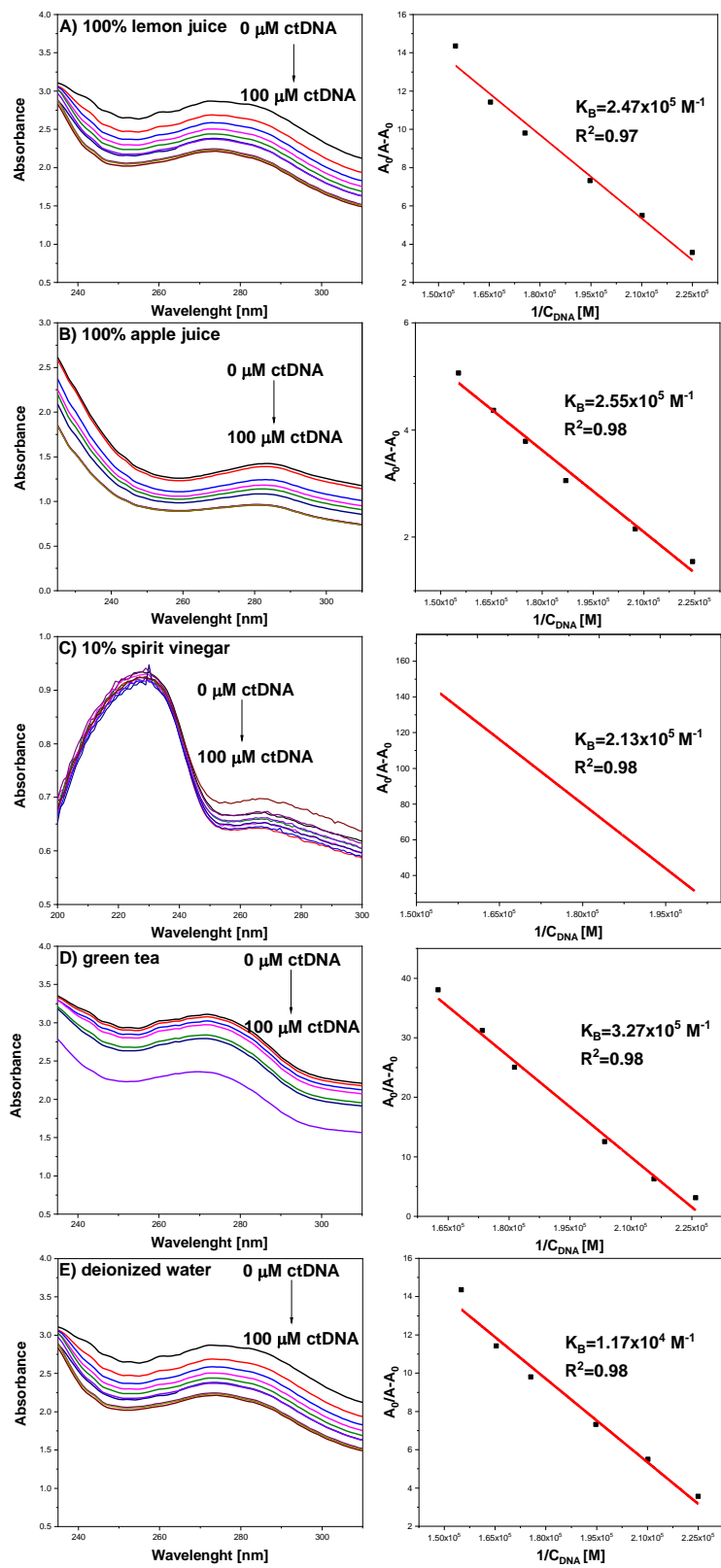
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358 **Figure 5.** UV-Vis absorption spectra of OTA in different comestible liquids and plots of  $(A_0/(A-A_0))$   
 359 versus  $1/[DNA]$ .





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### 361 3.4. Fluorescent Analysis of the ND-OTA complex with DNA

362 The fluorescence spectra were obtained for the whole suspension (solvent, diamonds, OTA, ct DNA)  
363 and were dominated by the fluorescence of diamonds which emit such intense radiation due to the  
364 nitrogen-vacancy (NV) fluorescent color centers contained therein (**Figure 6**). The fluorescence  
365 intensities decreased regularly with the increase of ct DNA concentration. The titration data were  
366 analyzed according to the Stern–Volmer equation and investigated on the interaction types of ct DNA  
367 with the five suspensions of the nanodiamonds. The fluorescent intensity significantly decreased with  
368 increasing concentration of ct DNA. Therefore, fluorescence data could be studied using the Stern–  
369 Volmer equation <sup>51</sup>:

$$370 \quad \frac{F_0}{F_0 - F} = \frac{1}{f_a K_{SV}} \frac{1}{[ct DNA]} + \frac{1}{f_a}$$

371 where  $F_0$  and  $F$  are the fluorescence intensities in the absence and presence of a quencher;  $f_a$  is the  
372 fraction of the accessible fluorescence;  $K_{SV}$  is the quenching rate constant of the biomolecules, and  $[ct$   
373 DNA] is a concentration of calf thymus DNA.

374 To elaborate on the fluorescence exhibited by the nanodiamonds containing the NV centers, it is  
375 advisable to concentrate first on the analysis of the spectra registered for the reference suspension based  
376 on deionized water. This suspension was selected as it is least likely to alter the signal emitted by the  
377 particles. Local maxima at 575 nm and 637 nm are the zero–phonon lines (ZPLs) of  $NV^0$  and  $NV^-$   
378 centers, respectively <sup>52</sup>. The much higher intensity of the ZPL of the negatively–charged centers  
379 indicates their quantitative advantage over their electrically neutral counterparts. While the ZPL of  $NV^-$   
380 centers is clearly visible in every fluorescence spectrum registered in the experiment, the ZPL of  $NV^0$   
381 centers is quenched by the liquid medium in the suspensions based on lemon juice, apple juice, and  
382 green tea. Broad peaks at 659 nm and 682 nm are the vibronic (phonon) sidebands of the  $NV^-$  centers <sup>53</sup>  
383 with the known energy shifts of 65 meV <sup>54</sup> and 63 meV <sup>52</sup>, respectively. It is worth noting that the  
384 majority of the signal emitted by the  $NV^-$  centers falls to the phonon sidebands, and not the ZPL, which  
385 is a common occurrence when the measurements are carried out at room temperature <sup>55</sup>.

386 In every fluorescence spectrum of the sample based on the lemon juice there is a very intensive, broad  
387 peak observed at 677 nm. This peak is a superposition of the 2<sup>nd</sup> phonon sideband of the  $NV^-$  centers,  
388 and the maximum already present in the signal emitted by the pure lemon juice. A broad peak at 585 nm  
389 registered for the suspensions based on the green tea comes from the dispersion medium itself. The same  
390 peak has been identified in the spectrum of pure green tea. Lin *et al.* <sup>56</sup> found that the emission intensity  
391 in the range of 500–600 nm varies significantly between various kinds of green tea.



392 The OTA–DNA binding mode can be determined by fluorescence spectroscopy and various analytical  
393 tools based on the fluorescence emission can also provide particularly useful information. Moreover,  
394 the effective interaction with DNA usually causes a significant change of the fluorescence intensity as  
395 a consequence of various factors. Representative spectra obtained for OTA in the absence and the  
396 presence of calf thymus DNA dissolved in the solution are shown in **Figure 6**. We observed that with  
397 the increasing DNA concentration, the emission spectra of OTA show remarkable distribution and  
398 hyperchromic effect.

399 Given that OTA could emit fluorescence under ultraviolet irradiation, a series of experiments based on  
400 the fluorescence spectroscopy were performed to explore the binding mechanism of mycotoxin to ct  
401 DNA. The fluorescence intensity of OTA–DNA complex is quenched upon the addition of ct DNA.  
402 OTA on the nanodiamond surface in lemon juice displayed strong emission spectra, with emission local  
403 maximum at 637 nm. After the addition of ct DNA in a small concentration to OTA solution, we  
404 observed that the position and shape of the peaks did not change. Thus, it can be assumed that OTA  
405 interacts with ds ct DNA through noncovalent bonds rather than covalent bonds<sup>57</sup>. The  $K_{SV}$  value of the  
406 OTA–DNA complex at 298 K in various nanodiamond suspensions is presented in **Table 3**. The highest  
407 value  $K_{sv}$  of the OTA–DNA complex was  $8.12 \times 10^5 \text{ M}^{-1}$  on surfaces of nanodiamonds suspended in  
408 green tea. A comparison shows that the  $K_{sv}$  of the OTA–DNA complex on nanodiamonds using green  
409 tea solution was greater than in the case of other solutions. The difference in  $K_{sv}$  depends mainly on the  
410 structural differences of the solution. Green tea contains other compounds less analeptic than caffeine,  
411 xanthine derivatives, including theobromine, theanine, and theophylline which belong to the group of  
412 polyphenols<sup>58,59</sup>. This effect indicates that the hexatomic ring of OTA immobilized on nanodiamond  
413 suspended in green tea has a larger dimension and stronger stability than the other nanodiamonds,  
414 resulting in a stronger DNA binding.

415



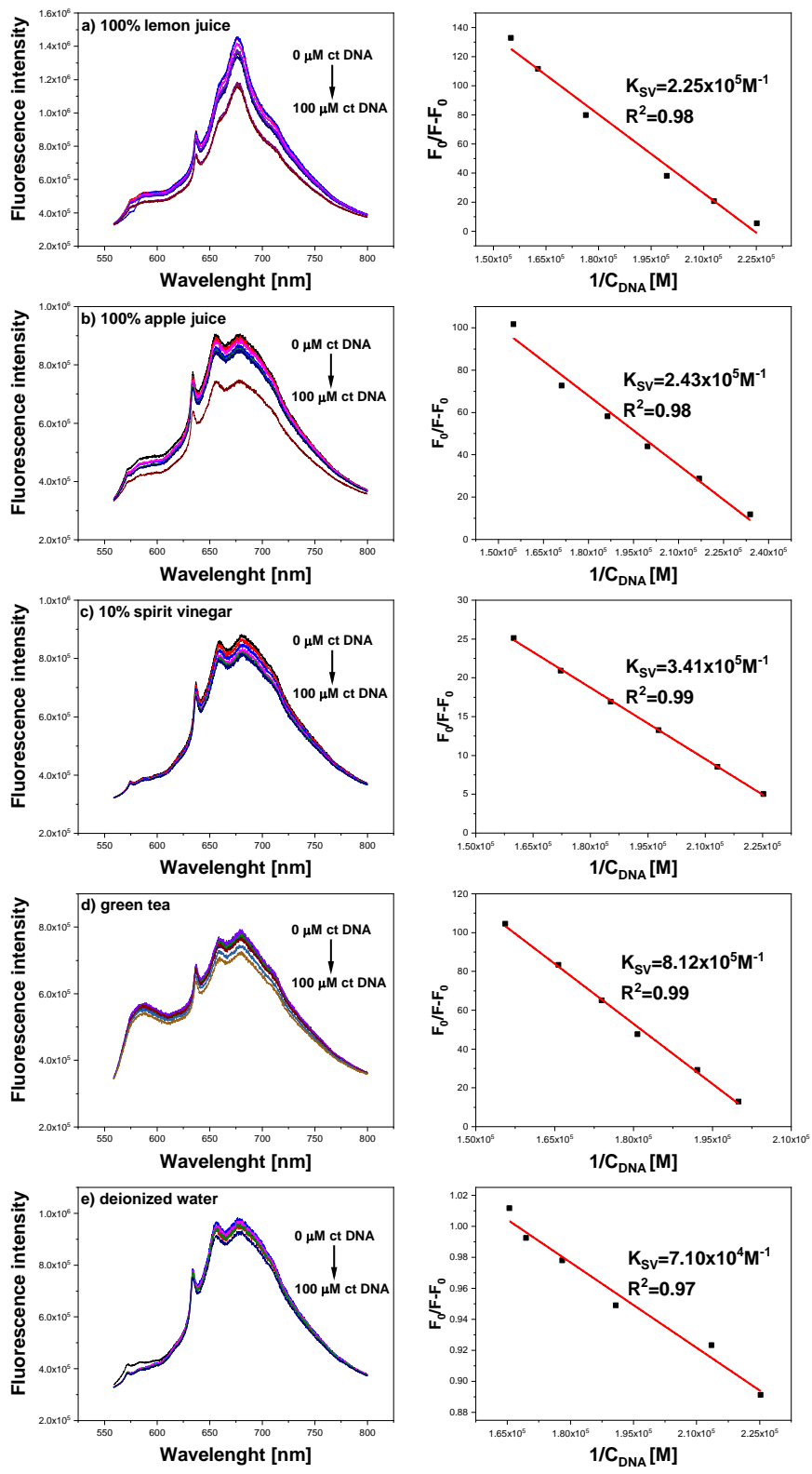
416 **Table 3.** Binding constants for OTA–ct DNA interaction.

<b>Nanodiamond suspensions</b>	<b>Binding constant <math>K_{sv}</math> [<math>M^{-1}</math>]</b>
Lemon juice	$2.25 \times 10^5$
Apple juice	$2.43 \times 10^5$
Deionized water	$7.10 \times 10^4$
Green tea	$8.12 \times 10^5$
Vinegar	$3.41 \times 10^5$

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**Figure 6.** Fluorescence emission spectra of OTA–DNA system in various comestible liquids and plots of  $(F/(F_0-F))$  versus  $1/[DNA]$ .

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### 3.5. Analysis of the Particles Electric Potential

Zeta potential measurements were conducted to investigate the variation of the surface charge at each experimental step (**Figure 7a**). The carboxylated NDs, OTA-coated NDs, and OTA-coated NDs in the presence of the DNA in the sample have been investigated in comestible liquids, namely vinegar, lemon juice, apple juice, green tea, and deionized water. **Table 1** presents the pH values of the utilized liquids.

First, we measured the zeta potential of carboxylated NDs. At low pH values (lemon juice, apple juice, and vinegar) the zeta potential oscillates around 0 mV, reaching a minimum value of ~ -5 mV for the apple juice. This behavior is attributed to a large amount of mobile H<sup>+</sup> ions present in these suspensions, making the oxidized NDs' surfaces electroneutral, thus, unstable and prone to aggregation.

With the increase in pH, the zeta potential is decreasing, reaching the values of -20 and -43 mV for the green tea and water, respectively. Once the pH increases, H<sup>+</sup> ions become depleted from the solution as well as from the carboxyl groups present on the NDs' surfaces, generating the negatively charged particles and, therefore, stabilizing them in the solutions <sup>60</sup>.

Next, OTA-coated NDs were measured. OTA contains phenolic and carboxylic groups (**Figure 9**), and therefore can exist in different ionization states: carboxy ionized (COO<sup>-</sup>), phenolic ionized (ArO<sup>-</sup>), dianionic form (both carboxylic and phenolic ionized), depending on the local pH. The reported pKa values of the carboxyl group and the phenolic group of OTA are in the range of 4.2–4.4 and 7.0–7.3, respectively <sup>61</sup>. Furthermore, knowing that the OTA's carboxyl group is involved in the covalent surface functionalization, the only part which can be ionized is the phenolic group. For this reason, ND-OTA complexes in acidic environments stay electroneutral and prone to aggregation as OTA exists in this condition in a neutral state.

However, in the case of water and green tea, considering their pH, both forms of OTA are simultaneously present in various ratios in these solutions (neutral and anionic form). Thus, for instance, in the case of water, we observed slight neutralization of the negative charge and an increase in zeta potential from -43 to -38 mV. In the case of green tea, we did not observe any changes, which can be caused by the involvement of two forms of OTA on the ND surface. Consequently, the final effect may be an average of these two. Another reason may be the influence of the co-existing green tea ingredients. Green tea contains as many as 200 bioactive compounds, such as polyphenols, caffeine, theanine, which also can neutralize OTA influence.

The last and the most complicated case was OTA-coated ND in the presence of ct DNA. There are many factors involved in the interaction of a compound with DNA, such as temperature, ionic strength, or

457 temperature. The picture is even more complicated if the compound—in our work OTA—exists in more  
458 than one form in the solution, as each form interacts with DNA differently. In general, DNA is a negative  
459 polymer, hence this molecule exhibits higher interactions with cationic species than the neutral species.  
460 On the other hand, these interactions are greater than in case of species that are negatively charged.

461 Zeta potential measurements in acidic liquids revealed no observable changes, although a neutral form  
462 of the OTA may bind the DNA. However, DNA can change its 3D structure in highly acidic  
463 environments, starting from an elongated coil state to a compact one, what is closely related to its charge  
464 neutralization<sup>62</sup>. Also, DNA may partially denature in such an extreme condition, as a result making the  
465 interaction of DNA with OTA impossible in both cases.

466 In the case of water, as mentioned before, neutral and anionic form of OTA are present on its surface,  
467 while DNA will exist in an elongated structure with a negative charge. Therefore, the interaction  
468 between OTA and DNA may be somehow impeded. However, as reported by Saha *et al.*<sup>63</sup>, the presence  
469 of the DNA in the examined sample can change the pKa value of the binding compound, in our case  
470 OTA immobilized on NDs' surface<sup>63</sup> and thereby changing the zeta potential of the surface. Another  
471 reason for the increase of the zeta potential value from -38 to -34 mV is the interaction (groove binding,  
472 or intercalation) of OTA with DNA, thus, neutralizing the negative charge of added DNA, as well as  
473 anionic phenolic group of OTA.

474 In the case of green tea, again, we did not observe any noticeable changes in the zeta potential. This  
475 effect can be justified by the same arguments as previously. In water, both forms of OTA are present;  
476 the pKa value of OTA can vary because of the DNA presence; interaction of OTA with DNA also may  
477 take place, however, the net charge may be neutralized by co-existing green tea ingredients.

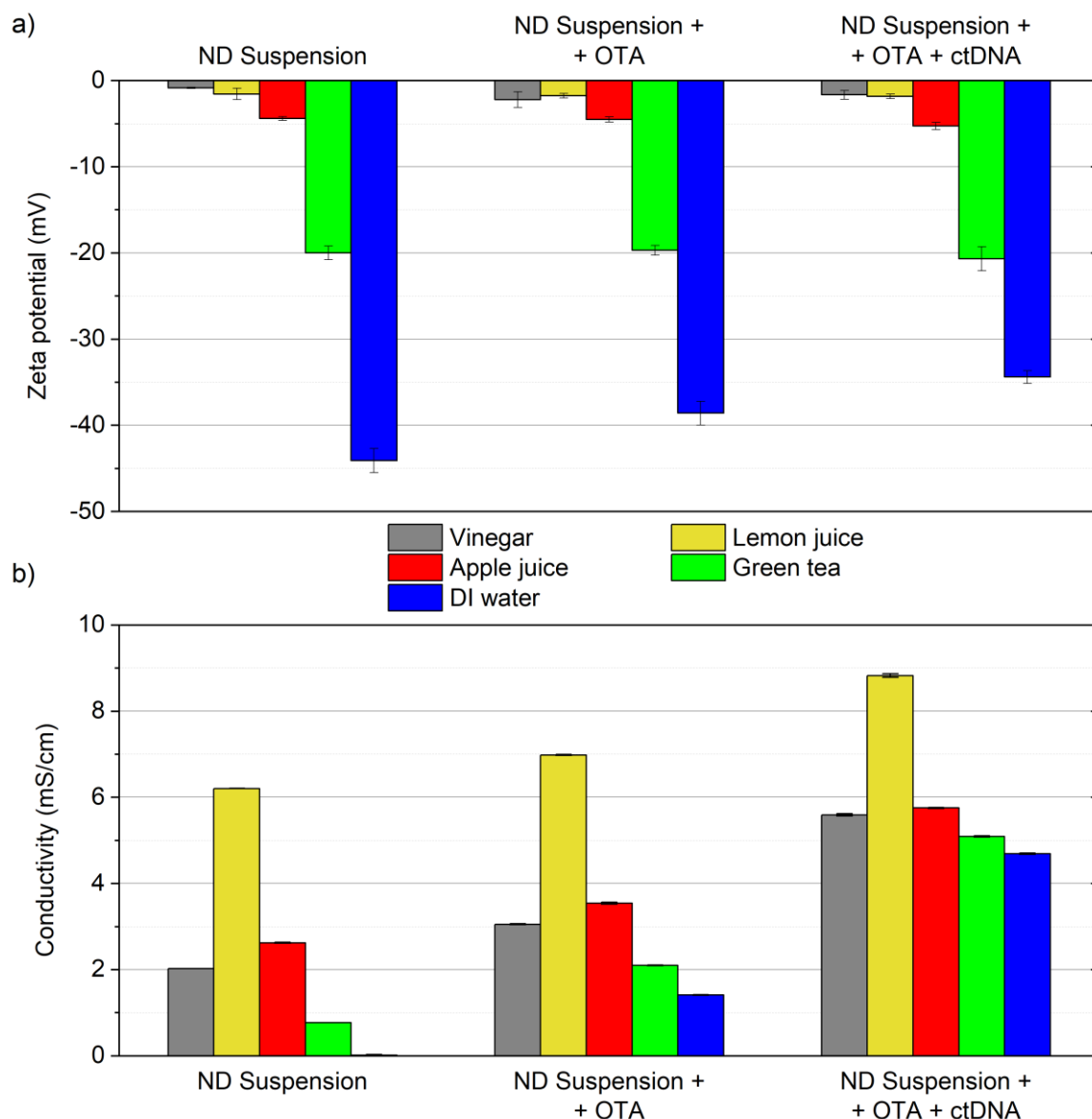
478 The conductivities of the nanodiamond suspensions at different stages of the particles modification are  
479 presented in **Figure 7b**. The deionized water is the only dispersion medium that exhibits virtually no  
480 electrical conductivity with suspended unmodified nanodiamonds. At the same stage, every other  
481 sample shows initial conductivity, with the suspension based on the lemon juice revealing the highest  
482 value, followed by the sample based on the apple juice, then on the vinegar, and finally on the green tea.  
483 This relation among the conductivities of the suspensions is preserved at every stage of the modification  
484 of the nanodiamonds. The attachment of the OTA to the surfaces of the diamond particles, as well as  
485 the addition of double-stranded ct DNA to the suspensions, increase the overall conductivities of the  
486 samples.

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489

490 **Figure 7.** The electrical properties of the nanodiamond suspensions based on the comestible liquids;  
 491 “ND Suspension” – the suspensions of unmodified nanodiamonds (0.1% concentration); “ND  
 492 Suspension + OTA” – the suspensions of the nanodiamonds with OTA attached to the surfaces of the  
 493 particles; “ND Suspension + OTA + ct DNA” – mixtures containing 2 mL of the nanodiamond  
 494 suspensions with OTA attached to the surfaces of the particles and 0.5 mL of the solvent containing ds  
 495 ct DNA; a) zeta potential; b) conductivity.

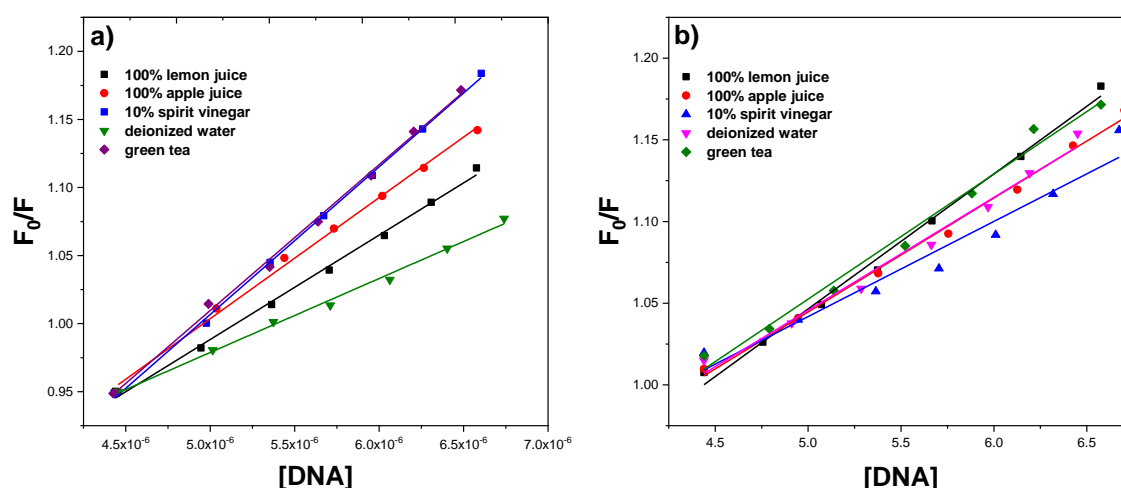
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### 497 3.6. Effects of Mycotoxins on the Characteristics of the Denatured DNA

498 A difference was observed in the quenching effect of single-stranded DNA (ssDNA) and  
 499 double-stranded DNA (dsDNA) when molecules were bound to DNA based on different interaction  
 500 modes. In the groove bounding mode, the quenching interactions of dsDNA should be stronger than for

501 ssDNA. Weaker quenching effects of dsDNA comparing with ssDNA also slightly differed if small  
 502 molecules intercalated between the DNA base pairs. The double strands were denatured. The quenching  
 503 effects of both ssDNA and dsDNA also slightly differed if small molecules interacted with the  
 504 phosphates groups of DNA based on electrostatic interactions<sup>64-66</sup>. The fluorescence quenching plots of  
 505 OTA upon the addition of different concentrations of dsDNA and ssDNA are shown in **Figure 8**. The  
 506 dsDNA exhibited a greater slope than ssDNA in OTA.

507



508

509 **Figure 8.** Fluorescence quenching plots of OTA for a) single-stranded DNA (ssDNA) and b) double-  
 510 stranded DNA (dsDNA) in different comestible liquids in,  $C_{OTA} = 2 \times 10^{-3} M$ .  $F_0$  is the fluorescence  
 511 intensity of OTA and  $F$  is the fluorescence intensity of OTA for the various ssDNA and dsDNA  
 512 concentrations.

513

## 514 Conclusions

515 In summary, the interactions of ND-OTA complexes with ct DNA were investigated using spectroscopic  
 516 methods applying nitrogen-vacancy rich nanodiamonds suspensions. The characteristics of the  
 517 interaction between OTA and ct DNA were studied in different comestible liquids. The fluorescent  
 518 measurements demonstrated that the intensity of the fluorescence significantly decreased with the  
 519 increasing concentration of ct DNA. The UV-Vis studies indicated that the increasing concentrations of  
 520 ct DNA lead to a decrease in the peak intensities.

521 We obtained the binding constants and the binding site sizes (0.97) of OTA with ct DNA at room  
 522 temperature. Moreover, we managed to form a complex with ct DNA where a part of OTA occupies a  
 523 space within the groove binding and the rest slips in between the stacked base pairs of duplex DNA via  
 524 the intercalation mode of binding.

525 The presence of the adsorbed species introduces additional states to the fundamental diamond bandgap.  
526 The energy states of NV<sup>-</sup> center lies near the states introduced by the adsorbed ochratoxin. Importantly,  
527 the states introduced by OTA lie in the bandgap of NV<sup>-</sup> center lowering its width and changing the  
528 optical absorption and emission spectrum of the center.

529 The present study provides detailed information on the binding affinity, the mode of binding interaction,  
530 the main forces of OTA–ct DNA interactions, and the structure of OTA–DNA complex, which enables  
531 the further elucidation of the DNA degradation mechanism.

532

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