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Light-Activated Zirconium(IV) Phthalocyanine Derivatives Linked to Graphite Oxide Flakes and Discussion on Their Antibacterial Activity

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Featured Application: Generation of reactive oxygen species combined with the antibacterial properties of graphene-based materials makes it possible to use them as antibacterial agents with enhanced activity under near-infrared light. Nevertheless, the antimicrobial effect depends on the kind of phthalocyanine derivatives and the type of microbial cells.

Abstract: In search of an effective antibacterial agent that is useful in photodynamic therapy, new derivatives of zirconium(IV) phthalocyanine (ZrPc) complexes were obtained and linked to graphite oxide flakes. In the syntheses of ZrPc derivatives, two bis-axially substituted ligands with terminal amino group and different lengths of linear carbon chain (C4 in 4-aminobutyric acid or C11 in 11-aminoundecanoic acid) were used. The optical properties (absorption and photoluminescence spectra) of ZrPcs and the composites were examined. Broadband red–near-infrared lamp was tested as an external stimulus to activate ZrPcs and the composites. Optical techniques were used to show generation of singlet oxygen during irradiation. Considering the application of graphite oxide-based materials as bacteriostatic photosensitive additives for endodontic treatment of periapical tissue inflammation, the antibacterial activity was determined on one *Escherichia coli* strain isolated directly from an infected root canal of a human tooth and one strain with silver and antibiotic resistance. Looking at the obtained results, modified levels of activity toward different bacterial strains are discussed.

Keywords: nanomaterial; photoactivation; photoluminescence; NIR irradiation; ROS generation; *E. coli*

1. Introduction

Optically active phthalocyanine (Pc) systems possess intense absorption bands and exhibit photoluminescence from the excited states. They are also very stable and resistant to chemical,

thermal, or photochemical degradation. Moreover, it is particularly interesting that they act as effective photosensitizers. Following their irradiation, singlet oxygen is generated, which is used in photodynamic therapy (PDT) to kill selected cells [1–3]. This kind of activity can also be used against microbial cells. For example, Nyamu et al. [4] reviewed antibacterial efficacy of phthalocyanines and noticed that Gram-positive bacteria are more sensitive to phthalocyanines than Gram-negative bacteria. This may be a result of differences in the cell structure. In general, the outer membrane present in Gram-negative species makes them more resistant. However, it is difficult to explain this phenomenon in detail without deeper understanding of the interactions, and it is impossible to generalize the antibacterial mechanism of the compounds. The interaction with bacterial cell and the cell's response strongly depends on the physicochemical properties of the materials (such as size, shape, structure, charge, etc.), as we have previously described [5]. One of the possible antibacterial mechanisms is interaction of the compounds with bacterial membrane [6], while the other is reactive oxygen species production [7]. In the case of phthalocyanines, Mikula et al. [6] underlined that cell binding is a key factor influencing their antibacterial activity. Thus, the susceptibility of bacteria depends on the kind of phthalocyanines. De Silva et al. [8] indicated that conjugation of phthalocyanines with antibiotics also enhances the antibacterial activity of phthalocyanine–sulfonamides conjugate against Gram-negative bacteria. In general, many articles about the antibacterial properties of Pcs as photosensitizers have already been published. However, there is lack of reports on their activity against multi-drug-resistant microorganisms.

Various carbon-based composites are fabricated to enhance the activity of materials, for example, in photocatalysis [9,10]. Phthalocyanines have also been incorporated into graphene or other carbon structures for photocatalytic or optoelectronic applications [11–13]. On the other hand, specific graphene-based systems have been found to possess good antimicrobial properties [14,15]. In our study, we wanted to combine antimicrobial effects caused by two different factors—graphite oxide flakes and phthalocyanines activated by light—and use them against bacteria causing infection of the root canals of teeth.

The application of ZrPc/GO-based composite (ZrPc with lysine ligand, LYS; GO, graphite oxide) for antibacterial PDT has already been shown by us [16], where the action against *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* (*E. coli*) ATCC 11229 was proven. In agreement with other studies, it was indicated that Gram-positive bacteria are more sensitive strains. However, the influence of irradiation on enhanced antimicrobial activity of bis(LYS)ZrPc/GO materials was observed, particularly in the case of *P. aeruginosa* (a multidrug resistant pathogen), where minimal inhibitory concentration (MIC) was equal to 0.32 mg/mL in comparison to 40 mg/mL needed for the nonirradiated sample [16].

The aim of this paper was to characterize two synthesized zirconium(IV) phthalocyanines, axially substituted with different ligands (hydrocarbon chains terminated with amino groups), which were used to obtain two hybrid materials with graphite oxide flakes. Optical properties of all systems were described, also showing singlet oxygen generation after red–near-infrared light exposition. The composite activities against two resistant *E. coli* strains were tested.

2. Materials and Methods

2.1. Material Syntheses

Synthesis methods of dichlorozirconium(IV) phthalocyanine complex ($ZrPcCl_2$) and axially substituted derivatives with two ligands have been described previously [17–19]. Substituted ZrPcs were obtained by direct interaction of $ZrPcCl_2$ with amines (proper amine was used for reaction instead of citric acid as described in [18]): 4-aminobutyric acid (GABA) or 11-aminoundecanoic acid (UDA). Respective complexes were bis(4-aminobutyrato)ZrPc [**bis(GABA)ZrPc**] and bis(11-aminoundecannato)ZrPc [**bis(UDA)ZrPc**].

The preparation of composites (highly oxidized graphite oxide with Pcs) was described and presented on a scheme in [16]. Briefly, ZrPc derivative was dissolved in dimethylformamide, where GO was added. The reaction mixture was ultrasonicated for 10 min and then mixed on a magnetic stirrer for 12 h in an ice bath with the addition of dicyclohexylcarbodiimide. The obtained dark-green suspension was centrifuged and washed carefully to remove excess of complex molecules (until colorless filtrate was observed). The materials were named **bis(GABA)ZrPc/GO** and **bis(UDA)ZrPc/GO**. All samples were kept in the darkness.

2.2. Material Characterization

The composition and structure of the complexes were confirmed by ^{1}H NMR and IR absorption analyses (Supplementary Materials). For absorption and emission measurements, solutions of ZrPcs at concentration of 0.25 mg/mL were prepared by dissolving the compounds in dimethyl sulfoxide (DMSO), whereas the concentration of composites was about 1 mg/mL.

Singlet oxygen generation was observed by indirect method, where 1,3-diphenylisobenzofuran (DPBF, 0.1 mg/mL) in DMSO medium was used as a probe added to ZrPc solution or ZrPc/GO suspension; a red–near-infrared lamp (Philips, 100W, wavelength range of around 600–1000 nm as shown in the Supplementary Materials Figure S1) served as a light source. During irradiation, the samples in cuvette were placed 30 cm from the source and irradiated for different times (from 10 s up to a maximum of 240 s). The spectrophotometer FLS980 (Edinburgh Instruments) with xenon lamp was used for photoluminescence measurements. The spectra were recorded with the respective lamp and detector corrections.

The absorption spectra of ZrPc solutions and ZrPc/GO suspensions were measured with the spectrophotometer Agilent CARY 5000 UV-Vis-NIR. Raman spectra were recorded using inVia^{TM} confocal Raman microscope (Renishaw, New Mills, United Kingdom), with 488 nm line of argon ion laser used as a light source. In case of GO-based compounds, the background was subtracted, and the spectra were normalized.

2.3. Antibacterial Tests

To check the sensitivity of bacterial strains to the studied composites, two *E. coli* strains were used: *E. coli* 6.2E (clinically isolated strain from root canal) and *E. coli* J53 (reference strain with determinants of silver resistance and selected classes of antibiotics).

The sensitivity of bacterial strains to GO-based composites was assessed using the serial dilution method in broth recommended for the determination of antibiotic susceptibility and chemotherapeutics by CLSI (Clinical and Laboratory Standard Institute) [20] and adjusted to graphene compounds by Kedziora. The aim of this method is to determine the lowest concentration inhibiting the growth of bacteria (MIC) and the lowest bactericidal concentration (MBC, minimal bactericidal concentration) of the composites used in the experiment.

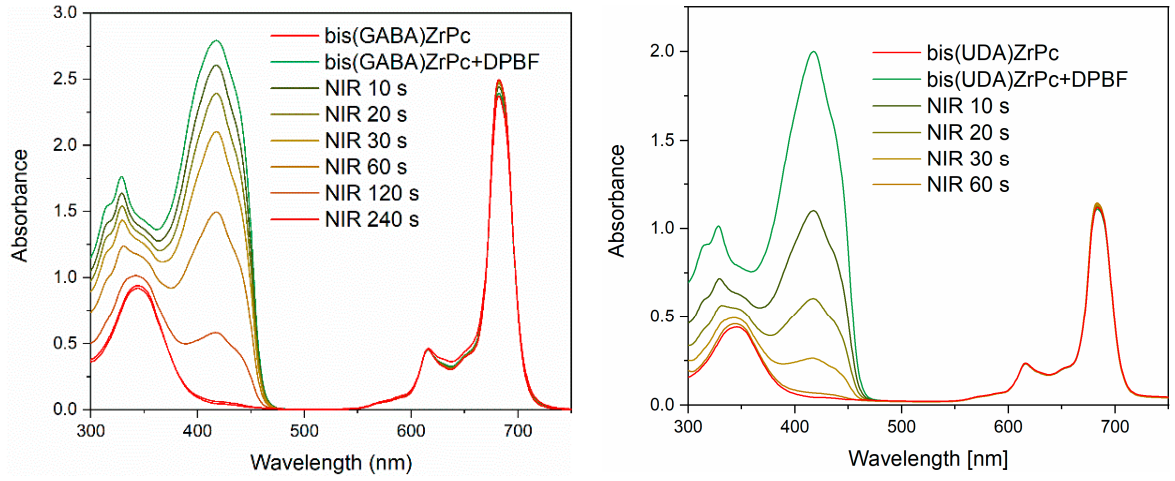
The day before the test, the bacterial strains stored at −70 °C were spread on a Mueller–Hinton agar (MHA) to verify their purity and vitality and incubated for 16–20 h at 37 °C. Bacterial susceptibility tests for the composites were carried out on 96-well titration plates. A series of dilutions of the materials were prepared in a Mueller–Hinton broth (MHB) liquid substrate. The range of concentrations was chosen experimentally, adjusting it to the course of the experiment, where the maximum concentration that was possible to test was 8192 µg/mL. To prepare bacterial suspensions, the bacteria were transposed into physiological saline at a density of 0.5 McFarland scale (corresponding to approximately 1.5×10^{8} cfu/mL) and then diluted tenfold. The prepared bacterial suspension was poured into each well of the titration plate containing the specified concentration of the composite, resulting in a final density of 1.5×10^{5} cfu/mL. Control samples were also included in the experiment. The negative test was a series of dilutions of composites in the broth without the addition of bacteria and pure medium. The positive control was MHB liquid medium with bacteria. Eventually, the selected plates were irradiated with the NIR lamp, shining at 50 cm from above, without a lid. During the

exposure, the tiles were placed in a cardboard box, and the lamp was placed in a hole cut out in this carton to reduce the impact of other physical factors and light on the tested samples. Tested exposure time was estimated at 5 min (continuous exposure) or 2 min, repeated 5 times with 1 min break to avoid possible medium heating. The titration plates were incubated for 16–20 h at 37 °C. After the incubation time, a MIC reading was performed taking into account control samples. The MIC value criterion was a hole in which turbidity or bacterial sludge was unnoticeable. The results of the MIC values were determined optically in the form of bacterial sediment at the bottom of a well of a round-bottomed titration plate. All results were repeated 3 times, taking into account twice the cultures in each replicate.

3. Results

3.1. Spectroscopic Studies and Singlet Oxygen Generation

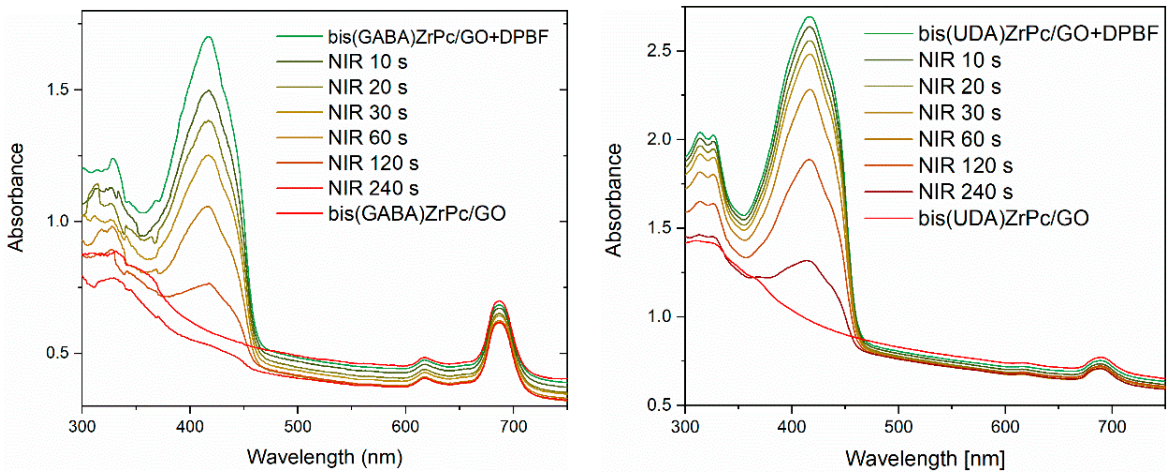
The obtained Pc compounds were of intense green color and had moderate solubility in DMSO. Their absorption spectra in DMSO are shown in Figure 1 (red lines). As for phthalocyanines, two broad bands were observed—one in the UV region (Soret, B band) and the second in the red spectrum range (Q band)—which resulted from the transition of electrons from the ground state (S_0) to the higher energy states (S_2 and S_1).



(**a**) (**b**)

Figure 1. Absorption spectra of (**a**) bis(4-aminobutyrato)ZrPc and (**b**) bis(11-aminoundecannato)ZrPc solutions in dimethyl sulfoxide (DMSO) before and after addition of 1,3-diphenylisobenzofuran (DPBF), showing DPBF bleaching caused by singlet oxygen generation with increasing time of irradiation.

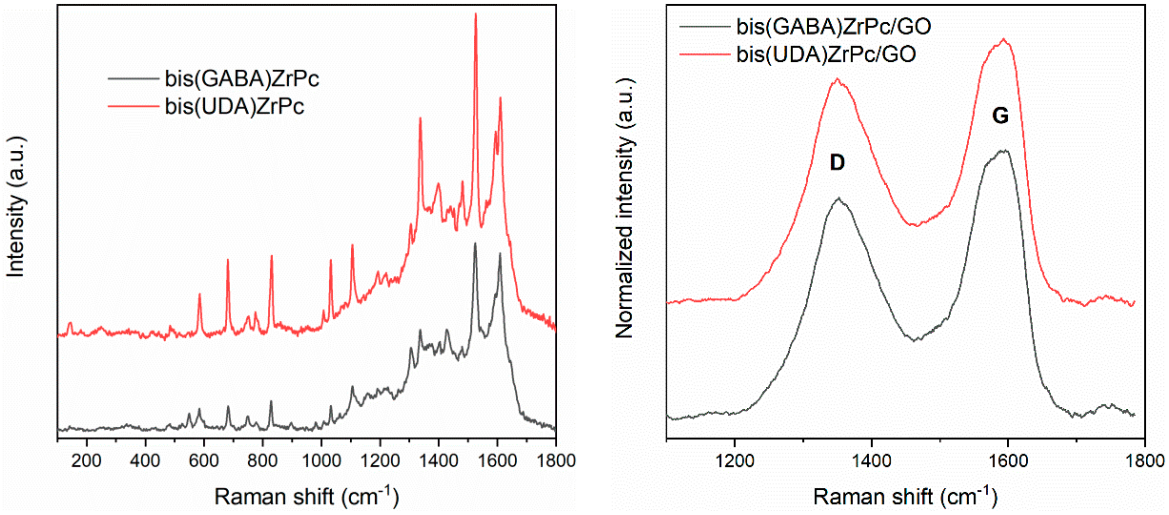
The graphite oxide flakes functionalized with Pcs were dark brown-green color. Their solubility in DMSO was very low, and most of the flakes formed suspension. To obtain samples that were stable enough to perform measurements in DMSO, ultrasonic treatment was applied. Nevertheless, partial sedimentation of the flakes occurred in time, which was registered as lower absorption intensity of GO during singlet oxygen generation measurements (Figure 2, see also Supplementary Materials and Figure S1). In the absorption spectrum of GO, characterized by lower transmission mainly in the UV–blue range, the Q band at 600–710 nm clearly appeared for both complexes (Figure 2).



(**a**) (**b**)

Figure 2. Absorption spectra of (**a**) bis(4-aminobutyrato)ZrPc/GO and (**b**) bis(11-aminoundecannato)-ZrPc/GO suspensions in DMSO and after addition of 1,3-diphenylisobenzofuran, showing DPBF bleaching caused by singlet oxygen generation with increasing time of irradiation.

Raman scattering spectra of both ZrPcs showed that the bands originated from macrocyclic rings and ligand chain vibrations (Figure 3a). These peaks were not recorded in the case of ZrPc/GO samples, where only D and G bands of graphite oxide were seen.



(**a**) (**b**)

Figure 3. Raman spectra of investigated (**a**) ZrPcs and (**b**) ZrPc/GO samples (λ = 488 nm).

The photoluminescence excitation spectra of bis(4-aminobutyrato)ZrPc and this complex after embedding on graphite oxide are shown in Figure 4a (monitoring emission at 700 nm). Similar spectra were recorded for the second complex and composite. The photoluminescence spectra of all investigated samples—two ZrPcs and their composites with GO—in the red–NIR range are shown in Figure 4b (excitation wavelength 620 nm), where the intensity has been normalized for better comparison.

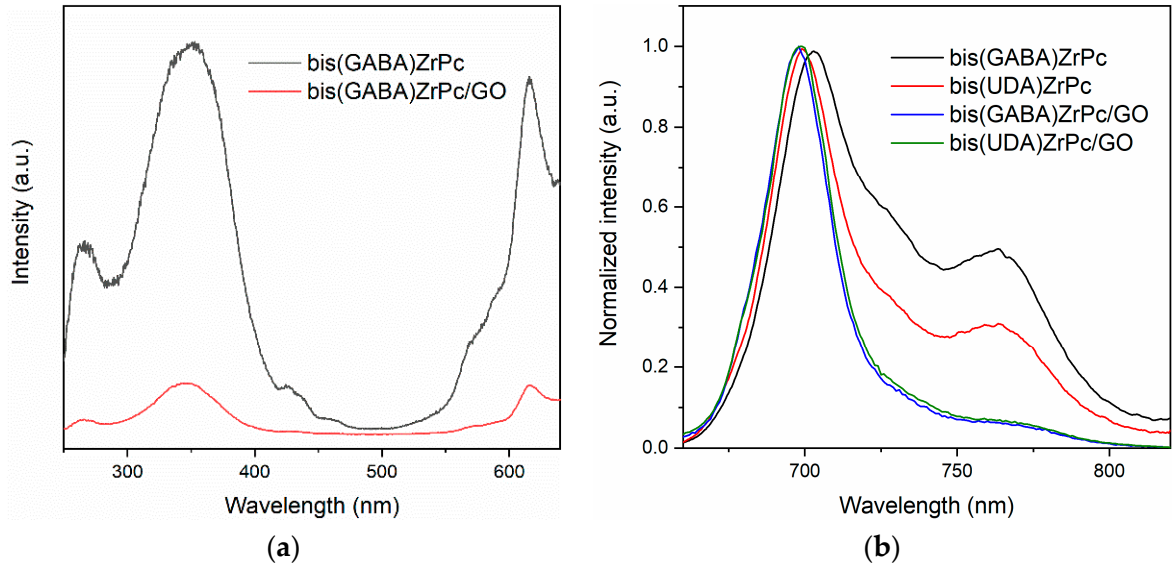


Figure 4. (**a**) Photoluminescence excitation spectra of bis(4-aminobutyrato)ZrPc before and after immobilization on GO (λ_{em} = 700 nm); (**b**) photoluminescence spectra of all investigated samples—two ZrPcs and their composites with GO (λ_{exc} = 620 nm).

1,3-diphenylisobenzofuran is a well-known indicator of singlet oxygen generation. The dye reacts in the presence of $^1O_2$ and forms an endoperoxide, which decomposes to give 1,2-dibenzoylbenzene. This effect can be followed by measuring the decrease in absorption or fluorescence intensity of DPBF [21]. For all tested samples, the absorption band of DPBF centered at around 420 nm and gradually decreased with time of red–NIR light exposition (Figures 1 and 2). The band intensity was not changed when the samples were not irradiated, when the sole DPBF solution was irradiated, or during irradiation of GO suspension (Supplementary Materials Figure S3).

3.2. Antibacterial Tests

To test the impact of light irradiation in the presence of composites on both bacterial strains, their survival was verified. After 5 or 10 min of irradiation, the number of *E. coli* (both 6.2E and J53 strains) cells has not been changed (Table S1) and was estimated at 1.5×10^5 cfu/mL, indicating that irradiation did not affect the viability of the bacteria at the tested compound concentration (≤8.2 mg/mL).

4. Discussion

In zirconium phthalocyanine complexes, chosen in the current study, the coordination number of metal (up to eight) is high enough for axial ligand substitution and extracoordination of one or two solvent molecules. Therefore, relatively high solubility of chosen Pcs complexes in water or DMSO can be expected without additional lateral substitution with hydrophilic moieties, as proposed for most known Pc-based PDT agents (e.g., tetrasulfo- or tetracarboxyphthalocyanines of Al or Zn). At the same time, ligands can assure covalent bonding of the complex molecule to the surface of graphite oxide through functional oxygen groups.

Phthalocyanine complexes tend to aggregate (dimerize), which greatly deteriorates their optical properties and significantly reduces their performance as photosensitizers. To avoid this effect on the surface of graphite oxide, it is important to obtain covalent bond between the complex and the GO through the formation of amide bonds of the ligand's terminal amino groups with the carboxyl groups of the carrier. However, lability of the system may cause the phthalocyanine ring itself to be attached to the carrier or other phthalocyanine rings. Therefore, two ligands with different chain lengths were used to tailor effectiveness of the interaction between the complex and the carrier. Carboxyl groups are unevenly dispersed over the surface of the carrier. With longer linear aliphatic ligands, the likelihood

that both ligands will be bounded by the carboxyl groups of the carrier increases. Such binding will be strong and hard to undergo hydrolysis. In the case of two short aliphatic ligands, only one of them will be bounded to the carboxyl group, but this connecting bridge should also prevent direct ring interaction with the carrier or other phthalocyanine molecules. Such binding will be less stable, and the phthalocyanine complex might be more easily discharged from the carrier. In both cases, no conformational changes resulting in modification of optical properties were observed.

Among the two studied phthalocyanine complexes, the one with 4-aminobutyric ligand has higher absorption coefficient, and absorption bands with higher intensity were thus observed for both **bis(GABA)ZrPc** and **bis(GABA)ZrPc/GO** in comparison to **bis(UDA)ZrPc** samples. Thanks to the high absorption coefficient, the absorption spectra confirmed the presence of phthalocyanine complexes on the GO flakes (Figure 2). On the contrary, axially substituted ZrPcs bands were not registered in the Raman spectra of the composites (Figure 3b), indicating low content of complexes in these samples.

The photoluminescence excitation spectra (Figure 4a) confirmed that the wavelengths of the absorption bands can excite the ZrPc molecules, causing light emission. The same spectrum shape but with lower intensity was recorded for the composites, confirming the presence of ZrPc derivatives on the carbon structures. The photoluminescence could be observed using excitation in both the UV–blue and red–NIR ranges. In the experiment, wavelength of 620 nm was used to stimulate and measure the emission of ZrPcs solutions and composite suspensions (Figure 4b). The intensity of the photoluminescence from the composites was much lower in comparison to ZrPc solutions but still clearly observed in the range of 650–840 nm.

The photosensitizing potential of the studied materials was verified through the test of singlet oxygen generation by an indirect method. Yellow DPBF indicator showed significant bleaching when all samples were irradiated with the lamp. Time needed to degrade dye molecules was below 5 min (slightly longer in the case of bis(UDA)ZrPc/GO). This proves that reactive forms of oxygen are formed when red–NIR light illuminates the samples and thus could possibly be used in photodynamic therapy.

Following our previous studies [16], the tested composites were taken to verify the antibacterial effect on two microorganisms that were selected among more resistant Gram-negative *E. coli* strains. *E. coli* 6.2E was clinically isolated from the root canal of a tooth that was previously treated with endodontic treatment, where elimination of biofilm, despite the use of antimicrobial agents, was unsuccessful, and reinfection of the root canal system and the formation of periapical lesion consequently occurred. *E. coli* J53 was taken as a strain known to be resistant to selected classes of antibiotics and silver ions. Unfortunately, the tested concentrations of composites did not affect the bacterial growth when the lamp was used for 10 min. Longer irradiation time would create more singlet oxygen, which should eventually affect bacterial cells. However, 10 min of light exposure is already quite long when one takes into account the medical application (such as irradiation of patient's tooth canals). Thus, we did not test longer irradiation of the tested systems. The minimal inhibitory concentration should appear above 8 mg/mL; however, that would be too high for an effective antibacterial agent.

As already reported, phthalocyanines show antibacterial activity at low concentrations [6,7,22]. For example, Mikula et al. indicated strong antibacterial efficacy of some cationic Pcs against *E. coli* for 2 mg/L [6]. In their work, the experiment was performed with Pcs not bonded to a carrier and with three hours of exposure under cool white light. In the materials studied here, Pcs concentration was much lower because it was deposited on GO, and the weight of the whole composite was taken into account and not just Pc (Pc content was lower than 1 wt.%). However, the different results confirm that the antibacterial activity of Pcs depends strongly on the individual properties of bacterial strains (not only bacterial species) and/or the structure of the phthalocyanines/compounds and their physicochemical properties. *E. coli* strains are one of the most variable species among bacteria, depending on their origin (human, animal, or environment). Therefore, each compound and, most of all, each composite should be considered as a separate factor with individual mode of action and efficacy.

As already mentioned, the effectiveness of the antimicrobial action depends strongly on the kind of phthalocyanine derivatives and the type of microbial cells (Gram-positive bacteria, Gram-negative bacteria, or yeasts) [23]. An antimicrobial agent efficient against one type, or even strain, can be useless for the others. Therefore, the research will be continued in terms of testing material activity against other bacteria and looking for better zirconia phthalocyanine derivatives. The studied composition, being ineffective as photosensitizer in the tested environment, can nonetheless be utilized for other purposes, such as catalysis, pollutant photodegradation, or filtering [11,24,25].

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/9/20/4447/s1: Lamp emission spectrum, data of ^{1}H NMR and IR absorption analyses of ZrPcs, test of ZrPc/GO solubility in DMSO, test of singlet oxygen generation by GO after irradiation, and results of antimicrobial test of composites against two *E. coli* strains.

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