

Nanostructured TiO₂ Catalyzed Oxidations of Caffeine and Isocaffeine and Their Cytotoxicity and Genotoxicity Towards Ovarian Cancer Cells

Xiaoxi Huang · Anandarup Goswami · Xiaoxin Zou ·
Stephanie Hayes · Vatsal Shah · Tamara Minko ·
Zhimin Tao · Tewodros Asefa

Published online: 11 January 2014
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Abstract Although caffeine is well-known as an antioxidant and a psychoactive stimulant, its antioxidative properties and biological activities under various conditions are still largely unknown. The same can be said about caffeine's isomer, isocaffeine, whose properties have been even less investigated. Furthermore, much remains unknown about the potential biological effects and anticancer properties of the oxidative products of caffeine and isocaffeine that can be formed in solutions under different conditions. Here, the oxidations of caffeine and isocaffeine in the presence of TiO₂ nanoparticles under ultraviolet (UV) irradiation are studied in different solvents [distilled water (dH₂O), phosphate-buffered saline (PBS), and ethanol] using ultraviolet-visible spectroscopy, ¹H NMR spectroscopy,

and electrospray ionization mass spectrometry. When exposed to colloidal TiO₂ nanoparticles and UV light, both caffeine and isocaffeine undergo oxidations in PBS and dH₂O. Moreover, in both cases the rates of their oxidations are much higher in PBS than in dH₂O. However, neither caffeine nor isocaffeine undergoes catalytic oxidations in ethanol under otherwise similar conditions. Compared with caffeine and isocaffeine, their oxidized products exhibit higher cytotoxicity and genotoxicity towards ovarian cancer cells. On the other hand, caffeine and its oxidized species show higher cyto- and geno-toxicity than isocaffeine and its oxidized products, respectively. This latter result clearly indicates that the simple structural difference by one methyl group in the xanthine backbone of these molecules causes these two molecules to exhibit distinct antioxidative properties and unique biological activities.

Electronic supplementary material The online version of this article (doi:10.1007/s12668-013-0120-7) contains supplementary material, which is available to authorized users.

X. Huang · A. Goswami · X. Zou · S. Hayes · Z. Tao (✉) ·
T. Asefa (✉)

Department of Chemistry and Chemical Biology, Rutgers, The State University of New Jersey, 610 Taylor Road, Piscataway, NJ 08854, USA

e-mail: zhimin.tao@rutgers.edu

e-mail: tasefa@rci.rutgers.edu

A. Goswami · X. Zou · Z. Tao · T. Asefa

Department of Chemical and Biochemical Engineering, Rutgers, The State University of New Jersey, 98 Brett Road, Piscataway, NJ 08854, USA

V. Shah · T. Minko

Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA

T. Asefa

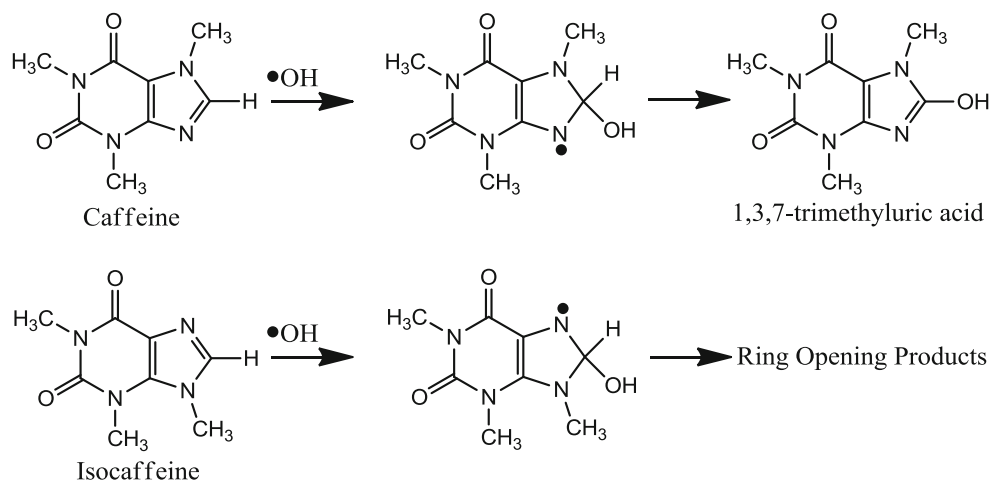
The Institute for Advanced Materials, Devices and Nanotechnology (IAMDN), Rutgers, The State University of New Jersey, 607 Taylor Road, Piscataway, NJ 08854, USA

Keywords Titanium dioxide nanoparticles · Photo-induced oxidation · Caffeine · Isocaffeine · Cytotoxicity · Genotoxicity · Ovarian cancer cells

1 Introduction

Caffeine, which is also chemically known as 1,3,7-trimethylxanthine (Scheme 1), is a purine-based alkaloid that exhibits a diverse range of biological activities. For example, while caffeine can function as a psychoactive stimulant to the central nervous system in mammals [1], it can also induce severe mitochondrial dysfunction, and sometimes cell death by increasing the concentration of cellular cyclic AMPs and flooding the cytoplasm with Ca²⁺ ions [2–7]. Moreover, caffeine can sabotage DNA repair processes by promoting the degradation of nucleotides [8], and this effect can be exploited or combined with many other DNA-targeting anticancer drugs for enhancing the therapeutic effects of the latter [9, 10]. Other

Scheme 1 Caffeine and isocaffeine undergo different oxidation processes when catalyzed by free radicals in solutions [18–20]. These processes can be expected to accelerate, or at least change, on the surfaces of photocatalytically active nanostructured colloidal particles, such as TiO₂; this was used as the hypothesis behind our present study



recent studies showed that caffeine can play an important role in minimizing the risks of some types of cancer and cardiovascular diseases by serving as an effective scavenger of reactive oxygen species (ROS), such as singlet oxygen and hydroxyl ($\bullet\text{OH}$) radicals [11–13]. This property of caffeine has actually recently generated a lot of interest among the scientific community as well as ordinary people, particularly in the context of effects of caffeinated drinks in the fight against cancers [14]. Despite some of these known properties of caffeine, our fundamental understanding of the antioxidant properties of caffeine is still in its infancy and requires further intensive studies, especially to unravel the interplay between its chemical structure and antioxidative properties as well as so-caused biological activities.

Similarly, little or even less is known about the antioxidative properties and the oxidation metabolites of isocaffeine—the 9-substituted isomer of caffeine, chemically known as 1,3,9-trimethylxanthine (Scheme 1). Moreover, there is little information available about its bioactivity (e.g., possible toxicity) [15] and none about the nature of its oxidation products, especially those formed with the aid of photoactive colloidal nanoparticles, and their corresponding biological activities.

Previous reports revealed that caffeine and isocaffeine undergo different oxidation processes in aqueous solutions as depicted in Scheme 1 [16, 17]. Generally, as stated earlier, caffeine tends to undergo oxidation by scavenging $\bullet\text{OH}$ radicals and then forming its corresponding radical intermediates while leaving its purine ring intact [16, 17]. On the other hand, isocaffeine tends to undergo oxidation through different routes and via ring-opening reactions, affording ring-opened intermediates and products [18–20]. By taking these pieces of information into consideration, we hypothesized that, in the presence of photoactive or photocatalytic nanomaterials such as TiO₂ nanoparticles, caffeine and isocaffeine could undergo more accelerated oxidation processes compared with their oxidation under normal conditions or in the absence of such

nanoparticles. More importantly, we hypothesized that the photoactive nanoparticles-induced oxidation products of caffeine and isocaffeine could exhibit different biological activities when compared with each other as well as their respective parent compounds before oxidation (i.e., caffeine and isocaffeine).

We herein investigated the oxidation reactions of caffeine and isocaffeine in the presence of photoactive colloidal nanoparticles and ultraviolet (UV) irradiation in different solvents [distilled water (dH₂O), phosphate-buffered saline (PBS), and ethanol]. Our results indicated that the two compounds went through distinct oxidation processes under these conditions. The rates of their oxidations upon exposure to UV irradiation and photo-activated colloidal TiO₂ nanoparticles were found to rely on the types of solvents used: The fastest catalytic oxidation was obtained in PBS, while no oxidation occurred in ethanol. Both caffeine and isocaffeine and their oxidized products also showed different biological activities, as observed from their distinct cytotoxicity and genotoxicity towards ovarian cancer cells. Notably, compared with their unoxidized counterparts, the oxidized products of caffeine or isocaffeine obtained through TiO₂ nanoparticle-photocatalyzed oxidation processes showed higher toxicity towards A2780 ovarian cancer cells.

2 Materials and Methods

2.1 Materials and Instrumentation

Caffeine, dimethyl sulfoxide and ethyl methanesulfonate (EMS) were obtained from Sigma-Aldrich. Isocaffeine was obtained from Santa Cruz Biotechnology. Cell counting kit-8 was purchased from Dojindo Laboratory. P25 TiO₂ nanoparticles were obtained from Evonik/Degussa. Ultraviolet–visible (UV-vis) spectra were measured with a Lambda 850 UV-Vis-NIR spectrometer (PerkinElmer). Solution proton nuclear

magnetic resonance spectra (^1H NMR) were acquired using 400 and 500 MHz Varian VNMRs spectrometers, and the spectra were recorded in parts per million (ppm) using D_2O (at 4.79 ppm) as an internal standard. The electrospray ionization mass spectrometry (ESI-MS) spectra were obtained with a Finnigan LCQ-DUO mass spectrometer equipped with Xcalibur software.

2.2 Photocatalytic Oxidation of Caffeine and Isocaffeine

First, different concentrations of caffeine and isocaffeine (0–150 μM) were freshly prepared in water, PBS, and ethanol, respectively, and their absorption spectra were immediately recorded from 800 to 190 nm wavelength. Using the results, standard or calibration plots of absorbance versus concentration for both caffeine and isocaffeine were obtained. Then, a series of experiments were performed to determine the oxidations of 100 μM caffeine and isocaffeine in three different solvents (dH_2O , PBS, and ethanol) with or without UV irradiation (125 W high-pressure mercury lamp with light intensity 90 mW/cm^2) and with or without nanostructured TiO_2 particles (0.5 mg/mL , P25). Some of these experiments were conducted to serve as control experiments. The mercury lamp we used here is widely employed as UV light source for similar experiments, and it can give off different wavelengths of ultraviolet light with wavelengths of 254, 303, 334, 365, and 366 nm in its emission spectrum. As these wavelengths of light match with the band gap of P25 TiO_2 nanoparticles, they can be absorbed by P25 TiO_2 nanoparticles and enable the nanoparticles to serve as photocatalyst for oxidation reactions. Quantitative information about the extent of oxidation was obtained by collecting samples from the mixtures at different time intervals and then separating the supernatants via centrifugation and measuring their UV-vis absorbance spectra.

2.3 Preparation of Oxidized Caffeine and Isocaffeine by Lyophilization

To prepare oxidized caffeine and isocaffeine solutions, 200 μM caffeine (or isocaffeine) was irradiated by UV light in the presence of 0.5 mg/mL P25 TiO_2 nanoparticles for different time periods. After 3 and 6 h, 200 μL of the solution was taken and centrifuged, and the UV-vis spectra of the supernatants were recorded to obtain the percentage of caffeine or isocaffeine left unoxidized in the solutions. The photocatalytically oxidized caffeine or isocaffeine solutions after 6 h of UV irradiation were then centrifuged to remove P25 TiO_2 nanoparticles. The supernatants were separated, frozen at -20 $^\circ\text{C}$ for overnight, and lyophilized to remove the solvents. The lyophilized powder containing oxidized caffeine or isocaffeine products were redissolved in dH_2O to give a solution with a concentration that corresponds to 25 mM unoxidized caffeine or isocaffeine.

2.4 Cytotoxicity Assessment

For cytotoxicity tests, 100 $\mu\text{L}/\text{well}$ cell-free medium or cell suspension was distributed into a 96-well plate, with a row of six wells under identical conditions for statistical purposes (i.e., $n=6$). A2780 cells were seeded at the density of 6,000 cells per well overnight. Caffeine, isocaffeine, their individually oxidized product, or the supernatant obtained after centrifugation of the suspension of the TiO_2 nanoparticles (a control sample) was then added into the cell media until the final concentration reached 5 mM corresponding to oxidized caffeine or isocaffeine (or a concentration equivalent to 5 mM of their unoxidized parent compounds). For a positive control experiment, 20 μM cisplatin was used. After incubating the cells at 37 $^\circ\text{C}$ with 5 % CO_2 for 24 h, 10 μL WST-8 reagent from a cell-counting kit was added into each well to measure cell viability.

2.5 Genotoxicity Assessment

The genotoxicity of caffeine, isocaffeine, and their oxidized products was determined by following a previously reported protocol [21, 22]. First, A2780 cells were seeded in six-well plate with 10,000 cells per well for 24 h. Then, ethyl methanesulfonate, caffeine, isocaffeine, and the oxidized products of caffeine and isocaffeine were incubated with the cells at 37 $^\circ\text{C}$ with 5 % CO_2 for 3 h. After this time, the medium was replaced with a fresh culture solution and incubation of the cells continued for 21 more hours. The ethyl methanesulfonate, which possesses a well-known genotoxicity to cells, was used as positive control, whereas fresh medium was employed as a negative control. At the end of 24 h, all the cells were washed with PBS and fixed with cold anhydrous methanol for 5 min. After removing the methanol, the nuclei in the cells were stained with 4',6-diamidino-2-phenylindole for 8 min. Finally, the solution was discarded and the cells were washed three times with PBS containing 0.05 % Tween 20. The PBS was used here not only to wash the samples but also to keep them moist enough so that the cells could be visualized with a fluorescence microscope and their micronuclei could be counted.

3 Results and Discussion

We investigated the photocatalytic oxidation caffeine and isocaffeine in presence of P25 TiO_2 nanoparticles (Degussa/Evonik), which are composed of 75 % anatase and 25 % rutile (see [Supplementary Materials](#) and [Fig. S1](#) for more details) [23, 24]. P25 TiO_2 was chosen because it has been widely shown to have excellent photocatalytic activity, in addition to being biocompatible, inexpensive, and stable under many different experimental conditions [25, 26]. When irradiated

with a high-pressure mercury light, colloidal P25 TiO₂ nanoparticles can readily absorb ultraviolet light and generate electron–hole pairs (also called excitons or charge carriers). These charge carriers can then react with the solvent molecules or other molecules in the solution, leading to reductive and oxidative processes, respectively. The photo-oxidative catalytic reactions, in particular, take place when the holes in the valence band of the P25 TiO₂ nanoparticles react with the molecules in solution (often solvent molecules), and subsequently generate free radicals that can oxidize substances like caffeine or isocaffeine.

In our experiments, first, different concentrations of caffeine and isocaffeine (0–150 μM) were freshly prepared in distilled water (dH₂O), PBS, and ethanol, respectively, and their UV-vis spectra were scanned between 800 and 190 nm. The results are shown in Figs. S1–S3 and Table S1. Only the spectra in dH₂O are described in detail below as the spectral patterns and absorption maxima of caffeine and isocaffeine in ethanol and PBS remain fairly similar to their corresponding spectra in dH₂O, except their peak intensities change differently as a function of time in the three cases.

In almost all the spectra of caffeine in dH₂O, two prominent absorbance peaks, at ~205 and ~273 nm, were observed. In addition, a shoulder in the wavelength region of 225–240 nm was evident in the spectra. However, a small red shift in the absorbance peaks, particularly the one at ~205 nm, was seen when the concentration of caffeine was increased. The red shift was more obvious especially for the caffeine solutions prepared in PBS and ethanol. This red shift can be attributed to the thermodynamically-driven self-assembly or π – π^* stacking of caffeine molecules when they are in relatively higher concentration in solution [17, 27, 28]. Because of this slight red shift in the absorbance maxima of caffeine, we adopted an integrated absorbance of the band, i.e., the sum of intensities under a number of wavelengths, instead of the intensity of a single peak in the spectrum to probe the changes in caffeine concentration. The inset in Fig. S1A shows the graphs of summed intensities for the band in the ranges of 205–210 nm (solid squares), 225–235 nm (solid triangles), and 270–275 nm (empty squares) as a function of concentration of caffeine. The integrated intensities in each case gave linear graphs with positive slopes with respect to the concentration of caffeine except in the ranges of 205–210 nm, in which case the peaks were beyond the maximum absorbance values of the instrument and thus unmeasurable. For the band in the range of 225–235 nm and 270–275 nm (solid triangles and empty squares, respectively), the integrated intensities also increased as the concentration of caffeine increased. Graphs of sum of intensities for each individual band vs. concentration of caffeine were made and the curves were fitted into a linear function passing through zero. The results obtained after curve fitting were compiled in Table S1.

Compared with the UV-vis spectra of caffeine, those of isocaffeine in dH₂O displayed some differences. First, whereas the spectra of caffeine exhibited an absorbance shoulder in the region of 225–240 nm, the spectra of isocaffeine showed a weak peak in the region of 235–240 nm. Here, also, the intensities from 235 to 240 nm, rather than a single peak's intensity, were summed up, and the summed intensity in the band ($S_{235-240}$) vs. concentration of isocaffeine was then fitted into a linear function. The results of the linear fit are compiled in Table S1. Second, the longer wavelength band in the spectra of isocaffeine appeared at a lower wavelength of ~268 nm (*cf.* the corresponding longer wavelength band for caffeine appeared at ~273 nm). The sum of intensities in the range between 265 and 270 nm was plotted as a function of concentration of isocaffeine, and the graph was then linearly curve-fitted (dashed line). The results are also compiled in Table S1. Furthermore, although the short wavelength band of isocaffeine in dH₂O appeared in a similar position in the range of ~200–294 nm as that of caffeine, its absorption maximum generally appeared at a smaller wavelength than that of caffeine. For example, 60 μM of caffeine showed peaks at 206 and 273 nm, while 60 μM of isocaffeine gave absorbance maxima at 204 and 268 nm. This difference can be attributed to the minor structural difference in the relative positions of the methyl groups in the two molecules. Specifically, compared with the methyl (–CH₃) groups in caffeine, the ones in isocaffeine, particularly the one in the xanthine moiety, lead to more resonance effects that contribute to more participation of C=N groups in the electronic structure of isocaffeine molecules [29]. This, in turn, results in the stabilization of isocaffeine molecules in their ground states. Thus, when excited, the augmented energy gap in the isocaffeine molecule drives a hypsochromic (red) shift in their absorption spectra [29]. The observed red shift in the absorbance maxima of the band in ~205–210 nm region with the increase of concentration can be due to the mutually vertical stacking of the planar rings of isocaffeine molecules in the same way as those of caffeine, as discussed above. Graphs of the summed intensities vs. the concentration of isocaffeine graphs were fitted into linear functions, and the results obtained from the curve fitting are compiled in Table S1.

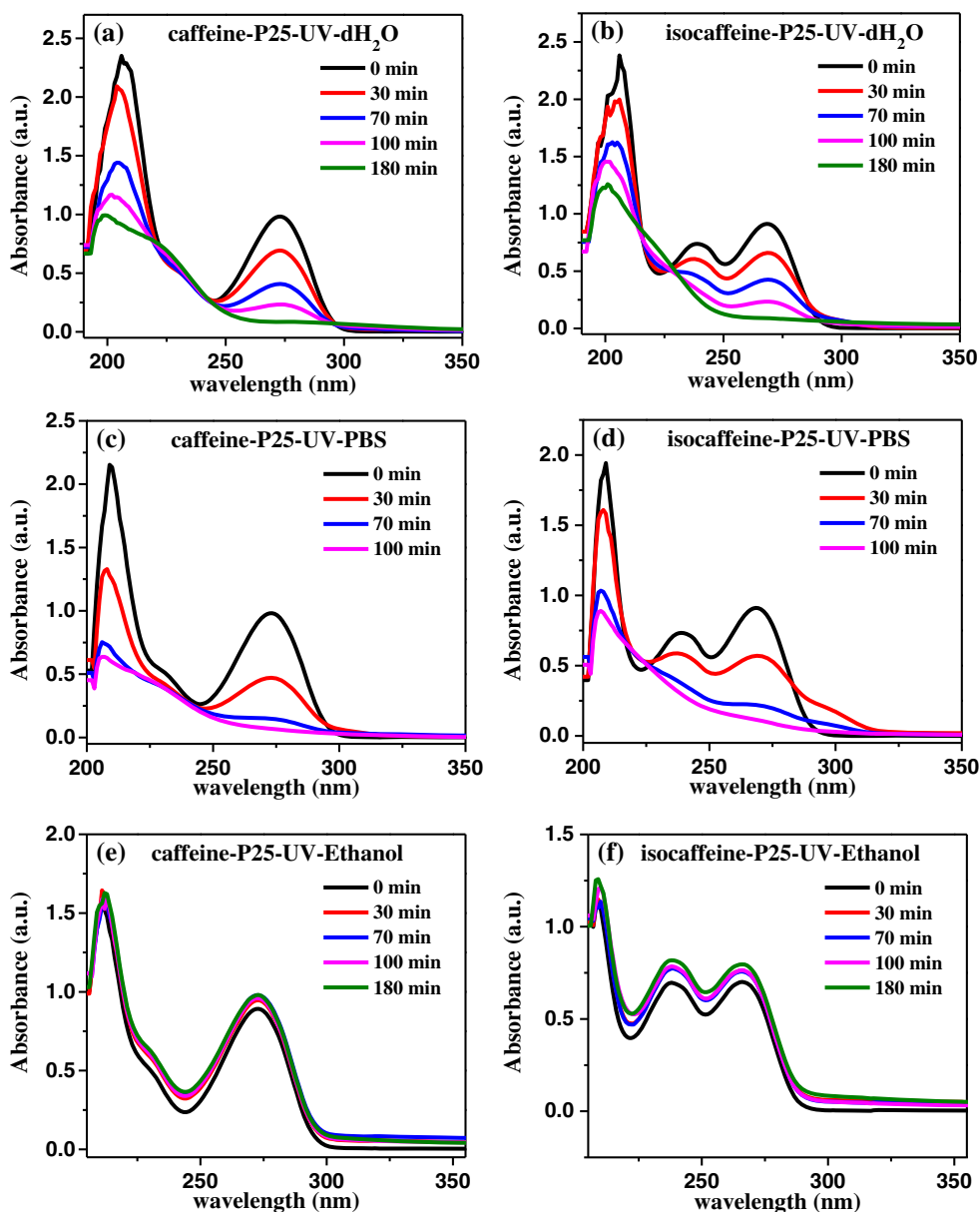
All these differences in the properties between caffeine and isocaffeine underline how the structural variations, or more specifically the spatial position of –CH₃ group in the xanthine skeleton of the two molecules, can significantly affect the physicochemical properties of these bioactive molecules.

Next, a series of experiments involving oxidations of 100 μM caffeine and isocaffeine with or without UV irradiation (90 mW/cm²) and with or without P25 TiO₂ nanoparticles (0.5 mg/mL) in the three different solvents was conducted. To monitor the progress of reactions, samples were taken from the solutions in different time intervals and centrifuged, and

the respective supernatants were then carefully separated and measured by UV-vis spectroscopy. The results and the absorption profiles are depicted in Fig. 1 (as no significant absorbance from 350 to 800 nm was observed in all the cases, only those between 190 and 350 nm are displayed). Generally, it was found that the oxidations of caffeine and isocaffeine occurred only in the cases where P25 TiO₂ nanoparticles were present in the solution and the solution was exposed to the UV irradiation. Moreover, the rates of oxidation of both compounds were found to be highly dependent on the type of solvent used for making the solution, as discussed in detail below. These results clearly suggest that caffeine and isocaffeine are susceptible to P25 TiO₂-assisted photocatalytic oxidation, and their oxidative processes are heavily dependent on the solvents present in the solution.

In dH₂O, P25 TiO₂ nanoparticles and UV irradiation caused both caffeine and isocaffeine to undergo quick oxidations as confirmed by the changes occurring in their absorption spectra (Fig. 1). Caffeine showed significant decrease in the absorbance of its two peaks at ~205 and ~273 nm upon exposure to P25 TiO₂ and UV light for 3 h (Fig. 1a). Specifically, its band at shorter wavelength became only 39.0 % as big as its original band, while its band at higher wavelength almost completely disappeared. In the case of isocaffeine in dH₂O, after 3 h of UV irradiation in presence of P25 TiO₂, isocaffeine’s two characteristic UV-vis absorbance peaks at ~235 and ~268 nm were barely visible, whereas its primary peak at ~205 nm decreased to 49.8 % of its original intensity (Fig. 1b). It is worth noting that the wavelength of the primary peak at ~205 nm in both cases also slightly blue-shifted after

Fig. 1 UV-vis spectra of caffeine or isocaffeine under different conditions. One hundred micromolars caffeine or isocaffeine was dissolved in different solvents (a, b are in dH₂O; c, d are in PBS; e, f are in ethanol), and the solution was mixed with 0.5 mg/mL P25 TiO₂ nanoparticles with magnetic stirring. While the solutions were exposed to UV irradiation, samples were taken at different time intervals as indicated, and measured by UV-vis spectroscopy



the photo-oxidation reactions. This is most likely due to the decrease in concentration, and thereby attenuated π - π^* stacking, of the caffeine or isocaffeine molecules [16, 17].

In PBS, similar oxidation features as those in dH_2O were observed for both caffeine and isocaffeine. However, the rates of oxidation of both caffeine and isocaffeine in the presence of P25 TiO_2 nanoparticles under UV irradiation were much faster in PBS than those in dH_2O (Fig. 1c, d). Upon UV exposure for 100 min, the intensity of the primary peaks of caffeine and isocaffeine at ~ 205 nm dropped to 39.3 and 50.0 %, respectively, of the intensity of the original peaks while the peak at a higher wavelength completely disappeared. In contrast, both caffeine and isocaffeine in ethanol underwent barely any change during the 3-h exposure to P25 TiO_2 /UV irradiation, with their absorbance peaks remaining almost unchanged (Fig. 1e, f). Please note that the slight fluctuation in intensity (a little higher than the original intensity) of the UV-vis absorbance bands in both cases could be due to the slight evaporation of ethanol by the heat generated due to UV irradiation and the concomitant gradual increase in the concentration of the solutions.

Hydroxyl ($\bullet\text{OH}$) radicals are often suggested to be the primary oxidant critically responsible for typical photocatalytic reactions catalyzed by nanostructured TiO_2 under UV irradiation. Thus, the fact that both caffeine and isocaffeine underwent faster oxidations in PBS than in dH_2O , but none in ethanol, is most likely to do with how readily $\bullet\text{OH}$ radicals form and/or remain stable in each case. In aqueous solutions (particularly in PBS), the facile formation of $\bullet\text{OH}$ radicals [30, 31] could make the oxidation of caffeine or isocaffeine quite feasible. However, in the protic organic solvent like ethanol, such $\bullet\text{OH}$ radicals either do not form or could get quickly quenched by the large amount solvent molecules before they have the chance to react with the caffeine or isocaffeine molecules.

Further information about the presence and identity of oxidized products of caffeine and isocaffeine, after being treated with P25 TiO_2 nanoparticles under UV irradiation, was obtained using ^1H NMR spectroscopy or probing the ^1H chemical shifts associated with the reactants and products in ^1H NMR spectra. For example, the ^1H NMR spectra of 10 mM of pure caffeine and isocaffeine in D_2O (Figs. S5 and S6) showed chemical shifts at 7.90, 3.95, 3.52, and 3.34 ppm and at 7.65, 4.01, 3.97, and 3.37 ppm, respectively. After the respective solutions underwent oxidations, catalyzed by P25 TiO_2 nanoparticles under UV irradiation, the aliphatic region of the spectra changed or became quite complex (Figs. S7–S10), suggesting the presence of many oxidized species in both cases. In the case of caffeine, a singlet peak at 8.46 ppm, which corresponds to pyridinic proton in the tautomer of 1,3,7-trimethyluric acid, was occasionally observed. This peak was not consistently observed because of the highly exchangeable nature of this proton in D_2O and the

very low concentration of the product(s) in the NMR tubes. Further study using electrospray ionization mass spectrometry (ESI-MS) indicated the presence of 1,3,7-trimethyluric acid, along with other possible oxidized products in photocatalyzed caffeine solutions (Fig. S11 and Table S2). The ESI-MS study also confirmed the presence of many types of oxidized isocaffeine products in the P25 TiO_2 photo-oxidized isocaffeine solution (Fig. S12 and Table S2).

To test the biological activities of the nanostructured TiO_2 /UV light-assisted photo-oxidized caffeine and isocaffeine species towards cancer cells, we performed cytotoxicity and genotoxicity experiments (Fig. 2). For these tests, we

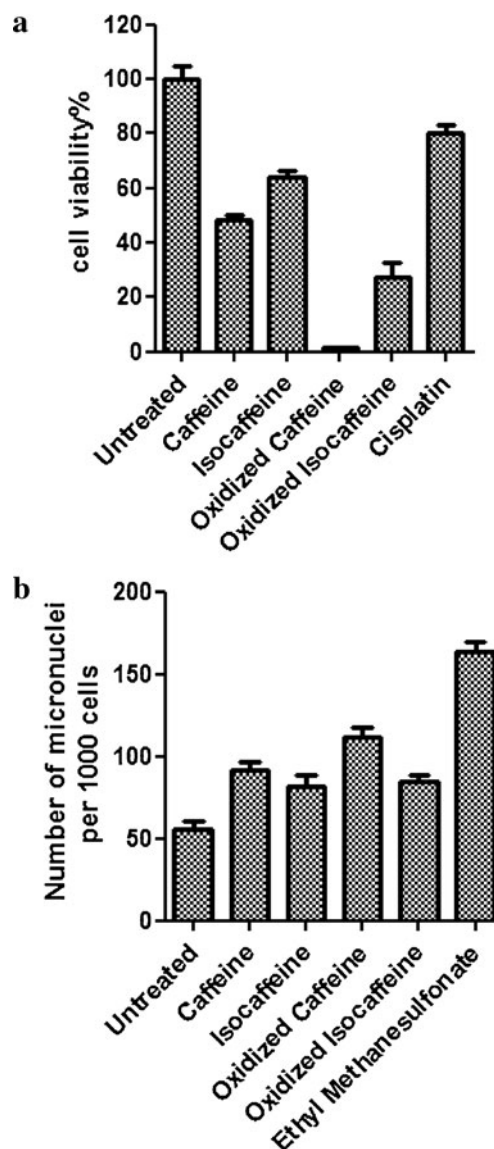


Fig. 2 **a** Cell viability of A2780 ovarian cancer cells and **b** The number of micronuclei per 1,000 cells under different conditions, as indicated on X-axes: when the cells were (1) untreated; or after the cells were treated with (2) caffeine (5 mM), (3) isocaffeine (5 mM), (4) oxidized caffeine (5 mM), and (5) oxidized isocaffeine (5 mM); and (6) cisplatin (20 μM) in **a** or (7) ethyl methanesulfonate (400 $\mu\text{g}/\text{mL}$) in **b**. Mean \pm SD are shown ($n=6$ for **a**; $n=3$ for **b**), $p<0.05$ in all the treated cells compared with the untreated ones

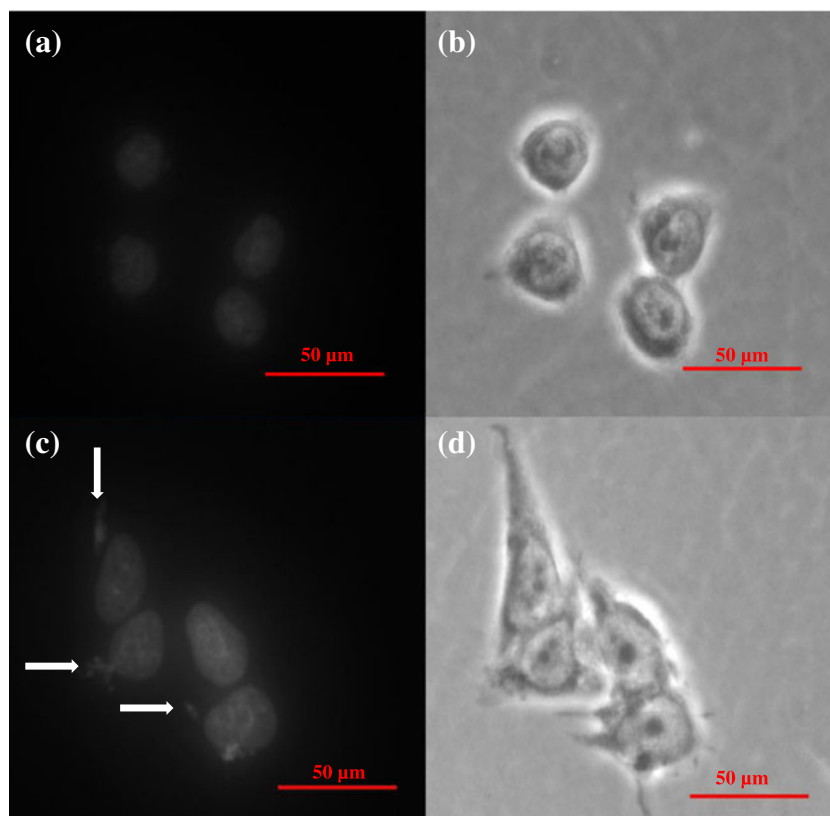
purposely chose A2780 ovarian cancer cells because there was precedence that caffeine intake could reduce the risk of ovarian cancers [32]. Before the tests though, appropriate samples were prepared as follows. First, 200 μM caffeine (or isocaffeine) was mixed with 0.5 mg/mL P25 TiO_2 . The solution was then irradiated with UV light for 6 h under stirring to induce the photocatalytic oxidations of caffeine (or isocaffeine). This was followed by centrifugation to remove the nanoparticles and lyophilization to concentrate the supernatant. The resultant lyophilized/powdered materials were redissolved in dH_2O to make high concentration stock solutions, as described below. For control experiments, the same lyophilization procedure was adopted to afford two other samples: (1) a solution containing 200 μM caffeine (or isocaffeine) (i.e., with no nanoparticles) and (2) the supernatant obtained after centrifugation of 0.5 mg/mL P25 TiO_2 after 6-h UV exposure. It is worth noting here that in our experiment, the oxidized products were used immediately after lyophilization, or kept at 4 $^\circ\text{C}$ to maintain their stability until further use. NMR spectroscopy showed that the oxidized products before and after lyophilization (after being redissolved in the same solvent) have no significant difference. The lyophilized samples in duplicate NMR tests also showed no difference. In other words, the oxidized products were fairly stable after lyophilization. The lyophilized samples were redissolved in dH_2O to make stock solutions

corresponding to the 25 mM unoxidized caffeine (or isocaffeine). These different solutions were then incubated with A2780 ovarian carcinoma cells with a final concentration corresponding to 5 mM unoxidized caffeine (or isocaffeine) for 24 h. The dosage of the oxidized products used for the cell treatment was normalized according to the corresponding/equivalent concentration of the original compounds before oxidation. For example, 5 mM oxidized caffeine here represents the oxidized products obtained from 5 mM caffeine.

The cytotoxicity results are shown in Fig. 2a. The results indicate that whereas untreated cells remained $100 \pm 4.9\%$ viable, those treated with 5 mM caffeine or isocaffeine gave $48.5 \pm 3.0\%$ and $64.2 \pm 3.8\%$ cell viability, respectively (please note that for caffeine-induced apoptotic cell death in a mouse epidermal cell line, IC_{50} was reported to be 2.7 mM [33]). In contrast, cells treated with 20 μM cisplatin had $80.3 \pm 4.7\%$ viability (please note that the IC_{50} of cisplatin for ovarian cancer cells is generally in the order of μM) [34, 35].

The viability of the cells treated with oxidized caffeine significantly dropped, reaching only $1.3 \pm 0.3\%$ (*cf.* the viability of the cells treated with caffeine is much higher or $48.5 \pm 3.0\%$) (Fig. 2a). In parallel, the viability of the cells treated with oxidized isocaffeine dropped to $27.6 \pm 5.4\%$ (lower than that of isocaffeine-treated cells), but the value still remained much higher than the viability of the cells treated with oxidized caffeine. This result clearly reveals that the oxidized

Fig. 3 Representative fluorescence microscopy images of A2780 cells incubated with media only (**a, b**) or ethyl methanesulfonate (**c, d**) for 3 h. The *arrows* indicate the typical formation of micronuclei. **a, c** Fluorescent images stained by 4',6-diamidino-2-phenylindole (DAPI) and **b, d** bright-field images

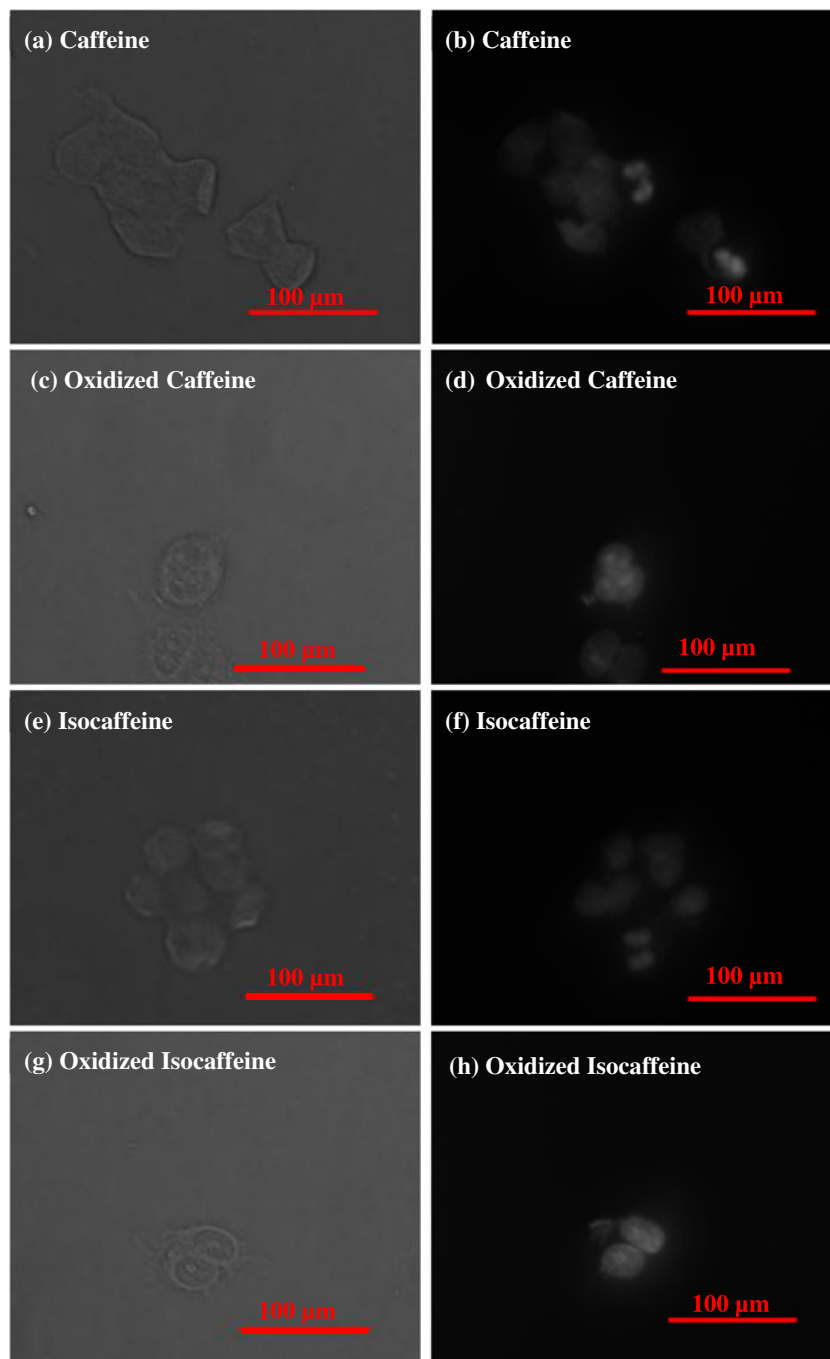


caffeine and isocaffeine species exhibit a more severe cytotoxicity to ovarian A2780 cells than their parent compounds. Moreover, the result indicates that the oxidized products obtained from caffeine induce higher cytotoxicity than those obtained from isocaffeine. It is worth noting here that the control experiment involving the supernatant collected from the suspension of TiO₂ under UV light irradiation for 6 h was not toxic to cells on its own as it gave cell viability of 116±7.3 %. This result thus rules out the possibility that the ROS possibly forming in the UV-exposed P25 TiO₂ solution might

have directly caused the substantial cytotoxicity exhibited by P25 TiO₂ photo-oxidized caffeine or isocaffeine solutions.

By following a previously reported protocol [21, 22], genotoxicity tests were then performed by examining the formation, and if so, the number of micronuclei in the cells after treatment with caffeine, isocaffeine or their corresponding oxidative products generated with the aid of P25 TiO₂/UV light. Generally, micronucleus is produced when a chromosome or a segment of a chromosome does not merge with one of the daughter nuclei during cell mitosis, resembling a

Fig. 4 Genotoxicity results and fluorescent images of A2780 cell after incubating with different samples: **a, c, e, and g** bright field and **b, d, f, and h** fluorescence field images



miniature nucleus in its shape [21, 22]. Once DNA was stained by fluorescent dyes, microscopic observation can be adopted to differentiate the micronucleus from normal cell nucleus based on their obvious size difference (Figs. 3 and 4). As a control, EMS was added to demonstrate the significant genotoxicity in A2780 cells. The genotoxicity was then determined by counting the number of micronuclei per 1000 cells (%). The results are shown in Fig. 2b. After 3-h incubation, the micronuclei formed in untreated cells reached $56 \pm 5\%$, whereas cells treated with 5 mM caffeine or isocaffeine showed a higher number of micronuclei, i.e., $91 \pm 5\%$ or $82 \pm 7\%$, respectively. In contrast, the photocatalytically oxidized caffeine or isocaffeine at an original concentration of 5 mM resulted in $112 \pm 6\%$ or $85 \pm 4\%$ micronuclei, respectively. The experiments were also repeated after longer incubation times; however, the higher cell death under this condition made quantification of the micronuclei formed or determination of their number difficult, as the measurement requires removal of apoptotic cells prior to sample preparation or DNA staining.

4 Conclusions

In summary, we investigated the photocatalytic oxidation properties of caffeine and isocaffeine in the presence of nanostructured TiO₂ particles and UV irradiation in different aqueous or organic solvents, including dH₂O, PBS, and ethanol. While both caffeine and isocaffeine underwent faster oxidations in PBS and slower oxidations in dH₂O in the presence of TiO₂ nanoparticles and under exposure to UV irradiation, they both did not undergo any catalytic oxidation in ethanol under otherwise similar conditions. The resulting oxidized products of caffeine or isocaffeine were shown to have higher cytotoxicity as well as genotoxicity on A2780 ovarian cancer cells than their unoxidized counterparts. It is also worth noting that caffeine or oxidized caffeine products were more cyto- and geno-toxic than their isocaffeine counterparts.

Acknowledgments TA gratefully acknowledges the financial assistance of the US National Science Foundation (NSF) under grant nos. NSF DMR-0968937, NSF NanoEHS-1134289, NSF-ACIF, and NSF Special Creativity grant.

References

- Fisone, G., Borgkvist, A., Usiello, A. (2004). Caffeine as a psychomotor stimulant: Mechanism of action. *Cellular and Molecular Life Sciences*, 61, 857–872.
- Rousseau, E., Ladine, J., Liu, Q.-Y., Meissner, G. (1988). Activation of the Ca²⁺ release channel of skeletal muscle sarcoplasmic reticulum by caffeine and related compounds. *Archives of Biochemistry and Biophysics*, 267, 75–86.
- Cavallaro, R. A., Filocamo, L., Galuppi, A., Galione, A., Brufani, M., Genazzani, A. A. (1999). Potentiation of cADPR-induced Ca²⁺-release by methylxanthine analogues. *Journal of Medicinal Chemistry*, 42, 2527–2534.
- Bauer, C. S., Simonis, W., Schonknecht, G. (1999). Different xanthenes cause membrane potential oscillations in a unicellular green alga pointing to a ryanodine/cADPR receptor Ca²⁺ channel. *Plant and Cell Physiology*, 40, 453–456.
- Acosta, D., & Anuforo, D. (1977). Acute mitochondrial toxicity of caffeine in cultured heart cells. *Drug and Chemical Toxicology*, 1, 19–24.
- Sardao, V. A., Oliveira, P. J., Moreno, A. J. (2002). Caffeine enhances the calcium-dependent cardiac mitochondrial permeability transition: relevance for caffeine toxicity. *Toxicology and Applied Pharmacology*, 179, 50–56.
- Jones, E., Penefsky, H. S., Souid, A.-K. (2009). Caffeine impairs HL-60 cellular respiration. *Journal of Medical Sciences*, 2, 61–72.
- Selby, C. P., & Sancar, A. (1990). Molecular mechanism of DNA repair inhibition by caffeine. *Proceedings of the National Academy of Sciences of the United States of America*, 87, 3522–3525.
- Fingert, H. J., Chang, J. D., Pardee, A. B. (1986). Cytotoxic cell cycle and chromosomal effects of methylxanthines in human tumor cells treated with alkylating agents. *Cancer Research*, 46, 2463–2467.
- Shinomiya, N., Shinomiya, M., Wakiyama, H., Katsura, Y., Rokutanda, M. (1994). Enhancement of CDDP cytotoxicity by caffeine is characterized by apoptotic cell death. *Experimental Cell Research*, 210, 236–242.
- Shi, X., Dalal, N. S., Jain, A. C. (1991). Antioxidant behavior of caffeine: efficient scavenging of hydroxyl radicals. *Food and Chemical Toxicology*, 29, 1–6.
- Devasagayam, T. P., Kamat, J. P., Mohan, H., Kesavan, P. C. (1996). Caffeine as an antioxidant: inhibition of lipid peroxidation induced by reactive oxygen species. *Biochimica et Biophysica Acta*, 1282, 63–70.
- Lee, C. (2000). Antioxidant ability of caffeine and its metabolites based on the study of oxygen radical absorbing capacity and inhibition of LDL peroxidation. *Clinica Chimica Acta*, 295, 141–154.
- Kawasumi, M., Lemos, B., Bradner, J. E., Thibodeau, R., Kim, Y.-S., Schmidt, M., et al. (2011). Protection from UV-induced skin carcinogenesis by genetic inhibition of the ataxia telangiectasia and rad3-related (ATR) kinase. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 13716–13721.
- Donoso, P., O'Neill, S. C., Dilly, K. W., Negretti, N., Eisner, D. A. (1994). Comparison of the effects of caffeine and other methylxanthines on [Ca²⁺] in rat ventricular myocytes. *British Journal of Pharmacology*, 111, 455–458.
- Jorge, R., & Annia, G. (1976). Is caffeine a good scavenger of oxygenated free radicals? *Journal of Physical Chemistry B*, 115, 4538–4546.
- Cesaro, A., Russo, E., Crescenzi, V. (1976). Thermodynamics of caffeine aqueous solutions. *Journal of Physical Chemistry*, 80, 335–339.
- Dalmazio, I., Santos, L. S., Lopes, R. P., Eberlin, M. N., Augusti, R. (2005). Advanced oxidation of caffeine in water: On-line and real-time monitoring by electrospray ionization mass spectrometry. *Environmental Science and Technology*, 39, 5982–5988.
- Telo, J. P., & Vieira, A. J. S. C. (1997). Mechanism of free radical oxidation of caffeine in aqueous solution. *Journal of the Chemical Society, Perkin Transactions*, 2, 1755–1758.
- Vinchurkar, M. S., Rao, B. S., Mohan, H., Mittal, J. P., Schmidt, K. H., Jonah, C. D. (1997). Absorption spectra of isomeric OH adducts of 1,3,7-trimethylxanthine. *Journal of Physical Chemistry A*, 101, 2953–2959.
- Dayan, N., Shah, V., Minko, T. (2011). Preliminary evaluation of the genotoxic potential of a hydrophilic polymer with three preservation systems. *International Journal of Cosmetic Science*, 33, 497–502.
- Shah, V., Taratula, O., Garbuzenko, O. B., Patil, M. L., Savla, R., Zhang, M., et al. (2012). Genotoxicity of different nanocarriers:

- Possible modifications for the delivery of nucleic acids. *Current Drug Discovery Technologies*, 10, 8–15.
23. Reeves, P., Ohlhausen, R., Sloan, D., Pamplin, K., Scoggins, T., Clark, C., et al. (1992). Photocatalytic destruction of organic dyes in aqueous TiO₂ suspensions using concentrated simulated and natural solar energy. *Solar Energy*, 48, 413–420.
 24. Zou, X., Tao, Z., Asefa, T. (2013). Semiconductor and plasmonic photocatalysis for selective organic transformations. *Current Organic Chemistry*, 17, 1274–1287.
 25. Hakki, A., Dillert, R., Bahnemann, D. W. (2013). Arenesulfonic acid-functionalized mesoporous silica decorated with titania: a heterogeneous catalyst for the one-pot photocatalytic synthesis of quinolines from nitroaromatic compounds and alcohols. *ACS Catalysis*, 3, 565–572.
 26. Du, J., Lai, X., Yang, N., Zhai, J., Kisailus, D., Su, F., et al. (2001). Hierarchically ordered macro-mesoporous TiO₂-graphene composite films: Improved mass transfer reduced charge recombination and their enhanced photocatalytic activities. *ACS Nano*, 5, 590–596.
 27. Guttman, D., & Higuchi, T. (1957). Reversible association of caffeine and of some caffeine homologs in aqueous solution. *Journal of the American Pharmaceutical Association*, 46, 4–10.
 28. Gill, S. J., Downing, M., Sheats, G. F. (1967). The enthalpy of self-association of purine derivatives in water. *Biochemistry*, 6, 272–276.
 29. Yanuka, Y., & Bergmann, F. (1986). Spectroscopic studies on caffeine and isocaffeine. *Tetrahedron*, 42, 5991–6002.
 30. Tao, Z., Wang, G., Goodisman, J., Asefa, T. (2009). Accelerated oxidation of epinephrine by silica nanoparticles. *Langmuir*, 25, 10183–10188.
 31. Chen, H., Nanayakkara, C. E., Grassian, V. H. (2012). Titanium dioxide photocatalysis in atmospheric chemistry. *Chemical Reviews*, 112, 5919–5948.
 32. Tworoger, S. S., Gertig, D. M., Gates, M. A., Hecht, J. L., Hankinson, S. E. (2008). Caffeine alcohol smoking and the risk of incident epithelial ovarian cancer. *Cancer*, 112, 1169–1177.
 33. He, Z., Ma, W. Y., Hashimoto, T., Bode, A. M., Yang, C. S., Dong, Z. (2003). Induction of apoptosis by caffeine is mediated by the p53 Bax and caspase 3 pathways. *Cancer Research*, 63, 4396–4401.
 34. Moufarij, M. A., Phillips, D. R., Cullinane, C. (2003). Gemcitabine potentiates cisplatin cytotoxicity and inhibits repair of cisplatin-DNA damage in ovarian cancer cell lines. *Molecular Pharmacology*, 63, 862–869.
 35. Wang, Q. E., Milum, K., Han, C., Huang, Y. W., Wani, G., Thomale, J., et al. (2011). Differential contributory roles of nucleotide excision and homologous recombination repair for enhancing cisplatin sensitivity in human ovarian cancer cells. *Molecular Cancer*, 10, 24.