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Aspects of Photodynamic Inactivation of Bacteria

Faina Nakonechny and Marina Nisnevitch

Abstract

Increasing resistance of bacteria to antibiotics is a serious worldwide problem, and to combat resistant bacteria, new antibacterial approaches are to be developed. One alternative to traditional antibiotic therapy is photodynamic antimicrobial chemotherapy (PACT). PACT is based on excitation of photosensitizers (PS) capable of transferring the absorbed light energy to dissolved molecular oxygen causing generation of reactive oxygen species, which irreversibly damage bacterial cell components. The overall efficiency of PACT has been proven for Gram-positive and Gram-negative bacteria. The effectiveness of PACT can be increased by encapsulation of PS in liposomes providing more concentrated delivery of PS, enhanced cytotoxicity, improved pharmacokinetic properties, sustained release, and prolonged action of the PS. For continuous and reusable application, PS can be immobilized in polymers. Chemiluminescence, sonodynamic treatment, and radiofrequency irradiation allow to perform excitation of PS in the dark without external illumination, opening prospects for combating internal infections. Combination of PS with antibiotics can gain a synergistic effect, allowing in some cases to overcome the resistance of bacteria to antibiotics.

Keywords: photodynamic therapy (PDT), photodynamic antimicrobial chemotherapy (PACT), photosensitizer (PS), chemiluminescent antimicrobial chemotherapy (CPAT), sonodynamic antimicrobial chemotherapy (SACT), targeted drug delivery, liposomes, immobilization

1. Introduction

1.1 History of photodynamic therapy

The therapeutic properties of light were observed already in ancient Greece, Egypt, and India. However, they were not widely used for many centuries [1]. The history of modern photodynamic therapy (PDT) dates back to 1900, when Oscar Raab discovered the toxic properties of the dye acridine red on *Paramecium* spp. [2]. He and his supervisor, Hermann von Tappeiner, noticed a positive effect of illumination on the toxic activity of this dye. In his later work, von Tappeiner and his colleagues applied this approach to inactivation of bacteria [3] and to treatment of skin cancer [4]. In 1909, von Tappeiner introduced the term "Photodynamic Action" and showed that oxygen is essential for this procedure [5]. PDT has been studied and developed as an anticancer therapy for a long time and was approved by the Food and Drug Administration in the 1990s for various applications in this area of medicine [6–8]. The antimicrobial properties of this approach were unfairly

forgotten for several decades. However, interest in antibacterial PDT has been rekindled and is continuously increasing because multidrug resistance of pathogenic microorganisms has become a serious threat to public health. Photodynamic antibacterial chemotherapy (PACT) has become a promising approach for combating bacterial infections, which are resistant to modern antibiotics.

1.2 Photosensitizers and their mechanism of action

PACT is based on the exposure of bacteria to photosensitive compounds—photosensitizers (PSs). When a PS located in the bacteria or on the bacterial surface is exposed to light (usually visible), it transfers from its low-energy ground state to an excited singlet state. Return of the PS to its ground state is accompanied by either emission of fluorescence or transition of the PS to a longer-living, higher-energy triplet state (PS*) via intersystem crossing. The PS* in turn reacts with surrounding molecules to form free radicals and hydrogen peroxide (Type I reaction) or transfers its energy to molecular oxygen to produce singlet oxygen and other highly reactive oxygen species (ROS; Type II reaction) [9, 10]. Type I and Type II reactions occur simultaneously, and the ratio at which they occur depends on both the PS type and the surrounding conditions. A detailed description of the photosensitization process can be found in the recent reviews of Castano et al. [11] and Cieplik [10]. ROSs formed in this process oxidize biomolecules, damage the cell membrane, and ultimately lead to cell death [12]. PACT usually proceeds predominantly through Type II processes. However, since Gram-negative bacteria are more susceptible to OH$^{\cdot}$ radicals than to singlet oxygen, the Type I reaction may be more efficient against such microorganisms [13, 14].

1.3 Photosensitizers for PACT

Hundreds of compounds are currently available for mediating PDT in various areas of medicine, where some have been shown to be suitable for antimicrobial applications. PSs employed for medical uses should be a single pure compound, stable at room temperature and inexpensive. The PS must have a strong absorption peak in the visible spectrum between 600 and 900 nm and should possess a high-triplet quantum yield that will provide high production of ROS upon illumination. It should not be toxic in the dark (especially to mammalian cells), mutagenic or carcinogenic [15–18]. In addition, when talking about PACT, it is very important that the PS will display preferential association with bacteria, accumulate within the cells, or bind to the bacterial cell envelope [14, 19].

PSs can generally be assigned to several chemical classes: tetrapyrroles (which include porphyrins, chlorins, bacteriochlorins, and phthalocyanines), synthetic dyes (phenothiazinium salts, Rose Bengal, squaraines, etc.), and naturally occurring compounds (such as riboflavin or curcumin). Cyclic tetrapyrroles present the most well-known class of clinically relevant PSs used mostly for anticancer applications [20]. This structure can be found naturally in such important biomolecules such as haem, chlorophyll, and bacteriochlorophyll. Unlike other types of PSs, most tetrapyrroles (except for bacteriochlorins) are more likely to react by a Type II reaction with the creation of singlet oxygen [16], whereas bacteriochlorins act *via* a Type I mechanism. Other well-known antimicrobial agents are phenothiazinium-based synthetic dyes, including methylene blue (MB) and toluidine blue O (TBO), which also act as anticancer agents in PDT. These structures can be synthesized more easily than tetrapyrroles but possess high-dark toxicity compared to other PSs [15, 21]. Another representative of synthetic dyes, Rose Bengal (RB), has already been used successfully in antimicrobial and anticancer applications for a long

time [16]. Photodynamic active compounds isolated from plants arouse particular interest. These natural compounds include curcumin, extracted from the rhizomes of *Curcuma longa*, which was found effective in eradicating oral pathogens [22]. Another representative of this group is hypericin isolated from St. John's wort, which exhibits photodynamic activity against Gram-positive and Gram-negative bacteria. Detailed descriptions of all PS classes can be found in the reviews published by Hamblin and colleagues [15, 16].

2. Photosensitizer activation modes

2.1 Dark activity

The name photosensitizer implies the need for illumination in order to activate PS molecules and trigger their action. However, PSs possess some so-called "dark activity" even in the absence of illumination, leading to cell death in the dark [23–29]. This feature depends on the PS concentration and manifests itself in different ways for various PSs.

Shrestha demonstrated dark toxicity of RB against Gram-positive *Enterococcus faecalis*. Exposure of the cells to 10 μM RB in the absence of illumination for 15 min led to a 0.5 $\log_{10}$ reduction in cell concentration [26]. Furthermore, a marked dark toxicity of RB against clinical isolates of Gram-negative *Pseudomonas aeruginosa* was observed by Nakonieczna [27]. Brovko compared the activity of various PSs against several types of microorganisms and noted high dark toxicity of RB, as well as of phloxine B against Gram-positive *Bacillus sp*. and *Listeria monocytogenes* (more than 5 $\log_{10}$ reduction in the bacterial concentration after 30 min of treatment with the dye) [30]. The toxicity of malachite green in the dark against the same microorganisms was very low (<0.1 $\log_{10}$ reduction in concentration after 30 min of treatment with the dye). High concentrations (>500 μg/mL) of acriflavin neutral in the absence of light were significantly toxic to *E. coli* (more than 6 $\log_{10}$ reduction in concentration after 30 min of treatment with the dye, both under illumination and
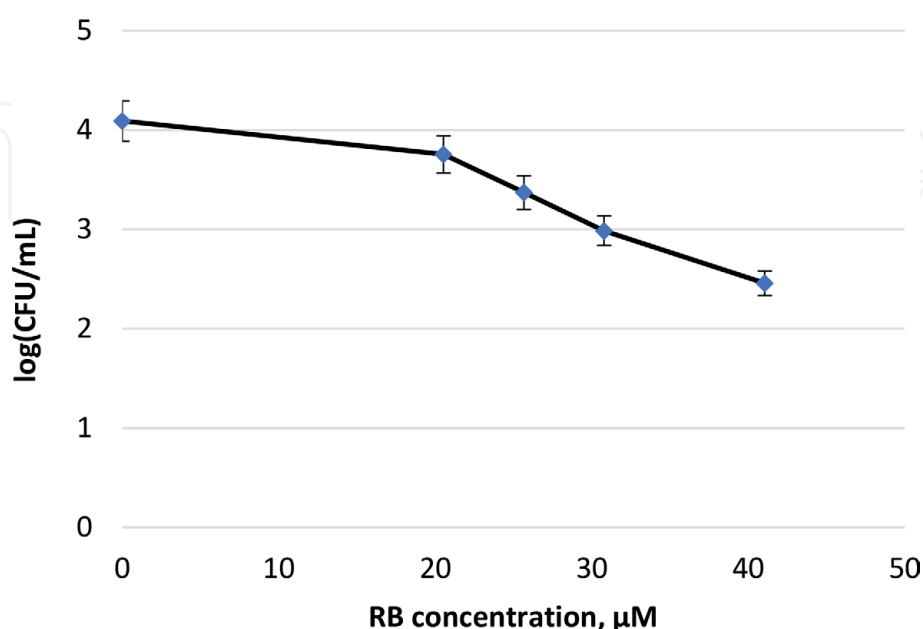

Figure 1.
Effect of RB concentration on its cytotoxic activity. S. aureus cells at the initial concentration of 104 CFU mL−1 were incubated for 3 min in dark conditions at various concentrations of RB. After the incubation, bacteria were tested by viable count. Error bars present standard deviations.

in the dark). However, illumination significantly enhanced its toxic effect against other tested microorganisms [30].

In our studies, we also noted the dark toxicity of various PSs against different types of bacteria (**Figures 1, 2, Table 1**). **Figure 1** shows the effect of various RB concentrations on *S. aureus* in the absence of light. The number of living cells decreases with increasing RB concentration in the dark. **Table 1** shows a comparison between dark and light toxicity of three PSs—malachite green oxalate (MGO), RB, and safranin O. The effect of MGO in the dark was the strongest, and a 0.87 μM concentration of MGO was sufficient for inhibiting the growth of *S. aureus*. The dark activity of RB and safranin O is noticeably weaker, and the minimal inhibitory
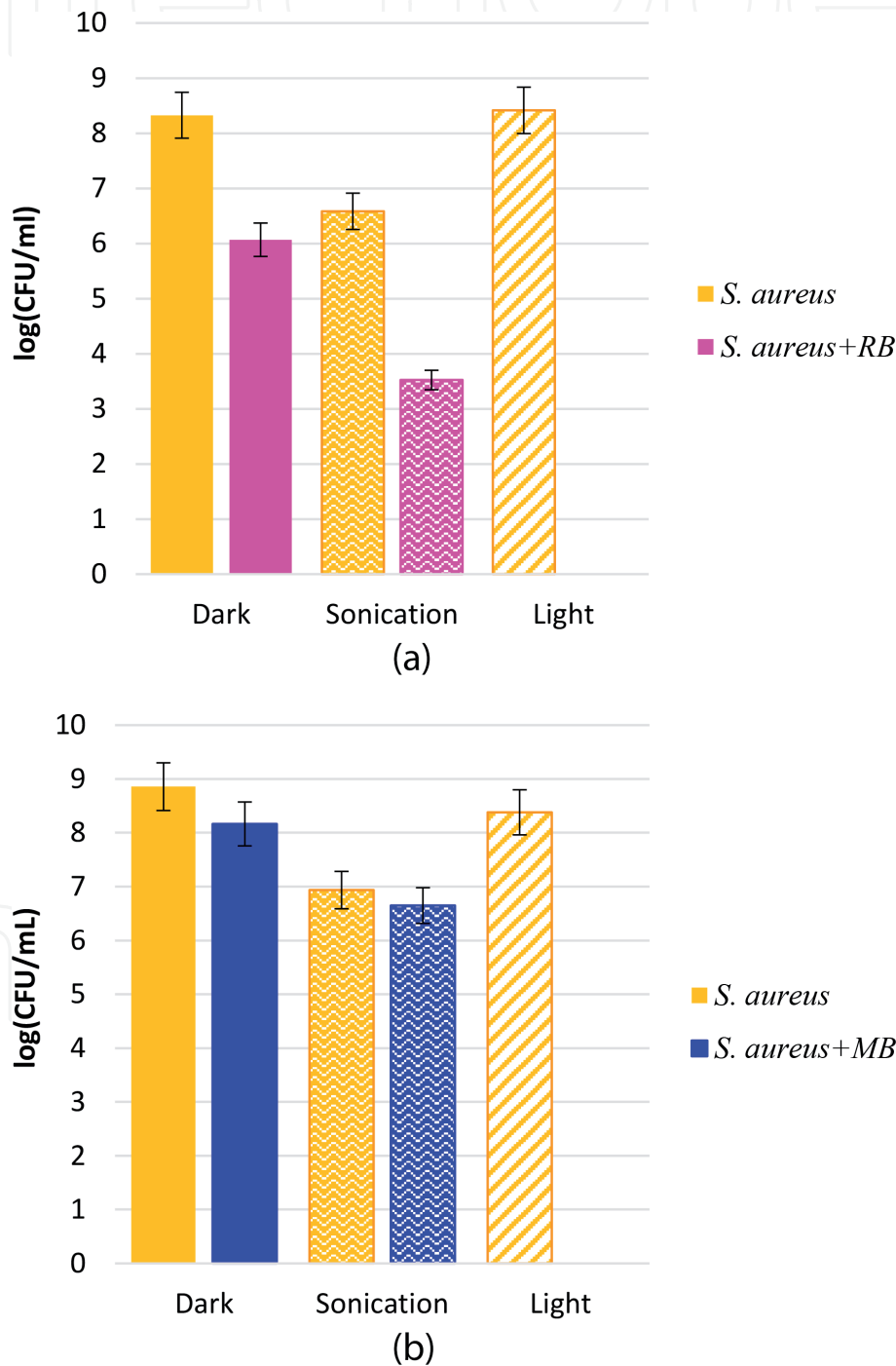

Figure 2.
SACT and PACT effect of MB on S. aureus. In SACT experiments, the cells at 108 CFU mL⁻¹ concentration were incubated with (a) 5 μM RB or (b) 30 μM MB in the ultrasonic bath for 1 h in the dark. In PACT experiments, the cells were illuminated for 15 min by 1.6 mW cm⁻² white light under the same conditions but without sonication. After the treatment, bacteria were tested by viable count. Error bars present standard deviations.

Photosensitizer	MIC, µM	
	Dark	**Illumination**
Malachite green oxalate	0.87	0.15
Rose Bengal	128	2
Safranin O	89	23

Table 1.
The MIC values of water-soluble PSs in the dark and under illumination. About 3 × 104 CFU mL^{-1} of S. aureus were treated by malachite green oxalate, Rose Bengal, and Safranin O at doubled dilutions, illuminated at room temperature by white light of 1.6 mW cm^{-2} intensity for 1 h, and incubated overnight in the dark by shaking at 37°C.

concentrations (MIC) for these PSs against *S. aureus* are more than 100-fold higher. **Figure 2** shows that *S. aureus* cells were completely destroyed by RB at a concentration of 5 µM and MB at 30 µM under illumination. These PSs also showed a cytotoxic effect when applied at the same concentrations in the dark, where MB reduced the bacterial concentration by one and RB by two orders of magnitude.

2.2 Illumination

Although PSs are known to possess a certain dark activity, illumination noticeably increases their cytotoxic effect [6, 14]. An example of the difference in antibacterial activity of different PSs with and without illumination is shown in **Table 1**. In this experiment, the MIC of three PSs was determined for the bacterium *S. aureus* in the dark and after 1 h of illumination. As a result of illumination, the MIC of the examined PSs decreased approximately 6-fold for MGO, 64-fold for RB, and 4-fold for Safranin O.

The main light sources used today for activation of PSs are lasers, light-emitting diodes (LED), and gas discharge lamps (GDL) [10, 31, 32]. There is no absolute advantage of one of these light sources over the others. The choice of light source depends on the specific application. Laser is a high-intensity monochromatic source. It can be easily coupled to a single optical fiber and installed on different lighting devices. LED lamps are cheaper and provide a wide emission spectrum. GDLs are also cheaper than lasers—both in acquisition and in maintenance and have a wide emission spectrum. However, GDLs transmit more heat to the illuminated area than lasers and LEDs, which can lead to tissue damage. In general, the emission spectrum and light intensity are more important for the excitation of a specific PS than the particular light source type [10, 31, 32].

2.3 Sonodynamic excitation of photosensitizers

Illumination is undoubtedly the easiest and most effective way to activate PSs. However, its use is restricted, due to limited penetration of visible light into tissues. There is an ongoing search for alternative methods of PS excitation in the dark in order to overcome this problem. Ultrasonic activation seems to be attractive as an alternative to illumination. As with light activation, ultrasound can be selectively focused on a specific area, thus activating only PS molecules located in the affected area. Ultrasound can also easily penetrate into tissues, which opens prospects for its application in treatment of internal lesions and infections, without the need for invasive devices [33, 34]. Ultrasonic irradiation of PSs initiates the formation of highly active cytotoxic species—ROS and free radicals—which lead to the death of pathogenic cells. It was found that some well-known PSs also have sonosensitizing properties. Among

them are porphyrins [35], RB [36, 37], chlorin e6 derivative, photodithazine [36], and curcumin [38]. Several studies found sonodynamic therapy (SDT) to be the promising treatment in various forms of cancerous tumors [39–43]. Sonodynamic therapy is also offered as treatment for atherosclerosis [44]. The applicability of sonodynamic antimicrobial chemotherapy (SACT) for the treatment of infectious diseases has been confirmed by various research groups [33, 34]. We have previously demonstrated the effectiveness of RB activated by ultrasonication for eradication of Gram-positive *S. aureus* and Gram-negative *E. coli* [29, 45, 46]. The effectiveness of SACT in inactivation of *S. aureus* by two other sensitizers—curcumin [38] and hematoporphyrin monomethyl ether [35]—was also reported. Alves et al. have recently reported on effective destruction of *Candida albicans* by photodithazine and RB in the dark under the ultrasonic excitation. A significant synergistic effect of the combination between PDT and SACT for combatting *C. albicans* biofilms was also found [36].

Figure 2 demonstrates the effect of ultrasonic activation that we showed on the antibacterial activity of two PSs—RB (**Figure 2a**) and MB (**Figure 2b**)—against *S. aureus* compared to photodynamic activation. **Figure 2a** shows that 15 min of sonication reduces the number of living cells by almost two orders of magnitude, from 2×10^8 to 4×10^6 CFU mL^{-1}. RB alone applied in the dark causes a two orders of magnitude decrease in the cell concentration. However, sonication in the presence of 5 µM RB exerts a much stronger effect, reducing the cell concentration by 5 orders of magnitude. It should be noted that RB at the same concentration under illumination by visible light of 1.6 mW cm^{-2} fluence causes complete eradication of *S. aureus* cells, whereas light alone does not cause any significant harm to these cells. However, MB applied under sonication at the concentration causing complete destruction of *S. aureus* cells in the light did not eradicate microbial cells more than sonication alone (**Figure 2**).

2.4 Activation of photosensitizers by radio waves

Another possible way for activating PSs in the dark is by using nonionizing radiofrequency electromagnetic waves. The ability of radiofrequency waves to heat human tissue has been known for a long time and has already been applied for local destruction of cancerous tumors [47, 48]. The effectiveness of this method can be significantly improved by using suitable sensitizers, which can be targeted to the affected area and activated by means of radiofrequency radiation for selective destruction of cells. Tamarov et al. proposed the use of crystalline silicon-based nanoparticles as sensitizers induced by 27 MHz radiofrequency waves for effective treatment of Lewis lung carcinoma *in vivo* [48]. Another approach involved using gold nanoparticles, which were heated by an electric field using 13.56 MHz radiofrequency, and effectively destroyed human pancreatic cancer cells *in vitro* [49]. The same frequency was used in other studies to activate fullerene [50] and transferrin [51] and to eradicate cancer tumors *in vitro* and *in vivo*. A possible mechanism of radiosensitization, according to Tamarov et al. [48] and Chung et al. [51], may be thermal activation of sensitizers by hyperthermia, caused by dissipation of electromagnetic energy, which leads to thermal damage of cancer cells.

In our studies, we tested the possibility of using radiofrequency radiation to sensitize PSs in order to destroy microorganisms [29]. For this purpose, we irradiated *S. aureus* cells in physiological saline alone and in the presence of RB with radio waves at different frequencies—from 1 to 20 GHz. *S. aureus* cells in physiological saline in the dark (without RB and without radiation), *S. aureus* cells treated with radio waves (in the absence of RB), and *S. aureus* cells in the presence of RB, but not exposed to radio waves, were used as controls. Radiofrequency radiation alone did not significantly affect the survival of *S. aureus*. RB in the dark applied at the same
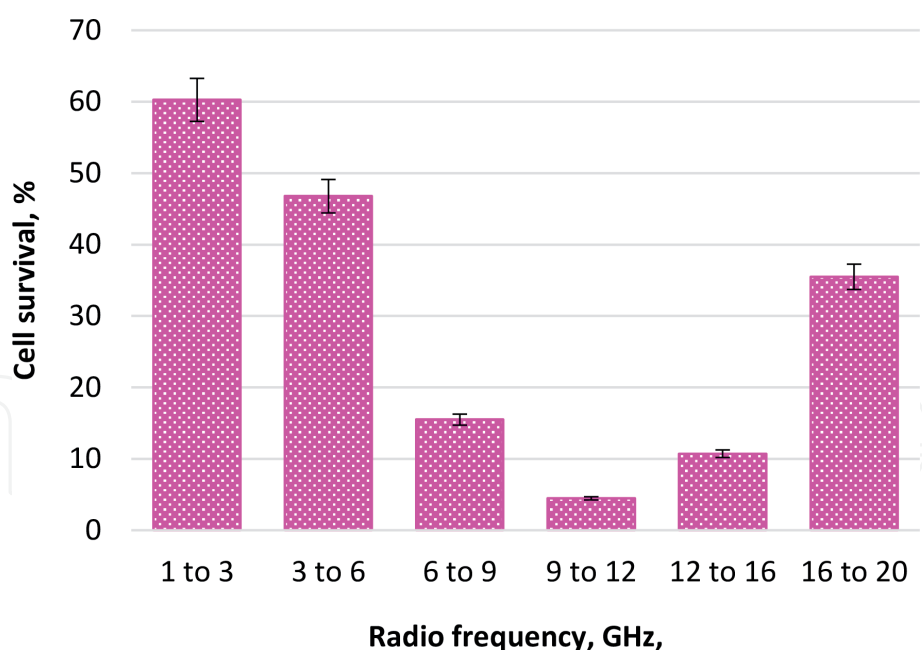
Figure 3.
Effect of RB at the 10 μM concentration under activation by radio waves at various frequency ranges on eradication of S. aureus at the initial cell concentration of 4.4×10^4 CFU mL^{-1} in the dark. Error bars present standard deviations.

concentration did not lead to any decrease in the bacterial concentration. However, exposure of *S. aureus* cells to radio waves in the presence of RB markedly reduced the number of live microorganisms. The rate of cell damage depended on the radio wave frequency. The most significant effect was observed in the frequency range of 9–12 GHz, where in the presence of RB, only 4.5% of the cells survived (**Figure 3**). For comparison, irradiation of cells treated by RB with radio waves in the frequency range of 1–3 GHz caused only a 40% reduction in the number of live cells.

To the best of our knowledge, our work was the first attempt to sensitize a PS by radio waves for destruction of bacteria. This topic naturally necessitates a broader and deeper study to understand the mechanisms of excitation and the possibilities of applying this method. The most likely mechanism of RB excitation by radio waves is conversion of electromagnetic energy into heat, which causes activation of RB, followed by energy transfer to dissolved oxygen and the formation of ROS, affecting the cells. We assume that when PSs are exposed to radiofrequency radiation, they actually behave like thermosensitizers excited by heat instead of light [29].

2.5 Chemiluminescent and bioluminescent excitation of photosensitizers

Another approach to overcoming the limitations of PACT in the treatment of deep infections is to replace the external light source by chemo- or bioluminescent light. Bioluminescence is a well-known phenomenon occurring in biological systems as a result of oxidation reactions of luciferins catalyzed by luciferases. This property is inherent in various microorganisms, worms, and insects, and the luciferins and luciferases of different organisms can be completely different. Bioluminescence is considered as a type of chemiluminescence, i.e., luminescence originating in the course of a chemical reaction. Bio- and chemiluminescence systems are used in various fields of medicine, pharmaceuticals, and bioanalytics [52, 53].

One of the well-studied and most effective chemical reactions involving light emission is oxidation of luminol [52, 54, 55]. Most applications of this reaction are associated with treatment of cancers [55–57]. Use of chemiluminescence as a light source for PACT has not been studied as extensively. Ferraz and colleagues
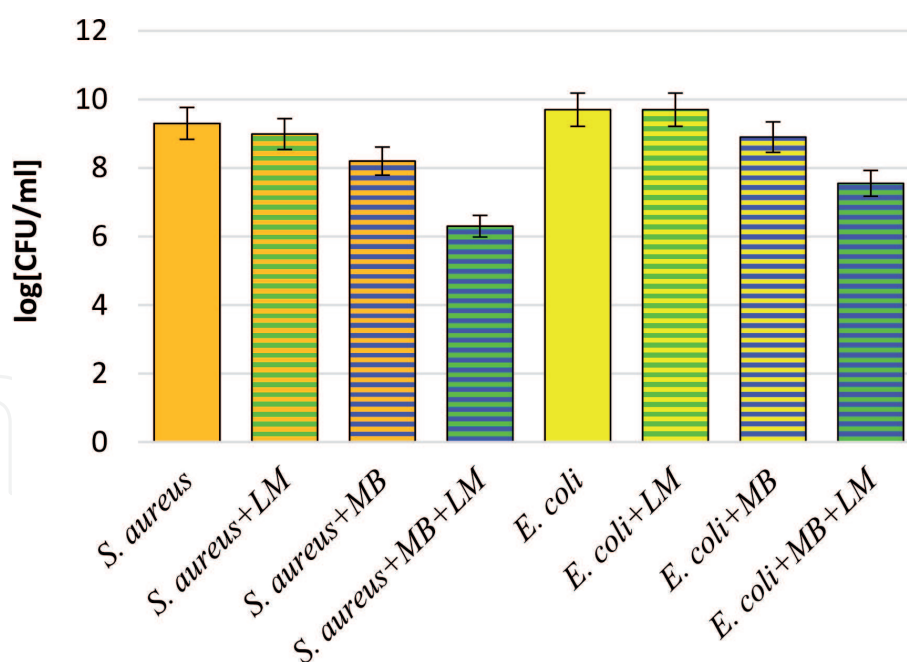
Figure 4.
Effect of chemiluminescent photodynamic antimicrobial treatment (CPAT) on the viability of S. aureus and E. coli. Cells were incubated with MB at 25 µM concentration in the presence of 0.7 mM luminol. After the treatment, bacteria were tested by viable count. Error bars present standard deviations.

evaluated the potential of chemiluminescent-excited photogem in killing *S. aureus* cells [58]. Our group demonstrated the effectiveness of chemiluminescent photodynamic antimicrobial therapy (CPAT) for destruction of *S. aureus* and *E. coli* by exposing these bacteria to the photosensitizer MB in the presence of luminol [46, 59, 60]. The results presented in **Figure 4** show that the rate of growth inhibition by MB increased in the presence of luminol compared to untreated cells or to cells exposed in the dark to MB only.

The dark effect of MB discussed in the above "Dark Activity" section can be seen in **Figure 4**, where the exposure of *S. aureus* and *E. coli* to 25 µM MB in the dark reduced the number of live cells by about 10-fold. Luminol alone had no toxic effect on the tested microorganisms. However, when combined with MB, it reduced the number of surviving bacteria by two additional orders of magnitude for *S. aureus* and 1.5 orders of magnitude in the case of *E. coli*. Thus, the use of chemiluminescence may expand the capabilities of PDT, allowing the use of PSs for the treatment of internal organs.

3. Encapsulation of photosensitizers in liposomes

Since PSs are usually inactive in the absence of excitation, focusing the beam of light, ultrasound or radio wave radiation on the affected area is the easiest way to achieve selective action of a PS. However, surrounding healthy tissues may also be affected by the PS, even under such focused processing. It is therefore very important to target the treatment directly to the infected site. Highly biocompatible and low immunogenic liposomes can serve as carriers for targeted delivery of PSs encapsulated into liposomes to the infected site [61–63].

Liposomes are spherical multi- or unilamellar vesicles consisting of phospholipids (e.g., phosphatidylcholines) with an internal hydrophilic cavity. They vary in composition, size, charge, and number of layers and can encapsulate and deliver both hydrophilic and hydrophobic compounds, which can be retained in the water core of liposomes or be encapsulated in the phospholipid bilayer, respectively.

A variety of methods have been developed for the production of liposomes with a controlled size and special properties. The most widely used method for producing liposomes is hydration of thin lipid films. In this case, lipids with or without active substances are dissolved in an organic solvent, which is evaporated on a rotary evaporator, producing a thin film on a flask wall. The lipid film is then rehydrated by an aqueous phase. Membrane extrusion and sonication methods are most commonly used for control of liposome size [64]. Advanced strategies for liposome preparation include charging the liposomes, attaching the ligands such as antibodies or lectins to their surface, or altering the physiological conditions such as increasing the temperature or changing the pH in the target tissues to produce heat-sensitive or pH-sensitive liposomes [65]. The works of Ghosh, Li, Bulbake, Abu Lila, and Alavi summarize the latest developments in the field of liposome design and optimization, including passive and active targeting, extended circulation, building multifunctional liposomes, and so on [62–66].

There exist several methods for PS encapsulation into liposomes (**Figure 5**). Hydrophilic PSs (e.g., MB, RB, or photofrin) are dissolved in aqueous buffer and are included into the internal cavity of liposomes. Hydrophobic compounds (such as temoporfin and bacteriochlorin a) are integrated in the phospholipid bilayer [62, 67]. Several groups have shown that encapsulation of PSs in liposomes improves their effectiveness against cancer *in vivo*. Back in 1983, Jori and colleagues reported that hematoporphyrin and its derivatives incorporated into liposomes on the basis of dipalmitoyl-phosphatidyl-choline are effective for systemic delivery of PSs to tumors in rats [68]. Enhancement of the photodynamic effects of photofrin encapsulated in a liposome carrier was later demonstrated on a human glioma implanted in rat brain [69]. A variety of PSs (temoporfin, zinc phthalocyanine, benzoporphyrin derivative monoacid, etc.) in various liposomal formulations, such
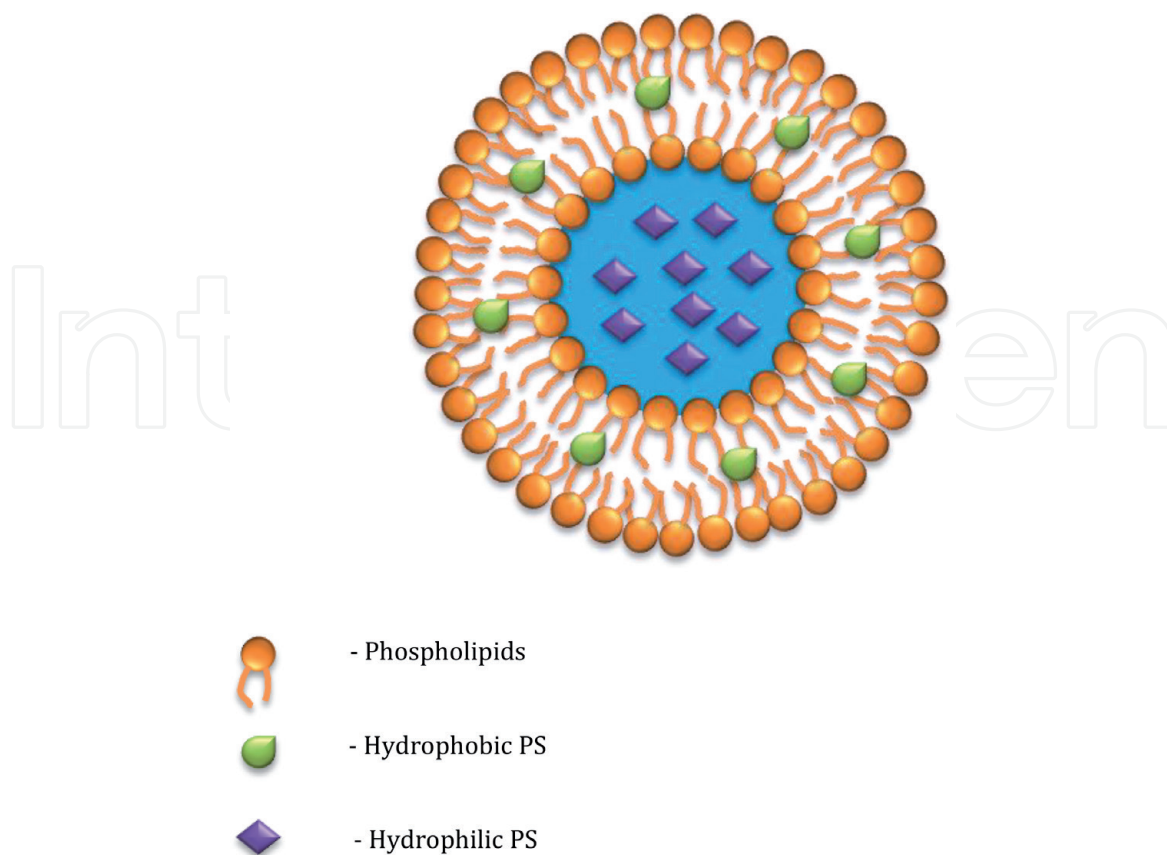
- Phospholipids

- Hydrophobic PS

- Hydrophilic PS

Figure 5.
Schematic representation of a liposome with PS entrapped in the internal aqueous phase and within the external phospholipid bilayer.

as dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, dioleoylphosphatidylcholine, and others, were found to be effective on HT29 and Meth A tumor models *in vivo* [62]. However, the only clinically approved liposomal PS drug to date is Visudyne, developed by QLT in Vancouver, and produced by Novartis AG, Switzerland. This formulation is produced from a derivative of benzoporphyrin monoacid encapsulated in unilamellar dimyristoylphosphatidylcholine/egg phosphatidylglycerol liposomes. The liposomes in this drug not only dissolve the
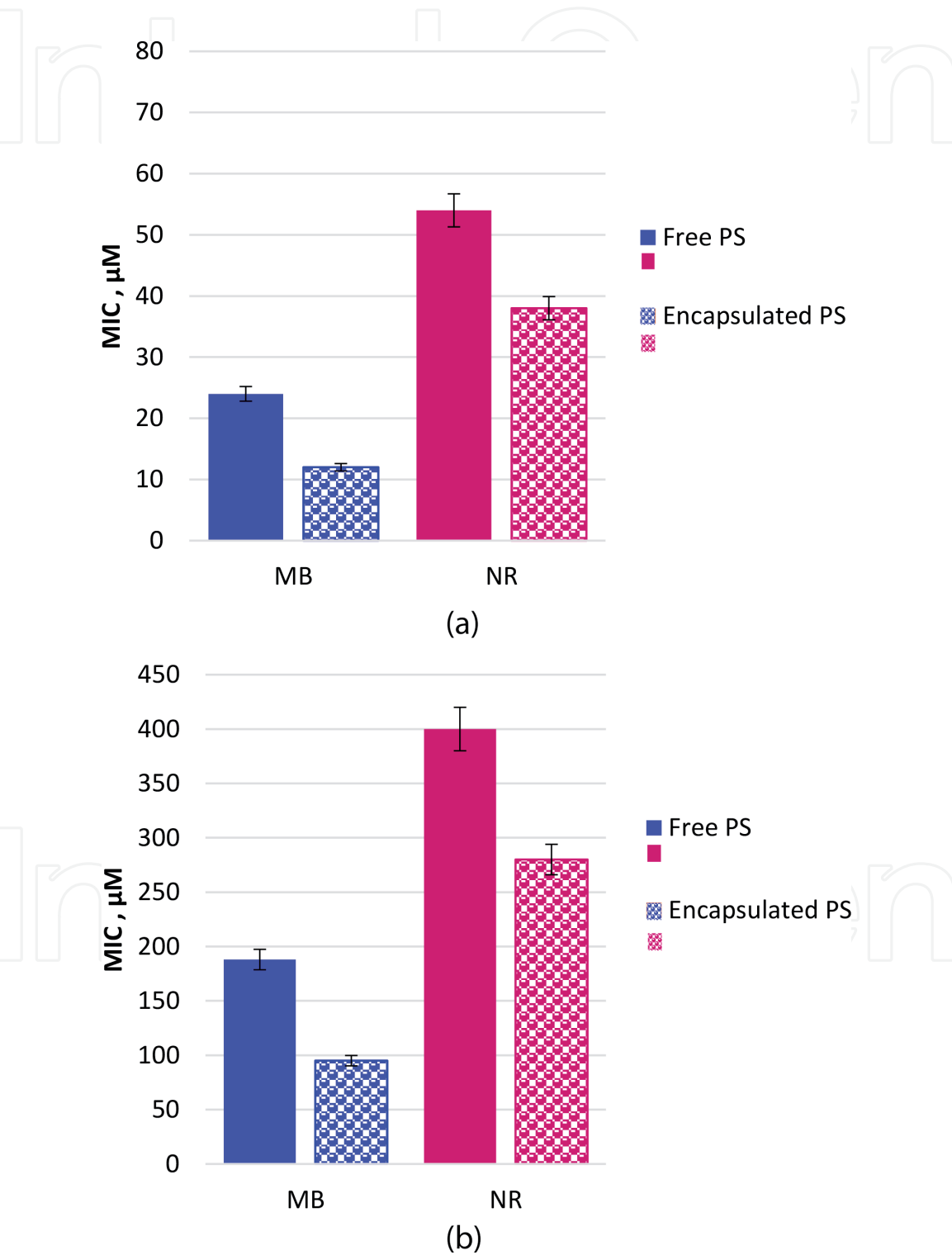

Figure 6.
MIC values of free and liposome encapsulated MB and NR determined against (a) S. aureus and (b) E. coli. Liposomes were prepared from dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol at 15 mg/ mL total lipid concentration by sonication for 10 sec. Bacteria at 3×10^4 CFU mL^{-1} concentration were treated by MB and NR at doubled dilutions, illuminated at room temperature by white light of 1.6 mW cm^{-2} intensity for 1 h, and incubated overnight in the dark by shaking at 37°C. Error bars present standard deviations.

lipophilic PS for intravenous administration but also contribute to its enhanced absorption in tumor tissues [62, 64].

Liposomal PS preparations are suitable for antibacterial applications. This approach ensures the delivery of the compound at a higher concentration, thus increasing the cytotoxicity of the drug. In addition, the local use of liposomal preparations provides a slow release of active components, which helps prolong their effect in infected tissues. In Gram-negative bacteria, fusion between liposomes and the outer cell membranes leads to the delivery of concentrated liposome contents directly into the cytoplasm [70–72]. In Gram-positive bacteria, the PS is probably released when liposomes interact with the external peptidoglycan and diffuse through the cell wall [72–74]. Various researchers have demonstrated the effectiveness of liposomal formulations of various PSs against Gram-positive and Gram-negative microorganisms and also against fungal infections *in vitro* and *in vivo*. Ferro et al. showed high efficacy of porphyrin incorporated into cationic liposomes against *S. aureus*, compared to the free drug [75, 76]. Tsai also showed an increase in the bactericidal efficacy of hematoporphyrin against a number of Gram-positive bacteria, including *S. aureus*, as a result of its incorporation into liposomes [77]. Yang proved the efficacy of chlorine e6 encapsulated in cationic liposomes against susceptible and drug-resistant clinical isolates of *C. albicans* both *in vitro* and for infected burn wounds *in vivo* [78].

In our studies, we tested the effect of different PSs in different liposome formulations on Gram-positive and Gram-negative bacteria. **Figure 6** presents a comparison between the MICs of free and dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol liposome-encapsulated MB and NR against *S. aureus* (**Figure 6a**) and *E. coli* (**Figure 6b**).
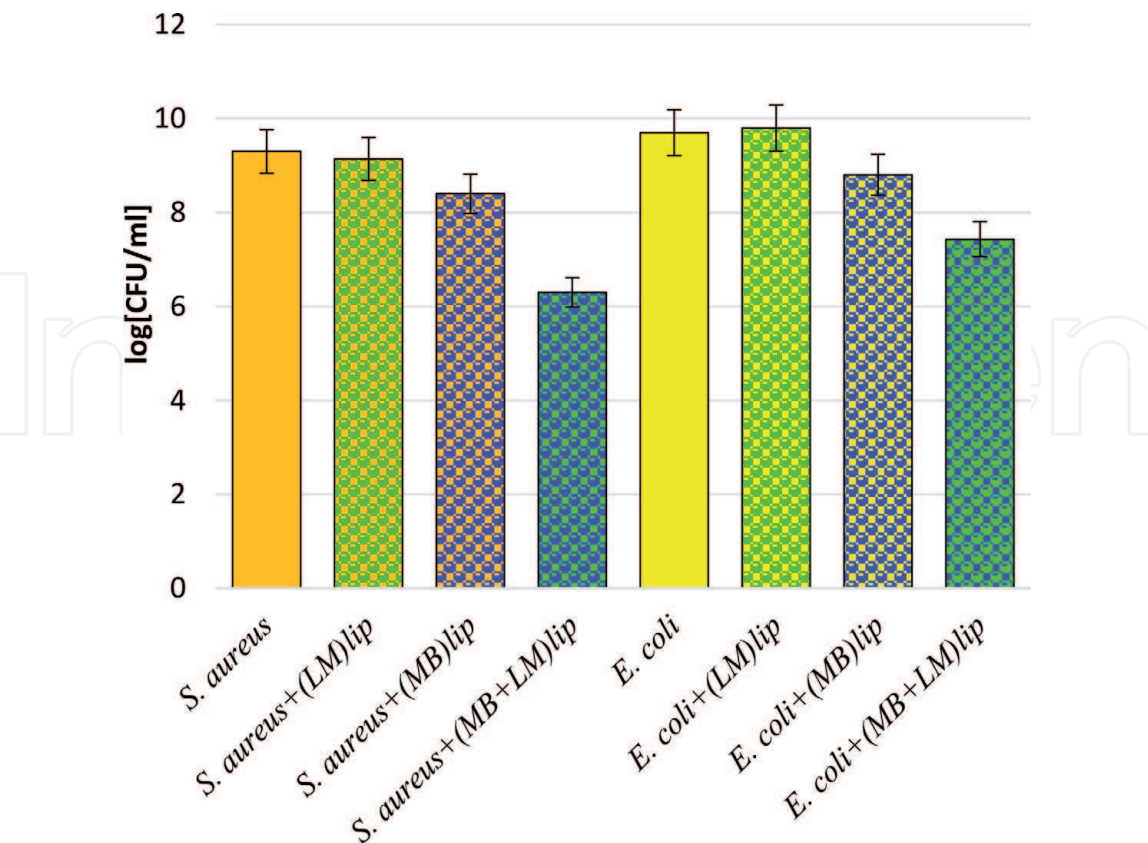

Figure 7.
Chemiluminescent photodynamic antimicrobial treatment effect on the viability of S. aureus and E. coli. Cells were incubated with 25 μM MB liposome (lip) encapsulated together with 0.7 mM luminol (LM). After the treatment, the bacteria were tested by viable count. Error bars present standard deviations.

As can be seen from the results, incorporation into liposomes significantly increased the antibacterial activity of MB and NR. Following encapsulation, the MIC of MB decreased by approximately 2-fold and that of NR by about 1.4-fold for both tested microorganisms (**Figure 6**). We tested the effect of liposome composition on the delivery of these PSs to cells and determined the conditions for efficient use of encapsulated PSs [74].

In addition, we tried to apply liposomal forms of PSs to CPAT by encapsulating not only PSs in liposomes but also luminol and introduced to activate PSs in sites inaccessible to external lighting [59]. We monitored the survival of the cells following their exposure to either liposomal MB or luminol, as well as to liposomes containing both compounds together (**Figure** 7) when the experiments were carried out in the dark.

It can be seen (**Figure** 7) that luminol itself did not lead to cell damage. MB in the liposomal form exhibited certain dark activity, similar to that in a free form discussed in the "Dark Activity" section. The addition of luminol to MB liposomes markedly increased its antibacterial activity toward *S. aureus* and *E. coli*. Liposomes were not targeted in this study. Targeting of liposomes can lead to an additional increase in the efficiency and specificity of this technique.

4. Immobilization

New prospects of using PSs are opened by the immobilization of PSs onto a solid phase. This approach may allow repeated or continuous use of PSs. PSs can be immobilized by adsorption and covalent bonding onto solid supports and by ionic bonding to ion-exchange resins or incorporation into polymer films. The photodynamic properties of immobilized PSs are reported to be retained for a long time [79–83]. PSs studied in the immobilized form include RB, MB, and TBO; the porphyrin derivatives 5,10,15,20-tetrakis (p-hydroxy phenyl) porphyrin, 5,10,15,20-tetrakis (p-aminophenyl) porphyrin, and zinc (II) phthalocyanine tetrasulfonic acid; and the ruthenium salts tris (4,4′-diphenyl-2,2′-bipyridine) ruthenium (II), tris (4,7-diphenyl-1,10-phenanthroline) ruthenium (II), tris (1,10-phenanthrolinyl-4,7-bis (benzenesulfonate) ruthenate (II), and tris (4,40-dinonyl-1,10-phenan throline) ruthenium (II). Solid supports applied for immobilization of PSs include polyethylene, polypropylene, polystyrene, polycarbonate, polymethyl methacrylate, polyester isophthalic resin, silicone, cationic nylon, porous silicones, poly (vinylidene difluoride), cellulose membranes, and chitosan [82–88]. Immobilized PSs demonstrated antibacterial properties against Gram-negative and Gram-positive bacteria in batch and continuous regimes and under reuse. Immobilized PSs were found more stable and resistant to photobleaching than in a free form [82, 86, 88].

Our group immobilized PSs in polymers using several techniques. The first method included mixing solutions of PSs in chloroform with solutions of polymers in the same solvent, followed by evaporation of the solvent, which yielded thin polymeric films with homogeneously incorporated PSs. This technique was applied to RB and MB immobilized onto polystyrene, polycarbonate, and polymethyl methacrylate [88–90]. In all cases, the obtained polymer films showed high antibacterial activity against Gram-positive and Gram-negative bacteria when exposed to an external source of white light. However, since this method involves using an organic solvent, it cannot be considered environmentally friendly. The second method is based on dissolution of PSs in a melted polymer under extrusion and does not require any additional chemical reagents [91]. The photosensitizers RB, Rose Bengal lactone, MB, and hematoporphyrin were immobilized in polyethylene and polypropylene using this method. The antibacterial efficiency of immobilized
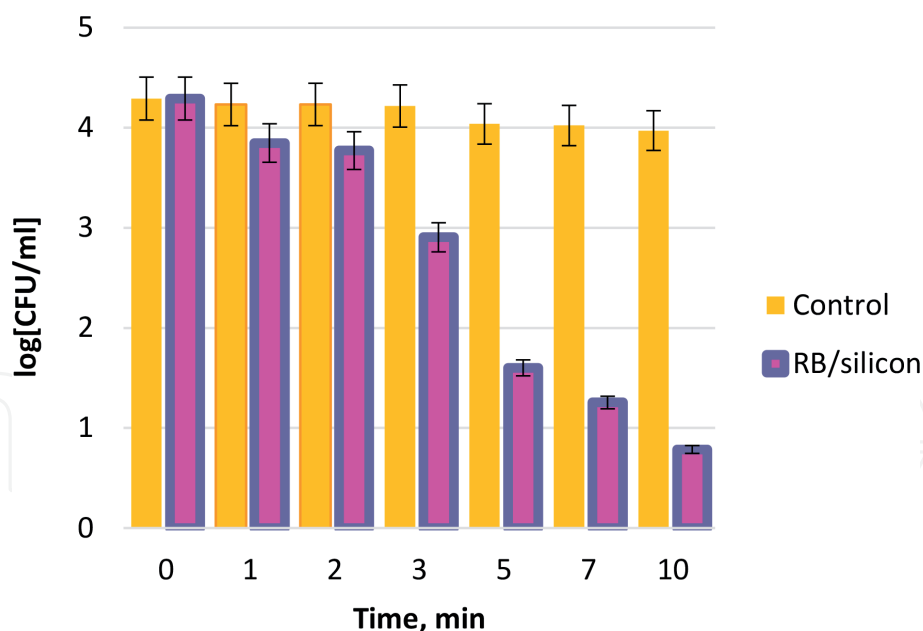
Figure 8.
Antibacterial activity of silicon-immobilized RB (5% w/w) under ultrasonic treatment in the dark.
Control—S. aureus cells treated by ultrasound only. After the treatment, bacteria were tested by viable count.
Error bars present standard deviations.

PSs obtained as polymeric strips and beads was tested against *S. aureus* and *E. coli* in batch and continuous regimes under white fluorescent light. All immobilized PSs significantly reduced the concentration of the tested microorganisms, up to their complete eradication [91].

Another immobilization technique was based on polymerization of silicon in the presence of RB as the photosensitizer. Silicon tablets produced by this method contained evenly distributed RB that was not bound to the support by covalent bonds [29]. The antibacterial activity of the immobilized RB was tested under illumination and using ultrasonic activation in the dark (**Figure 8**). **Figure 8** demonstrates the effect of immobilized RB on *S. aureus* cells when subjected to ultrasound in the dark. Silicone alone did not affect the microorganisms with and without sonication. However, the number of alive cells in samples subjected to immobilized RB under sonication decreased with sonication time and decreased by more than three orders of magnitude after 10 min of treatment.

Further development of immobilization methods and different PSs and polymers may expand the possibilities of this approach and yield the applications in various fields, such as the production of antibacterial surfaces and water disinfection.

5. Conclusions

Numerous studies show that photodynamic antibacterial chemotherapy is a powerful tool for killing microorganisms. Since this method requires external illumination, it can be successfully applied only to the treatment of local superficial skin and oral cavity infections. Development of new modes of PS excitation by ultrasound, radio waves, chemiluminescent, and bioluminescent light opens new prospects for their use in treating internal infections. Encapsulation of PSs in liposomes may solve the problem of using hydrophobic PSs with poor solubility in the aqueous phase. It can also provide delivery of a concentrated PS directly to the target site, thus increasing efficiency and reducing side effects of the treatment. Immobilization of PSs in a solid phase enables using them repeatedly or in

a continuous mode. It can be assumed that PSs have a good potential for various clinical and nonclinical applications.

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Conflict of interest

The authors declare no conflict of interest.

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