Synergistic Generation of Reactive Oxygen Species by Visible Light Activated TiO₂ and *S. Enterica* Interaction

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Abstract – A lot of human activities have negative impact on water quality and sometimes result in the biological water contamination. Currently used chemical (chlorine, ozone, and etc.) and physical (UV) water disinfection methods have strong environmental disadvantages or suffers from limited efficiency. To overcome these problems, scientists suggest to use photocatalyst activated advanced oxidation processes. One of the most studied photocatalysts which attracts a lot of research interest is titanium dioxide. TiO₂ application for the disinfection of water, air or surfaces is increasingly encouraged by researchers. However, to unlock its full potential it is highly desirable to make it suitable for the visible light activation. In the current study the effect of visible light assisted photocatalytic treatment to the outer membrane permeability of *Salmonella enterica* bacteria and how it changes under different titanium dioxide concentrations was analysed. The results from the treatment of relatively complex *Salmonella enterica* bacteria organism were compared to the visible light activated TiO₂ ability to oxidise considerably simpler objects like methylene blue molecules. The efficiency of TiO₂ photocatalytic disinfection process was evaluated using spread plate technique. Membrane permeability of the treated *Salmonella enterica* bacteria was determined by NPN uptake factor assay. Generation of intracellular reactive oxygen species was evaluated by Dichlorodihydrofluorescein diacetate fluorescence measurements. The key finding of this study was that intense wide spectrum visible light irradiation and TiO₂ powder synergistically inactivate *S. enterica* bacteria and halt its potential to form colonies. High amounts of intracellular reactive oxygen species could be seen as the main suspects for the observed inactivation of *S. enterica*.

Keywords – Disinfection; methylene blue; photocatalysis; *Salmonella enterica*; titanium dioxide; UV light; visible light

Nomenclature

AOPs	Advanced oxidation processes	–
CFU	Colony forming unit	–
DBPs	Disinfection by-products	–
DCFH-DA	Dichlorodihydrofluorescein diacetate	–
LB	Luria Bertani	–
MB	Methylene blue	–

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NPN	N-phenyl-1-naphthylamine	–
OD	Optical density	–
PMB	Polymyxin B	–
RFU	Relative fluorescence units	–
ROS	Reactive oxygen species	–
SP	Spread plate	–
UV	Ultraviolet light	–
Vis	Visible light	–

1. INTRODUCTION

The world is changing rapidly due to fast economic growth, industrial development and increasing population. At the same time the demand for clean water is increasing and it is one of the biggest challenges of the modern era. Ground waters, lakes and rivers are the major sources of drinking water. However, many of human activities have negative impacts on these water sources and result in the water contamination. Unfortunately, around the world one out of three people or 2.2 billion people in total lack safe drinking water [1] and one of the main causes is water contamination by pathogenic microorganisms and viruses. Water pollution alter biological, physical and chemical characteristics of the water and causes different diseases as cholera, typhoid, malaria, diarrhoeal infections, etc. [2]. Sedimentation, filtration and other water treatment methods are routinely used to remove solid and chemical contaminants. Along with these contaminants some microorganisms also are separated. But there are some specific methods to remove microorganisms from the water and they are known as disinfection methods.

Disinfection methods can be classified into physical (UV) and chemical (chlorine, ozone, and etc.). Unfortunately, both these groups have their limitations. Physical treatment, such as UV does not leave any active agents in treated water so it gives no protection against re-infection in the distribution network [3]. On the other hand, chemical treatments such as chlorination tend to form carcinogenic disinfection by-products (DBPs), and sometimes it also can be ineffective because there are some highly resistant waterborne pathogens. Due to the higher microorganisms' resistance to available disinfectants, higher doses of disinfectants are required, and that forms higher amounts of DBPs [4]. Fewer DBPs are formed by ozonation but this method has higher cost than chlorination and can form harmful bromates [3].

Reacting to the above-mentioned challenges scientists are working to find antibacterial agents which could effectively disinfect water pathogens without causing additional contamination by DBPs. Advanced oxidation processes (AOPs) have big potential to address these issues and attracts a lot of attention. AOPs involve in situ production of highly reactive and nonselective oxidants which are commonly known as reactive oxygen species or ROS. The most typical ROS examples are hydroxyl radicals ($\bullet\text{OH}$), superoxide radical anion ($\text{O}_2^{\bullet-}$), and hydrogen peroxide molecules (H_2O_2) which are capable to oxidise and mineralise a broad range of organic pollutants [5]. In aqueous solutions generation of usually short-lived ROS can be achieved by using specific catalysts and high energy irradiation. When photon energy surpasses photocatalysts band gap, some electrons are excited from the valence band to the conduction band. Negatively charged electrons (at conduction band) and positively charged holes (at valence band) enable chemical reactions which transforms neutral molecules (H_2O , O_2) and ions (OH^- , H^+) to ROS.

Titanium dioxide is arguably the most promising photocatalytic material. It is non-toxic and is widely used in various fields including water cleaning from organic pollutants, microorganisms and viruses [6]–[8]. Unfortunately, the band gap value of the pristine TiO_2 is larger than the energy of the visible light photons. In practice this means that unmodified TiO_2 can be excited only by the UV light. Meanwhile, ROS generation by using visible light is unattainable [9]. Photocatalytic efficiency of TiO_2 based catalysts usually is evaluated by using methylene blue dye and adopting relatively simple procedure described by the ISO 10678:2010 standard [10]. Efficiency assessment by photocatalytic treatment of live organisms such as bacteria is much more complicated. Accordingly, it is rarely used and up to now there is no common method which would be accepted as a golden standard for industrial or scientific laboratories.

Recently, we were experimenting with various TiO_2 modifications and several types of bacteria with the goal to develop new convenient bio-contamination based photocatalysis efficiency evaluation method. Interestingly, we accidentally noticed that under certain conditions combination of standard P25 TiO_2 powder and visible light source was capable to completely halt the formation of *Salmonella enterica* bacteria colonies. This observation was rather surprising. First, it was observed only for *Salmonella enterica*. Second, P25 TiO_2 powder is a well-documented, extensively used commercial product and is virtually inactive under visible light. We were not able to find any publications describing this phenomenon, therefore we decided to investigate it in more details. More specifically, we analysed the effect of visible light assisted photocatalytic treatment to the outer membrane permeability of *Salmonella enterica* bacteria and how it changes under different P25 titanium dioxide powder concentrations. The results from the treatment of relatively complex *Salmonella enterica* bacteria organism were compared to the UV and visible light activated TiO_2 ability to bleach considerably simpler objects like methylene blue molecules.

2. METHODOLOGY

2.1. Bacteria Cultivation

Gram-negative *Salmonella enterica* ser. Typhimurium SL1344 were obtained from prof. Séamus Fanning (Institute of Food and Health, University College Dublin, Ireland). For study cells were grown in 10 ml sterile Luria Bertani (LB) medium (LB-medium Roth, Karlsruhe, Germany) in 30 ml tube, incubated with shaking at 220 rpm for 18–22 h. The overnight culture was diluted with fresh LB medium to optical density (OD_{600}) of 0.15 and grown in 20 ml LB medium with shaking at 220 rpm until the OD_{600} of 0.8–1.0. The obtained bacterial suspension was centrifuged at 3000 g for 10 min at 4 °C (Heraeus™ Megafuge™ 16R, Thermo Scientific, Germany). The pellet was collected with 300 μl of PBS buffer, pH 7.4 (Roth, Karlsruhe, Germany).

2.2. Treatment of *S. enterica* Suspensions

For the photocatalytic treatment experiments we selected to use commercial P25 TiO_2 powder (Sigma-Aldrich, 99.5 % purity). P25 TiO_2 is a well know photocatalyst whose properties were widely reported in the literature [11], [12]. It has decent activity under UV light and is almost non-active under visible light irradiation [13].

Before each experiment TiO_2 powders were stored in the dark for at least 24 h. Then 4.5 mg or 15 mg of powders were added to 15 ml of the PBS buffer with supplement of glucose 0.1 % (pH 7.4) to reach TiO_2 concentration of 0.3 mg/ml or 1 mg/ml respectively. Magnetic stirring was used to saturate the medium with oxygen and to maintain homogeneous conditions.

Before the addition of *S. enterica* bacteria and their photocatalytic treatment, vessel with PBS buffer and photocatalyst was thermostated at 37 °C and irradiated by selected light source for 10 min. Lastly, nominally $2 \cdot 10^6$ cfu of *S. enterica* cells were added to the PBS buffer to obtain $3 \cdot 10^7$ cfu/ml initial bacteria concentration.

Photocatalytic treatment of *S. enterica* suspension was realised using UV (Thorlabs M365LP1-C1) and full spectrum visible (Vis) light (Thorlabs SOLIS-3C) LED sources. The distance from the light source to the suspension surface was 10 cm. The intensity of UV and visible light radiation was 30 mW/cm² and 40 mW/cm², respectively.

In order to identify individual and combined effects of irradiation and TiO₂ powder, three different treatment types were applied:

1. TiO₂ powder was not added to the *S. enterica* suspension and the treatment was done solely by light (treatment by light);
2. TiO₂ powder was added to *S. enterica* suspension but irradiation was not applied (treatment by TiO₂);
3. TiO₂ powder was added to the suspension and the light source was on (combined treatment).

In total 8 different treatment modes were applied (Table 1). In all cases individual control samples were prepared from the same day culture of *S. enterica* bacteria. Control samples were prepared at the same time as the corresponding test samples and were kept at identical conditions just without addition of TiO₂ and without irradiation by light.

TABLE 1. SUMMARY OF THE APPLIED TREATMENT MODES

Mode number	Treatment by one factor	Combined (photocatalytic) treatment
1	TiO ₂ powder (0.3 mg/ml)	
2	TiO ₂ powder (1.0 mg/ml)	
3	Ultraviolet light irradiation	
4	Visible light irradiation	
5		Ultraviolet light irradiation and TiO ₂ powder (0.3 mg/ml)
6		Ultraviolet light irradiation and TiO ₂ powder (1.0 mg/ml)
7		Visible light irradiation and TiO ₂ powder (0.3 mg/ml)
8		Visible light irradiation and TiO ₂ powder (1.0 mg/ml)

Efficiency of the process was evaluated by spread plate (SP) method after 60 min of treatment. Samples were diluted by PBS buffer (1250 times) and spread on Luria Bertani agar (LB-Agar, Roth, Karlsruhe, Germany) Petri dish (approximately 300 bacteria cells). For each treatment type three sets of samples (test + control) were obtained. Colony formation was calculated manually after 18–22 hours of incubation at 37 °C. In addition, after the treatment by TiO₂ and/or light irradiation as described above, the remaining bacteria suspensions from the vessels were centrifuged at 3000 g for 5 min at 4 °C (Heraeus™ Megafuge™ 16R, Thermo Scientific, Germany). The pellet was collected with PBS buffer, pH 7.4 to obtain $3 \cdot 10^8$ cfu/ml (OD₆₀₀ of 1). Such suspensions were further used for the outer membrane permeability estimation by NPN uptake factor assay and for the intracellular ROS formation (oxidative stress) evaluation by DCFH-DA fluorescence measurements.

2.3. NPN Uptake Assay

N-phenyl-1-naphthylamine (NPN) is a hydrophobic fluorescent probe. The fluorescence of NPN is significantly enhanced in a hydrophobic environment (e.g., membrane lipid bilayer),

rendering it an appropriate dye to probe outer membrane integrity of gram-negative bacteria [14]. It is known that TiO_2 can affect the integrity of bacteria outer membrane [15], therefore *S. enterica* cell wall permeabilization was assayed by the cellular uptake of NPN compound. After the treatment by TiO_2 and light samples of prepared bacteria suspensions (as described in 2.2) were mixed and transferred into 96-well flat-bottom black plates, 100 μl per well by adding 10 μM of NPN. The relative intensity of fluorescence (excitation 350 nm, emission 460 nm) was instantly measured in *TECAN GENios ProTM* (Männedorf, Switzerland) plate reader. The plate was shaken 5 s before each registration point. The 1-NPN cell uptake factor was calculated as a ratio between intensity of fluorescence values of the bacterial suspension incubated [14] with and without irradiation and TiO_2 . Three – six independent experiments were performed in triplicate.

2.4. DCFH-DA Fluorescence Measurements

Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, Germany) is good indicator of intracellular hydroxyl radicals ($\text{HO}\cdot$), hydrogen peroxide (H_2O_2) and peroxy radicals ($\text{ROO}\cdot$) [16]. DCFH-DA hydrolysis to DCFH was performed as described elsewhere [17]. Namely, 0.01 M of NaOH (Chempur, Poland) was added to 50 μM of DCFH-DA and incubated in the dark for 30 min at 37 °C temperature. After preparation of this probe, 100 μl of prepared bacteria suspensions (as described in 2.2) were mixed and transferred into 96-well flat-bottom black plates, 100 μl per well by adding 10 μM of DCFH. Relative intensity of the fluorescence was measured in *TECAN GENios Pro* (Männedorf, Switzerland) plate reader (excitation 492 nm, emission 535 nm) for 15 min. The plate was shaken 5 s before each registration point.

2.5. Statistical Analysis

The amount of colony forming units and NPN uptake factor values are indicated as means $\pm$ standard deviation. Methods of statistical analysis are described in our previous publication Kuliesiene *et al.* [7]. Tukey Test was used to pick out the significant differences within each group.

3. RESULTS

3.1. *S. enterica* Treatment by Individually Applied TiO_2 and UV/Vis Light Irradiation

The experiments were started by treating *S. enterica* cells only by adding TiO_2 powder or by applying UV/Vis light irradiation without addition of TiO_2 . NPN accumulation and *S. enterica* cells viability data for variously treated samples are shown in Fig. 1. The obtained results indicated that UV light increased the uptake of NPN by more than two times (Fig. 1A), meanwhile visible light and titanium dioxide just by half of that. Two different TiO_2 concentrations (1 mg/ml and 0.3 mg/ml) were used in order to determine the importance of TiO_2 factor for the efficiency of bacteria inactivation and to determine if higher concentration of TiO_2 directly affects the viability of bacteria. Colony formation test showed that 1 mg/ml of TiO_2 decreased the viability of *S. enterica* by 50 %, while 0.3 mg/ml – by 35 %. On the other hand, no significant difference was found between these two concentrations to NPN uptake into cell. Also, it should be noted, that UV light decreased the ability of bacteria to form colonies by approximately 20–30 %, while the NPN uptake to the cells treated by UV light was the highest amongst all tested samples. This means that, although UV light permeabilise the outer membrane stronger than TiO_2 , overall UV light has smaller effect on

bacteria ability to multiply than integral effects caused by TiO_2 .

Fig. 1. Effects of individually applied TiO_2 and UV/Visible light irradiation on *S. enterica* cells: A) Permeability of outer membrane; B) bacteria viability.

* – statistically significant difference compared to control group, using one-way ANOVA; ♦ – statistically significant difference compared with each other, using Tukey test, $p < 0.05$ ($n = 3$).

3.2. Photocatalytic Bacteria Inactivation

3.2.1. *S. enterica* treatment by the UV light activated TiO_2 powder

Efficiency of UV light activated photocatalytic *S. enterica* disinfection was measured using two TiO_2 concentrations: 1 mg/ml and 0.3 mg/ml (Fig. 2). Polymyxin B (PMB) was used as control for dead cells. Due to its ability to permeabilize the outer membrane and depolarize the cytoplasmic membrane [18] PMB is a good positive control for maximal NPN uptake. We used 50 $\mu\text{g/ml}$ addition of PMB compound which depolarized the cytoplasmic membrane and showed that maximal NPN uptake factor is approximately 3.5. After UV assisted photocatalytic treatment with 1 mg/ml TiO_2 concentration NPN uptake statistically did not differ from the control sample (Fig. 2A). However, under the same irradiation lower concentration of TiO_2 had statistically significant effect. Here, the uptake factor of NPN was 0.7 units higher than in the sample with 1 mg/ml TiO_2 . Viability test (Fig. 2(b)) showed that during combined treatment there was no difference between the two TiO_2 concentrations. But if we compare the result to the UV and TiO_2 effects separately (Fig. 1B) we can see, that without UV light 0.3 mg/ml TiO_2 reduces viability by 35 %, whereas in combination with UV light the reduction increases to approximately 50 %. Surprisingly, presence of UV light increased the inactivation efficiency of solution containing 0.3 mg/ml TiO_2 but not the one with 1 mg/ml.

In our previous study [7] bacteria inactivation by UV light and 1 mg/ml of TiO_2 at 23 °C temperature in 40 min reached more than 90 %. In current study we did not reach such high efficiencies and this could be related to the difference in temperature. At 23 °C metabolism and protective mechanisms in bacteria are relatively weak. Meanwhile, at 37 °C antioxidant system better protects bacteria from damage, especially when cells are grown at this temperature. The enhanced photocatalytic efficiency at lower temperature was also noticed by Kim M. J. and Yuk H. G. [19].

Fig. 2. The effect of UV light activated photocatalytic treatment of *S. enterica* cells: A) Permeability of the outer membrane; B) Bacteria viability.

* – statistically significant difference compared to control group, using one-way ANOVA; ♦ – statistically significant difference compared with each other, using Tukey test, $p < 0.05$ ($n = 3$).

3.3. *S. enterica* Treatment by the Visible Light Activated TiO_2 Powder

It is well known that TiO_2 has photocatalytic activity under UV light [13] and results presented in section 3.2.1. confirmed it. Nevertheless, it would be more useful to have photocatalyst which can be activated by the visible light. To compare the achieved results to the efficiency of the visible light activated TiO_2 , we used the same conditions as in previous section and just changed the UV light source for the wide spectrum daylight LED.

NPN assay showed that when assisted by the visible light irradiation both TiO_2 concentrations had a significant bactericidal effect (Fig. 3). NPN uptake factors were 2.5 and 2.8 when concentrations of TiO_2 were 1 mg/ml and 0.3 mg/ml respectively. The viability of *S. enterica* cells also decreased to 30 % when TiO_2 concentration was 1 mg/l, and down to approximately 7 % – when 0.3 mg/ml TiO_2 concentration was used. These results indicate that at both concentrations visible light irradiated TiO_2 had significant efficiency. Also, it was determined that similarly to the UV assisted photocatalysis 0.3 mg/ml TiO_2 concentration was more effective than 0.3 mg/ml. The later result can be explained by the assumption that higher concentration of TiO_2 particles shields the light at the surface of the solution and less of them become excited to produce Reactive Oxygen Species (ROS). On the other hand, the result that visible light assisted treatment had higher inactivation efficiency than UV light was rather surprising because it is well known that photocatalytic efficiency of P25 TiO_2 powder under visible light is weak [13]. To understand this observation we had to extend our study and to account for the specific differences between live bacteria and rather simple test markers which are traditionally used for the assesment of photocatalytic activity of tested photocatalysts.

Fig. 3. The effect of the combined (visible light activated TiO_2) treatment on *S. enterica* cells: A) Permeability of the outer membrane; B) viability of cells.

* – statistically significant difference compared to control group, using one-way ANOVA; ♦ – statistically significant difference compared with each other, using Tukey test, $p < 0.05$ ($n = 3$).

3.4. Intracellular ROS Generation

It is generally accepted, that photocatalytic TiO_2 activity is based on the production of ROS in the solution and their oxidative stress caused to the bacteria [13]. After observing the unexpectedly high bacteria inactivation under visible light we decided to measure concentration of the intracellular ROS following the fluorescence of DCFH dye (Fig. 4). NPN uptake and viability tests showed that 0.3 mg/ml TiO_2 concentration increased the OM permeability and decreased the viability of bacteria cells more efficiently than it was achieved with 1 mg/ml TiO_2 . The ROS levels in cells were measured in parallel with NPN assay, using the same concentrations of TiO_2 . It was reported that 5 and 15 min are optimal for this protocol [16], because it gives a stable measurement over time of unmodified ROS. Longer incubation times could lead to modifications in cells states and in intracellular ROS levels [16]. Accordingly, the selected 15 min incubation time is an acceptable compromise between longer incubation period and the need for the stable fluorescent measurement by successful DCFH diffusion and ROS-reaction inside cells.

When bacteria suspension treatment was performed only by TiO_2 powder (i.e no irradiation), final DCFH fluorescence was approximately 40 000 RFU (Relative Fluorescence Units) and 50000 RFU for TiO_2 concentrations of 0.3 mg/ml (Fig. 4A) and 1.0 mg/ml (Fig. 4B), respectively. Combined treatment by 0.3 mg/ml of TiO_2 and visible light irradiation resulted in the fluorescence level of 40 000 RFU which did not differ from the corresponding TiO_2 control. But when the sample with the same TiO_2 concentration was irradiated by the UV light, the fluorescence level was lower by approximately 10 000 RFU.

Combined treatment by 1 mg/ml TiO_2 and both irradiation types reduced ROS level in cells down to approximately 40 000 RFU. Noteworthy, with visible light irradiation ROS amount in cells was nearly equal for both titanium dioxide concentrations. Meanwhile, when TiO_2 was combined with UV light, the obtained ROS level was not the same. In comparison to the sample treated using lower TiO_2 concentration, the fluorescence for 1 mg/ml TiO_2 concentration was higher by approximately 8000 RFU.

Fig. 4. Intracellular ROS generation in variously treated *S. enterica* cells. Curves in chart (A) were obtained using TiO_2 concentration of 0.3 mg/ml; curves in chart (B) – with 1 mg/ml.

3.5. Photocatalytic Bleaching of Methylene Blue

The presented results showed that TiO_2 and visible light irradiation had a stronger effect on *S. enterica* cells although in nearly all of the reported studies it is stated that P25 TiO_2 is more efficient under UV light. Considering the results of intracellular ROS estimations, we presumed that bacteria itself can be the source of free radicals. To verify this assumption, we used the same conditions as in our previous experiments, but instead of PBS buffer we used a solution of methylene blue (MB). During this group of experiments (Table 2), MB solution (initial concentration 7.4 mg/l) was irradiated by UV or visible light for 60 minutes and samples were taken every 30 minutes. When applied initial concentrations of TiO_2 and *S. enterica* were 0.3 mg/ml and $3 \cdot 10^8$ cfu/ml, respectively. Phage PRD1 (10 000 pfu/ml) and UV light were used as control.

As expected, without TiO_2 or bacteria bleaching of MB under UV and visible light is weak. The minor reduction is caused only by the photolysis, which is naturally stronger for the visible light. Also, it can be noticed that addition of TiO_2 enables the photocatalytic processes and this in turn increases the bleaching of MB solution significantly. The strongest effect of photocatalytic MB bleaching is observed when all ROS generated by UV and TiO_2 interaction are consumed only by the MB (i.e. when there are no additional bacteria or viruses).

TABLE 2. METHYLENE BLUE SOLUTION BLEACHING DURING ITS TREATMENT BY UV AND VISIBLE LIGHT IRRADIATION IN THE PRESENCE OF DIFFERENT ADDITIVES

Light source type	UV			Visible light		
	0 min	30 min	60 min	0 min	30 min	60 min
MB photolysis (control)	0.34	0.35	0.32	0.38	0.30	0.29
TiO_2	0.38	0.13	0.05	0.37	0.32	0.26
<i>S. enterica</i>	0.37	0.35	0.34	0.39	0.35	0.30
<i>S. enterica</i> + TiO_2	0.30	0.27	0.18	0.36	0.09	0.04
PRD1 + TiO_2	0.35	0.24	0.16	0.37	0.28	0.27

Under visible light irradiation the colour of methylene blue solution changed weakly and was nearly identical to the bleaching caused by the visible light photolysis alone. The only exclusion was the sample with *S. enterica* bacteria and TiO₂. This combination demonstrated the largest reduction of MB optical density amongst all tested combinations including MB treatment by TiO₂ under UV light. Observation that three factors, namely visible light, TiO₂ and *S. enterica* bacteria, acting separately have nearly no effect, whereas their combination provides the strongest MB bleaching is clear indication of their synergistic interaction.

4. DISCUSSIONS

P25 TiO₂ powder are well known as efficient UV light photocatalyst whose activity in visible light is limited [11]. Nevertheless, the key finding of this study was that under intense wide spectrum visible light irradiation TiO₂ powder enables strong inactivation of *S. enterica* bacteria and halts its potential to form colonies. To understand this phenomenon, several specific factors have to be considered.

First, scientific studies indicate that under applied conditions *S. enterica* bacteria indeed have potential to generate ROS. For example, at 37 °C temperature *S. enterica* bacteria are known to carry out metabolism and due to their respiration reactive oxygen species potentially can be formed. After formation, internal ROS can be extruded from the cell to the media by active efflux pumps [20]. On the other hand, phototherapy with 405–800 nm light also activates bacterial porphyrins to generate ROS, which cause intracellular damages [21], [22]. We used powerful visible light source with wide spectrum. Accordingly, high amounts of intracellular ROS potentially could have been generated. Obviously, both of these mechanisms can be significant factors in ROS generation and *S. enterica* bacteria inactivation. However, it is important to note that both of them can proceed without the presence of titanium dioxide. Whereas, more detailed analysis of *S. enterica* bacteria treatment by visible light with and without TiO₂ powder revealed that inactivation enhancement by TiO₂ exceeds the sum of individual effects caused by light or TiO₂ alone.

The provided evidence of relatively strong intercellular ROS generation inside *S. enterica* bacteria under visible light (Fig. 4) suggests, that the key role of TiO₂ might be the increasing of the permeability of the outer membrane (Fig. 1, Fig. 2 and Fig. 3). In this model the intense visible light irradiation would stimulate *S. enterica* bacteria to generate intracellular ROS, which are extruded to the media by breathing (i.e. driven by active export by efflux pumps and porins). As indirect evidence for the validity of this model and the release of ROS, we take on the observation of unexpectedly strong bleaching of MB under the visible light (Table 1). Alternative reaction models with more direct involvement of TiO₂ can be considered as well. For example, it is possible that under strong irradiation *S. enterica* bacteria generates peroxide which enters solution, interacts with TiO₂ and creates ROS similarly to the mechanism reported at [23]. Viruses on the other hand, are not metabolically active and do not produce ROS. Therefore, the result that combination of PRD1 + TiO₂ powder did not bleach MB solution gives no ground for the last alternative and provides strong support for the former model.

5. CONCLUSIONS

In current study we analysed how *S. enterica* bacteria was affected by TiO₂, UV/Vis light and their combination. Looking at the effects caused by the individual factors, we determined, that UV light permeabilized the outer membrane stronger than visible light or TiO₂. But higher bacteria viability reduction was induced by TiO₂. During combined treatment with UV

and visible light irradiation lower titanium dioxide concentration (0.3 mg/ml) caused higher permeability of bacteria outer membranes and significantly reduced the viability of cells. Surprisingly, under visible light the damage to the cells was considerably stronger than under the UV light. Considering the applied conditions traditional photocatalytic activity of titania under visible light should be low, therefore synergistic interaction model between the wide spectrum visible light, *S. enterica* bacteria and titanium dioxide powder was proposed. According to the model, the main bactericidal effect of combined titanium dioxide and visible light irradiation treatment is enhanced formation and at least partial release of the intracellular reactive oxygen species to the media.

As far as we know, the proposed model works well only with *S. enterica* bacteria and was not yet reported for any other type of bacteria. Naturally, the combination of visible light irradiation and standard TiO₂ powder can be proposed only for the disinfection of water restrictively contaminated by *S. enterica*. However, the experiment with Methylene blue, visible light, TiO₂ and *S. enterica* bacteria allows to assume that potential range of applications can be much wider. Indeed, reactive oxygen species are universal oxidants and if during treatment *S. enterica* bacteria is stimulated to release ROS to the solution, when ROS can oxidise other available organic and inorganic contaminants as well. Altogether, findings of this study suggest that it might be not necessary to modify TiO₂ to make it suitable for the visible light assisted disinfection. Instead, it might be interesting to investigate holistic approach combining effects caused by TiO₂, wide spectrum visible light and *S. enterica*.

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