CHAPTER 3

Photodynamic Treatment: A New Efficient Alternative for Surface Sanitation

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Contents

Abstract

A novel and promising technology—photodynamic treatment (PDT), aimed for surface cleaning and sanitation in food industry—is presented. It is based on the treatment of surfaces with nontoxic dyes (photosensitizers), followed by illumination of the surface with regular white light. The method is currently used in the medical field and was proved to have wide specificity against a variety of bacterial and viral pathogens as well as against yeasts and protozoa. An additional advantage of this approach is that development of resistance of microorganisms to PDT was shown to be unlikely.

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The theoretical basis of light-induced antimicrobial treatment is described, followed by examples of its application for the cleaning and disinfection of surfaces. All available information supports the idea that PDT could offer a very efficient and cost-effective way to combat microbial contamination of foods. The advantages and pitfalls of the technique are discussed. Directions of future research needed for bringing the technology to commercial reality are identified.

I. INTRODUCTION

The development of novel cost-effective strategies for minimizing the pathogenic contamination of foods is of significant importance. Major routes for secondary contamination are processing surfaces and utensils. Commonly used sanitizers very often are not effective against bacterial spores and biofilms as well as against viruses. Besides, recent research has indicated that pathogens can acquire resistance to sanitizers, and as a result of such adaptation, cross-resistance to antibiotics has been observed (Romanova *et al.*, 2006). The emergence of multi-antibiotic-resistant pathogens is a risk to animal and human health as well as to the safety of food products. The problem of removing bacteria from food-processing surfaces is compounded by the fact that microorganisms growing in a biofilm secrete extracellular polymeric substances - exopolysaccharides (EPS), which can remain attached to the cell in a capsular form or, alternatively, be released as a slime in which the cells form a complex multicellular structure (Marsh *et al.*, 2003; Wang *et al.*, 2004). Bacteria in biofilms are more resistant to regular sanitizers. Detergents are formulated to remove particular types of soils, for example, proteinaceous, fatty, carbohydrate, or mineral soils, rather than to remove microorganisms. Gibson *et al.* (1999) reported that detergents did not significantly improve the removal of attached Gram-positive and Gram-negative organisms from food contact surfaces. Many commonly used enzymatic cleaners also fail to reduce the viable bacterial load or remove the bacterial EPS from surfaces (Augustin *et al.*, 2004; Vickery *et al.*, 2004). That is why it is necessary to investigate new approaches for surface decontamination and sanitation that will result in a minimized risk of infections, reduced bacterial loads on ready-to-eat foods, improved food quality, and a decrease in economic losses due to spoilage and recalls.

The bactericidal effect of photodynamic treatment (PDT) has been known for a long time (for reviews see Dai *et al.*, 2009; Wainwright, 1998, 2004). The method relies on illumination of microorganisms treated with nontoxic photosensitizers (PSs) by low-power visible (red, blue, or white) light. The interaction of light with PS produces highly active,

short-lived free radicals that are able to destroy cell components in close vicinity of the dye. This results, in some cases, in a 5–7-log cycle reduction in bacterial counts, indicating the promise of the approach. Development of resistance to antimicrobial PDT has not been reported, and was shown to be very unlikely (Lauro *et al.*, 2002), which makes this approach more attractive for investigation. As visible light can penetrate through thick layers, PDT may also provide a method for eradicating biofilms *in situ.*

Although PDT is gaining increasing acceptance as a novel therapeutic option, its applications for environmental cleaning and disinfection are still in their infancy. The goal of the following review is to summarize the available information on the scientific principles of antimicrobial PDT and present the current state of research and the future possibilities for application of this promising new technology to food scientists and food industry professionals.

II. INTERACTION OF LIGHT WITH MATTER AND HISTORY OF PDT

When light encounters matter, several different phenomena are observed, including reflection, transmission, refraction, scattering, and absorption. Among them, absorption is the main method of conversion of the energy of light, which represents a form of electromagnetic radiation, to other forms of energy such as heat and/or chemical energy.

The interaction of light and molecules is covered by a rather complex interdisciplinary science which includes the fields of photophysics, molecular spectroscopy, physical organic chemistry, and many others. In the simplest way, it can be divided into two separate topics, one being *photophysics of organic compounds*, and the other *photochemistry of organic compounds.*

Organic *photophysics* covers the interactions of light and organic molecules, resulting in net physical changes, but does not involve any net chemical changes. In general, the process can be visualized as the following (Eq. (1)):

$$M + h\nu \rightarrow M^* \rightarrow M \tag{1}$$

where M is an organic molecule in a ground state, that absorbs a quantum of light ($h\nu$) whose frequency (ν) is specific to the molecule, and M* is an electronically excited state of the molecule M that is formed as a result of light absorption. Subsequently, the electronically excited form M* is converted back to the ground state; sometimes, this process involves the release of the extra energy in the form of light. This phenomenon is called fluorescence or phosphorescence depending on the electronic features of the molecule.

Organic *photochemistry*, on the other hand, involves the process presented in Eq. (2):

$$M + hv \rightarrow M^* \rightarrow P \tag{2}$$

where M absorbs a photon (hv), and an electronically excited molecule (M*) is formed. But unlike in the photophysical process, M* is converted to the product (or products) P of a photochemical reaction. Thus, the energy of light is converted to chemical energy which in turn can be used in further processes. The goal of this chapter is to describe the possible applications of the highly reactive products of photochemical reactions to destroy and kill harmful bacteria in their vicinity.

The first step in a photochemical reaction is the same as in a photophysical reaction and involves the formation of an electronically excited state. Excitation in general proceeds through the promotion of an electron from one of the Highest energy Occupied Molecular Orbitals (HOMOs) to achieve one of the Lowest energy Unoccupied Molecular Orbitals (LUMOs). Unlike the ground-state entity, a molecule in its excited state survives for only a very short time. The short life-time is a consequence of the high energy of the molecule in the excited state, which exceeds that of the ground state, mostly by 150–600 kJ/mol. For comparison, the typical activation energy of a chemical reaction is $\leq$30 kJ/mol. The schematics of energy profiles for regular thermal and photochemical reactions are presented in Fig. 3.1.

The excess of energy in the excited molecule opens some new reactive channels, and the chemistry of the excited state is usually quite different from that of the parent ground-state species. Many properties of the excited-state molecule differ from those in the ground state, including electronic configuration, electronic density distribution, charge distribution, geometric structure, bond lengths and strengths, redox behavior, acid-base behavior, and magnetic properties. These differences not only make the excited-state entities short-lived, but often their reactivity would be completely unlike that of the ground state. Generally, the initial photochemical products are unstable and undergo secondary thermal and/or photochemical reactions, yielding more stable photoproducts. The main types of photochemical reactions are presented in Fig. 3.2.

Among the reactions presented in this figure, *Photosensitized reactions* are involved in the process of photodynamic killing of live cells. These reactions represent another way to dissipate the extra energy of the excited state, namely, to transfer it to another molecule(s). The species absorbing and transferring the radiant energy of light are called PSs. Both organic and inorganic molecules were shown to be effective PSs, for example, dyes, pigments, aromatic hydrocarbons, and transition metal complexes.

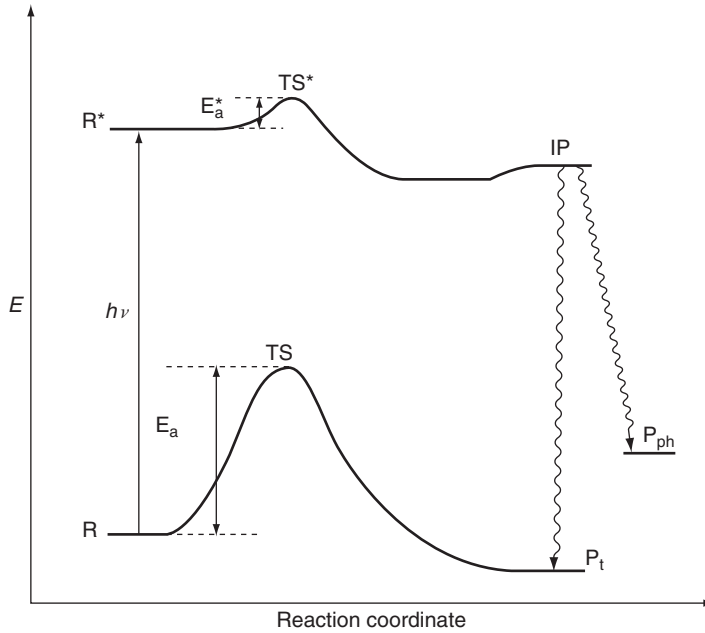


FIGURE 3.1 Comparison of the energy profile of thermal and photochemical reaction courses. R and R* are reactants, TS and TS* are transition states, E_a and E_a*, activation energies for the ground and excited states, respectively; IP, intermediate photochemical product; P_t and P_{ph}, products of thermal and photochemical reactions, respectively.

Photosensitized oxidations involving molecular oxygen are especially important in the biological field because of their impact on living organisms. In 1900, it was discovered by chance by a medical student that unicellular protozoa—paramecia were killed when exposed to light in the presence of acridine (a coal tar derivative) (Stables and Ash, 1995). Very soon, it was shown that many other living cells, including tumor cells, microorganisms, and viruses, can be killed in the presence of light, molecular oxygen, and PS. This discovery led to therapeutic applications of the interaction between PS and light to treat tumors. In 1907, the term ''Photodynamic'' was introduced to name the phenomenon of light-induced killing of cells in the presence of PS (Hamblin and Mroz, 2008). However, only from the middle of 1980s can the true explosion of interest in Photodynamic Treatment (PDT) be seen. Since then, multiple photodynamic treatments were developed and approved for clinical applications.

Despite the evident success of PDT in the medical field, the application of the principles of PDT to control pathogens in the environment and during food processing and handling was practically scarce. Only recently, several articles have been published on the use of PS dyes in combination with light for the removal of pathogens from contact surfaces

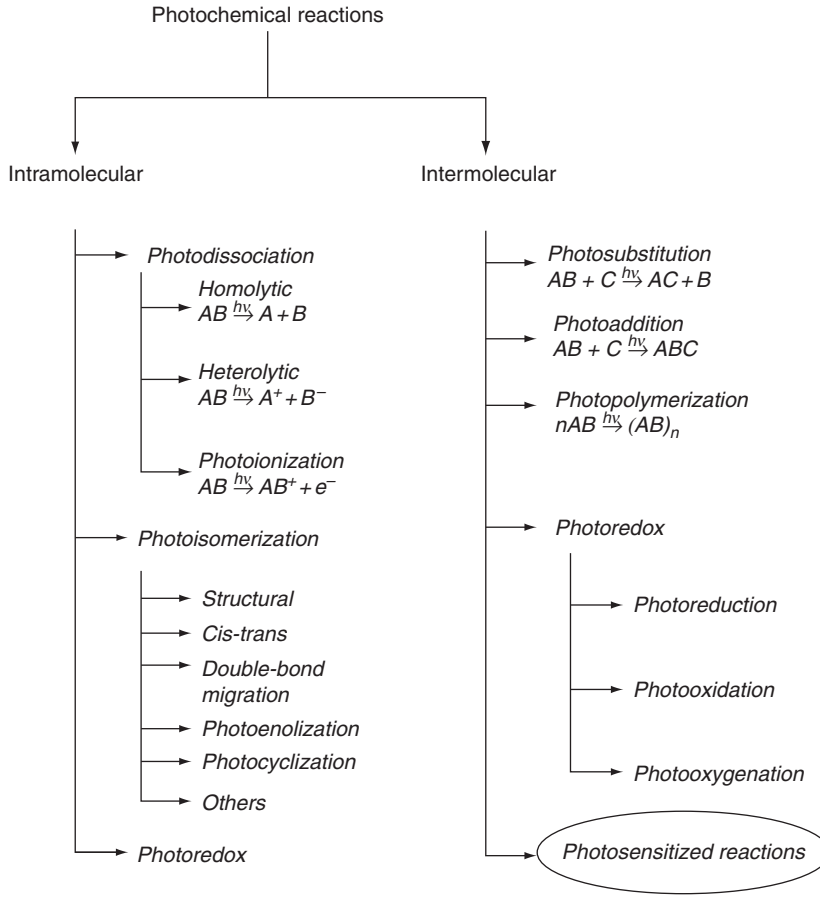


FIGURE 3.2 Main types of photochemical reactions (adapted from Stochel *et al.*, 2009).

(Brovko *et al.*, 2005, 2009). This chapter describes the mechanisms underlying PS-mediated killing of microorganisms by incident light and discusses the possible application of this phenomena for surface sanitation, control of pathogens in the environment, and the construction of self-cleaning materials.

III. MECHANISMS OF PHOTODYNAMIC PRODUCTION OF CYTOTOXIC SPECIES

In a majority of cases, the environment where live cells exist is oxygen-rich. The process involved in the photodynamic destruction of cells is, by its nature, photosensitized oxidation. A simplified illustration of the

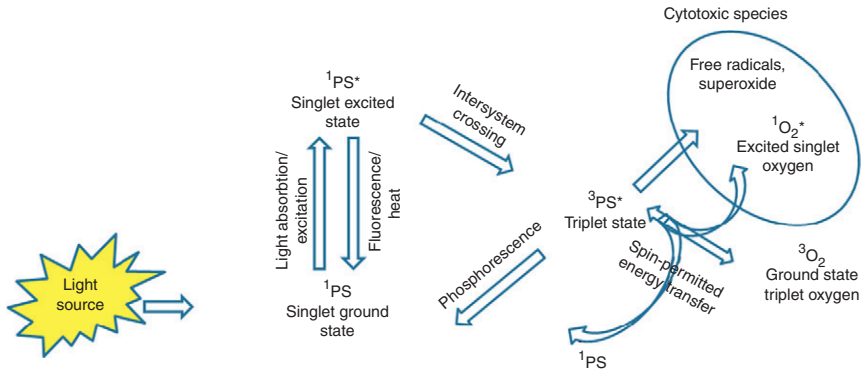


FIGURE 3.3 Scheme of photophysical and photochemical processes involved in the production of cytotoxic species during photoexcitation of photosensitizer (PS; adapted from Castano *et al.*, 2004).

processes of light absorption and subsequent energy transfer that lead to the formation of highly reactive species causing cell death is presented in Fig. 3.3. Following the absorption of light by the PS, one of its electrons is boosted into a high-energy orbital keeping its spin orientation antiparallel and thus converting the molecule from the ground singlet state (^{1}PS) to the first excited singlet state (^{1}PS*). Within nanoseconds, this species can lose its energy by emitting light (fluorescence) or by internal conversion into heat. The excited singlet state PS may also undergo the process known as intersystem crossing when the spin of the excited electron inverts to form the relatively long-lived (micro- or milliseconds) excited triplet state (^{3}PS*) that has electrons with parallel spins. The relatively long life-time of the excited triplet state is explained by the fact that the loss of excitation energy of the triplet by the emission of light in this case (phosphorescence) is a ''spin-forbidden'' direct conversion from a triplet to a singlet state.

During this delay time, the PS-excited triplet can undergo two kinds of reactions. In a Type I reaction, ^{3}PS* can react directly with a substrate that is located in its close vicinity and transfer a proton or an electron to form a radical anion or radical cation, respectively. These free radicals may further react with molecular oxygen to produce reactive oxygen species (ROS). Alternatively, in a Type II reaction, the excited ^{3}PS* can transfer its energy directly to oxygen, which is a ''spin-allowed process,'' as molecular oxygen itself is a triplet in its ground state. As a result, excited state singlet oxygen ($^1O_2$*) is formed. Both Type I and Type II reactions can occur simultaneously, and the ratio between these processes depends on the structure of PS, and the availability and concentration of the substrate and oxygen.

The Type I reaction often results in the formation of superoxide anion via electron transfer from the excited triplet PS to molecular oxygen. Though superoxide is not particularly damaging to the cell, it can react with itself, producing hydrogen peroxide and oxygen in the enzymatic reaction catalyzed by superoxide dismutase, or it can produce highly reactive hydroxyl radical (HO*).

These ROS, together with singlet oxygen produced in Type II reaction, are oxidizing agents that can directly react with many biological molecules, such as proteins and nucleic acids, causing their damage and leading to cell death.

Because of the high reactivity and short life-time of the ROS produced due to the interaction of light with the PS, only molecules and structures that are in close proximity to the localization of PS are directly affected. Considering that the estimated half-life of singlet oxygen in biological systems is <40 ns, the affected area would not exceed 20 nm (Moan and Berg, 1991). This feature of photosensitized oxidation has a major effect on the way that the photodynamic process can be applied to be effective in killing live cells and viruses. In order for photooxidative cell damage to occur, all required participants of the reaction must be located in close proximity to each other. Considering that the supply of oxygen is in excess in the regular environment and may not be a limiting factor, the main focus should be on interaction of PS with the target substrate—biomolecule or cell organelle essential to cell integrity and/or metabolic activity.

Formation of a tight complex between the PS and target at the moment when the photon of light is absorbed is crucial to the process of photodynamic killing of cells. Another important point to consider is a method of light delivery. The photon of light should be available in the right spot at the right moment for the photodynamic killing to occur. These are the key issues that should be addressed when methods for PS-mediated photokilling of microorganisms are being developed.

IV. MECHANISMS OF PHOTODYNAMIC KILLING OF BACTERIA AND VIRUSES

A. Interactions of cells with PSs

There are two main routes for PS to interact with the target cell—it could form a tight complex with the surface receptors of the cell wall and/or could be transported inside the cell where it would associate with the molecule/organelle essential for survival. In the first case, the oxidative damage is localized on the cell wall, which could lead to its disintegration and the leakage of intracellular material, resulting in cell death. When the

PS is inside the cell, it could attach to certain molecules/organelles and, after absorption of light, could cause their damage. This results in the disruption of essential metabolic pathways and subsequently causes the death of the cell. Both processes can proceed simultaneously. The input of each process into the photokilling most probably depends on the type of cell and PS, as well as on the environment.

It was observed from early on that there were substantial differences in the susceptibility to PDT between different types of microorganisms—Gram-positive and Gram-negative bacteria, viruses, fungi, and yeasts. It was found that, in general, neutral, anionic, or cationic PS molecules could efficiently kill Gram-positive bacteria, whereas only cationic PS or supplementation of PDT with permeabilizing agents are able to produce a significant kill of Gram-negative species. These differences were further explained by the differences in their physiology. In Fig. 3.4, a schematic of cell-wall structures for Gram-positive bacteria, Gram-negative bacteria, and fungi are presented together with an indication of their relative thickness.

In Gram-positive bacteria, a cytoplasmic membrane is surrounded by a thick (20–80 nm) but rather porous layer composed from peptidoglycan and lipoteichoic acid. This allows PS to easily interact with cell-wall components and to cross the cell wall. Positively charged PS could be incorporated into the cell wall through an interaction with negatively charged teichoic and lipotechoic acids similarly to the process of Gram staining of bacteria with crystal violet. When this molecule of PS is excited

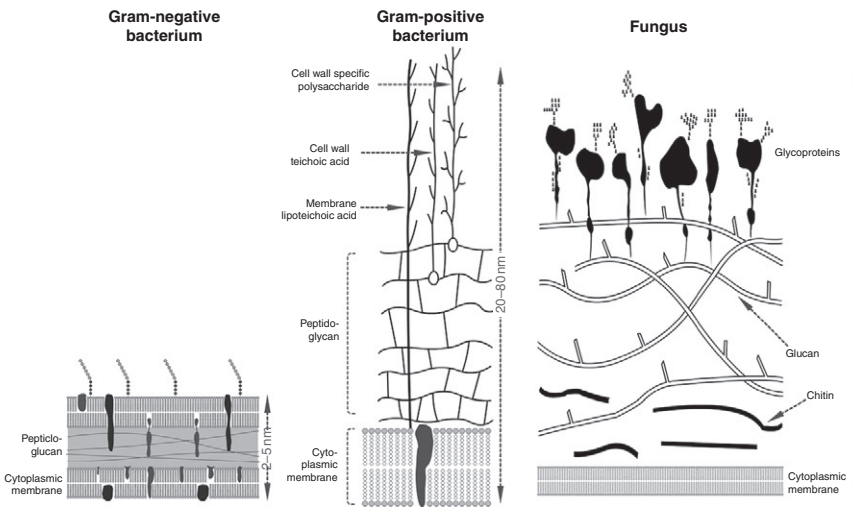


FIGURE 3.4 Structures of cell walls for Gram-positive, Gram-negative bacteria and fungi.

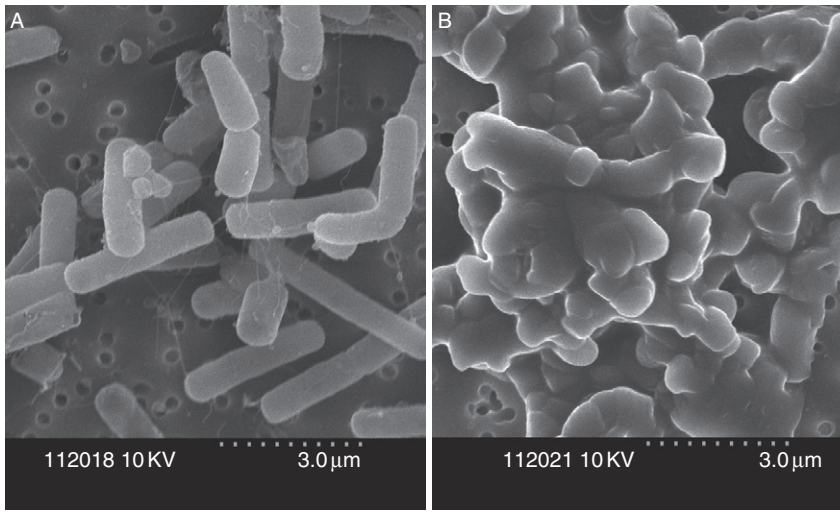


FIGURE 3.5 Electron microphotographs of *Bacillus cereus* cells before (A) and after (B) photodynamic treatment with Toluidine Blue O (20 μg/ml). Illumination with red light (660 nm, 7.6 mW/cm^2) for 1 h (Brovko *et al.*, 2005).

by a photon of light, ROS are generated and photooxdative damage of the cell wall occurs. This in turn leads to formation of pores or to disintegration of the cell and the leakage of intracellular material. In Fig. 3.5, electron photomicrographs of *Bacillus cereus* are presented before and after PDT with Toluidine Blue O (TBO). It is clear that, in this case, the PDT resulted in the total collapse of cells walls. The morphological changes induced by the photodynamic action of TBO in *Staphylococcus aureus* and *Escherichia coli* were studied using atomic force microscopy (Sahu *et al.*, 2009). For Gram-positive *S. aureus*, the PDT resulted in a light dose-dependent increase in surface bleb formation, suggesting breakage of contact between the cell wall and the membrane with no significant change in the cell dimensions. Photosensitization of Gram-negative *E. coli*, on the other hand, produced surface indentations, a significant reduction in mean cell height, and flattening of the bacteria. These results indicate damage to the bacterial membrane and a loss of cytoplasmic materials. Such morphological changes are consistent with the previously reported data that, for Gram-negative bacteria, the time course of release of intracellular ATP correlated with the reduction in live cells numbers. For Gram-positive bacteria, there was a delay between the beginning of viability loss and detection of the released intracellular ATP, which was explained by the thickness of the cell wall in Gram-positive bacteria (Romanova *et al.*, 2003).

The cell wall of Gram-negative bacteria consists of an inner cytoplasmic membrane and an outer membrane consisting of a phospholipid bilayer with incorporated lipopolysaccharides (LPS) directed outside. The two membranes are separated by a thin, though dense, peptidoglycan layer. The total thickness of the cell wall for Gram-negative bacteria is low (2–5 nm); however, it forms an effective permeability barrier between the cell and its environment. Due to the much smaller volume and dense structure of the cell wall in Gram-negative bacteria, the binding of PS is limited. Nevertheless, the net negative charge of the cell surface facilitates the binding of cationic PS providing the basis for photodynamic destruction of Gram-negative bacteria using cationic PS. When the efficiency of photodynamic killing was compared for Gram-positive and Gram-negative strains in similar conditions, it was shown that, in both cases, a multilog reduction in the number of live cells was reached. However, the difference in killing efficiency for different types of bacteria could be significant (Brovko *et al.*, 2009). In Table 3.1, data are presented on the observed reduction in the numbers of bacterial cells in suspension due to photodynamic killing using various dyes. In the majority of cases, the killing effect for Gram-positive bacteria (*Listeria monocytogenes* and *Bacillus* sp.) was greater than for Gram-negative species (*E. coli* and *Salmonella* Typhimurium).

TABLE 3.1 Killing effect of the photodynamic treatment observed for microorganisms in suspension (adapted from Brovko *et al.*, 2009)

	Mean log reduction in count after 30 min treatment with the dye				
Dye, μg/mL	EC	ST	BS	LM	SC
AF 5	1.13		0.55	>6	0.01
50	>5		>5	>6	1.5
500	>6		>5	2.2	>5
RB 5	0.2	2.0	>5	>6	1.01
50	>6	>6	>5	>6	>6
500	>6	>6	>5	>6	>6
MG 5	0.09	−0.01	−0.09	0.04	0.21
50	0.05	0.02	−0.02	>6	0.39
500	0.69	2.39	>5	1.93	0.68
PhB 50	0.16	−0.21	>6	>6	0.33
500	0.45	0.93	>6	>6	1.14
5000	−0.05	0.06	>6	>6	1.15

EC, *Escherichia coli*; ST, *Salmonella* Typhimurium; BS, *Bacilllus* sp.; LM, *Listeria monocytogenes*; SC, *Saccharomyces cerevisiae*; AF, Acriflavin Neutral; RB, Rose Bengal; MG, Malachite Green; PhB, Phloxine B.

Fungal cells and yeasts have a relatively thick cell wall (60–80 nm) comprised of a layer of beta-glycan and chitin that is coated on the outer side by an array of glycoproteins (Fig. 3.4). This creates a permeability barrier intermediate between Gram-positive and Gram-negative bacteria. However, the net charge of the cell wall in yeasts is close to zero (Vergnault *et al.*, 2004), thus making the electrical charge of PS nonessential for the ability to bind to the cell wall. This may explain the significantly lower average efficiency of photodynamic killing observed for *Sacccharomyces cerevisiae* as compared to bacteria species (Table 3.1). Nevertheless, under some conditions, the observed reduction in the number of yeast cells due to PDT was quite substantial. For example, when *S. cerevisiae* (10^7 CFU/ml) were treated with Rose Bengal (50–500 μg/ml), the live cells were practically eliminated from the suspension after 30 min of illumination with white light (Brovko *et al.*, 2009).

Some bacteria have the ability to protect themselves from adverse conditions by synthesizing an EPS that forms a 3D structure surrounding cells. These structures are called biofilms and are usually formed on contact surfaces. One of the important features of such environments is increased resistance of bacteria within biofilms to detergents and antibiotics, as the dense extracellular matrix and the outer layer of cells protect the interior of the community. The survival of bacteria in biofilms very often presents a challenge for sanitation processes applied in food industry. Nevertheless, it was shown that PDT is capable of removing biofilms and destroying the bacteria in them (Brovko *et al.*, 2005; Sharma *et al.*, 2008; Zanin *et al.*, 2005). The efficiency of PDT for biofilm destruction was similar or slightly less than for vegetative bacterial cells in suspension (Table 3.2).

Another way in nature to protect various forms of life is the formation of spores. Many bacteria, fungi, plants, algae, and protozoan are known to form spores as a strategy to survive for extended periods of time in unfavorable conditions. The high resistance of spores to chemical and physical agents is explained by their multilayered structure (Fig. 3.6). This structure is practically impermeable for cytotoxic chemicals. Besides, endospores have only 20–30% of the water content of vegetative cells

TABLE 3.2 Comparison of the efficiency of photodynamic treatments for killing bacteria in vegetative form and in biofilms (Brovko *et al.*, 2005)

Log reduction in bacterial cell numbers after photodynamic treatment			
Salmonella Typhimurium		*Listeria monocytogenes*	
Vegetative cells	Biofilm	Vegetative cells	Biofilm
2.08	1.92	1.66	1.25

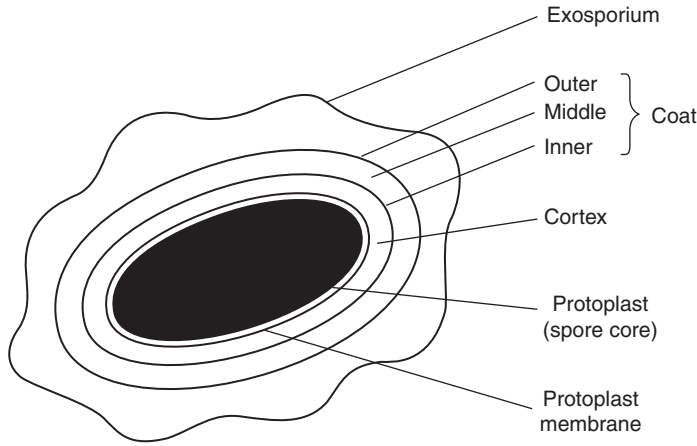


FIGURE 3.6 The structure of a bacterial endospore.

and so they are enzymatically dormant and able to resist long periods of desiccation. Some spore-forming bacteria produce special proteins that protect the DNA of the spores.

Generally to destroy spores, much harsher conditions are applied when compared with vegetative cells. Among sporicidal treatments, heat ($>121\,^{\circ}C$), strong hypochlorite solutions, chlorine dioxide, and ionizing or UV radiation are most commonly used. These conditions are not always compatible with a food-processing environment. It was shown recently that the effective destruction of bacterial endospores can be achieved by mild PDT (Brovko *et al.*, 2005; Demidova and Hamblin, 2005b, Oliviera *et al.*, 2009). The susceptibility of spores for PDT was shown to depend on the type of microorganism and on the nature of the PS used. For example, the treatment of *B. cereus* spores with TBO under red light illumination was shown to effectively decrease the number by more than 4 orders of magnitude. On the other hand, spores of *B. megaterium* remained intact under the same conditions (Demidova and Hamblin, 2005b). Some cationic porphyrin derivatives were quite efficient in photodynamic killing of bacterial spores, while others provided no effect. Though the presence and number of positive charges in the porphyrinic molecule was shown to be important for photodynamic inactivation, these were not the only factors contributing to the killing efficiency (Oliviera *et al.*, 2009).

Unlike in bacteria and fungi, viruses do not have a protective coat that separates essential proteins and nucleic acids from the environment. The majority of viruses consist of nucleic acid polymers (DNA or RNA) enclosed within a protein coat (capsid). Sometimes, viruses pick up a lipid membrane (envelope) from the host cell that surrounds the capsid. The average size of viral particles is in the range 10–300 nm. The most common

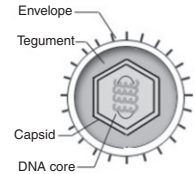
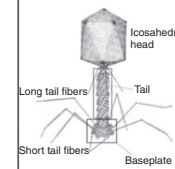
Helical non-enveloped, e.g., Tobacco mosaic virus	Polyhedral non-enveloped, e.g., Adenovirus	Enveloped helical, e.g., Paramyxovirus	Enveloped polyhedral, e.g., Herpesvirus	Complex virus, e.g., bacteriophage T4
				
20×300 nm	60–90 nm	150–300 nm	100–200 nm	30–200 nm

FIGURE 3.7 Structures of virus families.

structures of viruses are presented in Fig. 3.7. Viruses do not have the metabolic machinery necessary for growth and propagation. For supporting their life cycle, viruses use the resources of host cells. They cannot naturally reproduce outside of a host cell. Thus, the first step in their life cycle is attachment to the host which is followed by penetration or injection of DNA into the cell. The attachment is mediated by a specific binding between the viral surface proteins and their receptors on the host cellular surface. This specificity determines the host range of a virus. The relatively small size of viral particles, exposure of receptors essential for virus propagation, as well as the lack of protective coat such as a cell wall makes viruses to some extent more susceptible to photodynamic killing than other microorganisms. Virus inactivation in blood products using PDT is a well-established technique approved in many countries since its first introduction in 1992. It was reported that hepatitis B, hepatitis C, and human immunodeficiency viruses were totally inactivated in plasma products by PDT with micromolar concentrations of Methylene Blue (MB; Mohr *et al.*, 1997).

Recently, it has been shown that photoactive fullerene derivatives (C_{60}) were very effective in the photodynamic inactivation of bacterial viruses (bacteriophages). The treatment of water with C_{60}-based PS under illumination resulted in a 2-log reduction in the number of bacteriophages in the water within only 2 min (Lee *et al.*, 2009).

Despite the variability in susceptibility of microorganisms to PDT, it can be concluded that, in the majority of cases, a PS can be identified that will effectively destroy the target microorganism by PDT.

B. Modes of light delivery

For PDT to be effective, it is important to ensure that light could reach the target substance. Light is either scattered or absorbed when it enters the sample. The extent of both processes depends on the optical properties of the sample and the wavelength of light. Scattering is generally a more important factor in limiting light penetration in most samples. For turbid samples, light intensity could decrease 100–1000 fold per each cm. Thus, the highest efficiency of photodynamic inactivation of microorganisms could be achieved for surfaces and for layers directly underneath the surface with a penetration depth of several centimeters for clear solutions.

The absorbance of light by PS itself can also limit light penetration. This phenomenon has been termed ''self-shielding,'' and is particularly pronounced with PSs that absorb very strongly at the treatment wavelength (Dougherty and Potter, 1991). Thus, for better results, the concentration of PS should be optimized considering not only attachment to the target substance, but also the optical properties of the molecule such as light absorption.

The variety of light sources used for PDT includes lasers, photo diodes, and regular mercury or halogen lamps. The intensity of light reported as being sufficient for effective photodynamic killing of microorganisms is in the wide range between 0.5 and 200 mW/cm^2. The time of exposure varies from seconds to tens of minutes, depending on the intensity of the used light source. For comparison, according to the World Meteorological Organization, the intensity of direct sunlight is $\geq$12 mW/cm^2 (Anon, 2008). In general, the light intensities used for PDT are low and do not cause any thermal effects. Thus, it can be concluded, that the illumination required for food-processing areas (220–540 lux) would be sufficient to initiate the photodynamic killing of microorganisms.

V. EXAMPLES OF PHOTOACTIVE DYES USED FOR PHOTODYNAMIC KILLING OF MICROORGANISMS

Two major features that govern the choice of PSs for the photodynamic killing of microorganisms are their ability to efficiently form the triplet excited state upon illumination with the light of a specific wavelength, and their high affinity to the life-essential molecules or organelles. The field of application of the PDT is also very important when choosing the proper PS. For medical and therapeutic applications, the PS should have

minimal dark toxicity, be rapidly excreted by the body, and have strong absorbance with a high extinction coefficient in the 600–800-nm range where light penetration in live tissues at its maximum (Detty *et al.*, 2004). For applications in environmental cleaning, for example, for surface sanitation in food-processing facilities, the most important factors that define the choice of PS besides their photodynamic properties are their low dark toxicity, availability (cost), compatibility with food industry requirements, and minimal effect on food organoleptic qualities (Brovko *et al.*, 2009).

Historically, most of the PS tested for their antimicrobial properties were already known to be effective for cancer treatment. They include the following classes of organic dyes: porphyrin-related structures, phthalocyanines, phenothiazinium dyes, xanthylium dyes, and cationic fullerenes (Dai *et al.*, 2009) (Table 3.3).

In Fig. 3.8, examples are presented of structures representing the main classes of dyes used for antimicrobial PDT. The most investigated among them are cationic porphyrin derivatives (Alves *et al.*, 2009). They were shown to be effective in the killing of both Gram-positive and Gram-negative bacteria in suspension as well as in biofilms (Demidova and Hamblin, 2004; Di Poto *et al.*, 2009). Yeast cells (*Candida albicans*) were also shown to be susceptible to photodynamic inactivation when treated with tri- and tetracationic porphyrin derivatives (Cormick *et al.*, 2009).

TABLE 3.3 Physicochemical and photochemical properties of the photoactive dyes (adapted from Brovko *et al.*, 2009)

Dye	Absorption wavelength, nm (ε, $M^{-1}cm^{-1}$)	Current applications
Rose Bengal (anion)	525, 540 (7.28×10^4)	Biological stain, eye drops to assess the damage of conjunctiva and corneal cells; treatment of certain cancers
Malachite Green (cation)	629 (15.0×10^4)	Dye for silk, leather, and paper, biological stain, topical antiseptic, treatment for parasitic, fungal, and bacterial infections in fish
Phloxine B (anion)	524 (10.1×10^4)	Colorant for food, cosmetics and drugs, biological stain, disinfection and detoxication of waste water, toxicant for fruit fly, bacteriocidal agent in plants

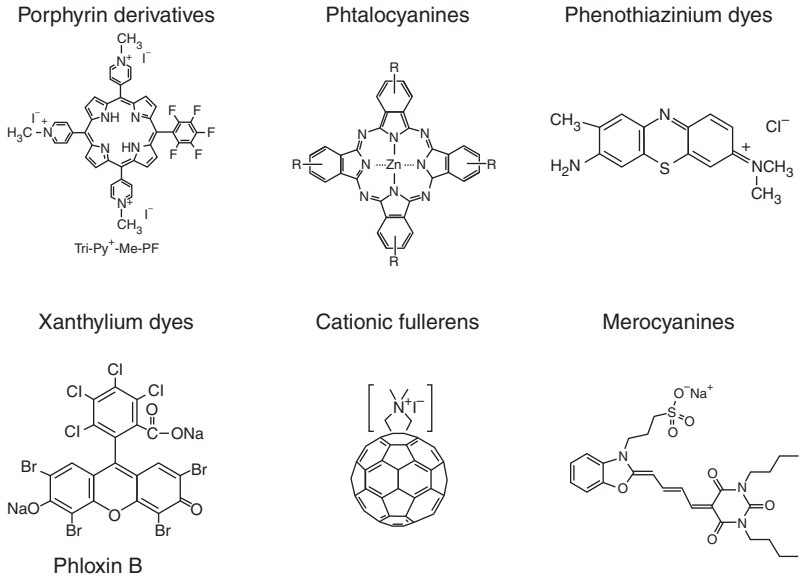


FIGURE 3.8 Major classes of photosensitizers used for antimicrobial treatment.

The photodynamic inactivation capacity of porphyrin-based PSs was confirmed for both cells in suspensions and on the surface of agar plates. The complete inactivation of viruses (>99.9999%) with tetracationic porphyrins under low intensity white light illumination was observed by Costa *et al.* (2008). The treatment of microbiologically polluted aquaculture waters with submicromolar doses of cationic porphyrins combined with the action of visible light (including sunlight) was shown to be effective in the inactivation of a mixture of bacterial and fungal pathogens (Magaraggia *et al.*, 2006). The conjugation of porphyrins with polymers (Demidova and Hamblin, 2004; Bonnett *et al.*, 2006; Xing *et al.*, 2009) or their incorporation into polymeric films (Funes *et al.*, 2009) or liposomes and micelles (Ferro *et al.*, 2007, 2009; Tsai *et al.*, 2009) resulted in a further enhancement of the photokilling effect.

In general, porphyrin derivatives demonstrated a wide specificity and high efficiency in photokilling. One disadvantage of this class of PSs is the high cost and limited availability of certain porphyrin derivatives.

Another way of initiation of porphyrin-mediated photodynamic killing of microorganisms is to use endogenous porphyrins synthesized by some bacteria. It was observed that the addition of 5-aminolevulinic acid (ALA)—the precursor on porphyrin biosynthesis, resulted in the accumulation of sufficient amounts of intracellular porphyrins to initiate the photodynamic killing of microorganisms upon illumination (Hamblin

and Hasan, 2004). Recently, it was shown that the important food-borne pathogen *B. cereus* can effectively produce endogenous PS from exogenously applied ALA even at very low concentrations (3 mM) (Luksiene *et al.*, 2009). Subsequent illumination of the cell suspension with blue light (20 mW/cm^2) for 15–20 min resulted in a 6.3-log reduction in the number of vegetative cells and a 3.1-log reduction in the number of spores.

Another group of compounds often used for the photodynamic killing of microorganisms is phenothiazinium dyes. These include such PSs as TBO, MB, 1,9-dimethyl-methylene blue (DMMB), and new methylene blue (NMB). TBO is probably the most frequently used member of this class of PSs for the photokilling of bacteria and fungi (Tseng *et al.*, 2009; Usacheva *et al.*, 2001; Wainwright *et al.*, 1998). A significant inactivation of biofilms was observed when staphylococcal biofilms were exposed to TBO and lasers simultaneously (Sharma *et al.*, 2008). The most potent form of TBO was reported by Gil-Thomas *et al.* (2007). They conjugated TBO with tioponin gold nanoparticles. The light-activated antimicrobial activity of this conjugate was at least four times higher when compared with free TBO at the same concentration. The improved performance was explained by the enhanced extinction coefficient of the conjugate which facilitates the formation of the excited TBO and thus of the cytotoxic oxygen species.

MB also demonstrated very wide photosensitizing activity (Peloi *et al.*, 2008; Prates *et al.*, 2009). It was reported to be effective against bacteria and fungi. A methylene blue based PDT was shown to eradicate *Pseudomonas aeruginosa* both in planktonic and biofilm cultures (Street *et al.*, 2009).

A strong photokilling effect was reported when cationic Zn(II) pyridyloxyphthalocyanine derivatives were used as PSs (Kussovski *et al.*, 2009; Scalise and Durantini, 2005; Spesia and Durantini, 2008). It was shown that both noncharged and cationic derivatives of phthalocyanine were readily bound to *E. coli* cells. But only cationic derivatives produced a significant photoinactivation with a 2.5–4.5-log reduction in the number of live cells after 30 min of illumination. However, the variety of microorganisms tested for their susceptibility to photodynamic killing by phthalocyanine-based PSs is very limited. It can be partially explained by the fact that they are not readily available commercially.

A group of newly emerging PSs is based on supramolecular carbon nanostructures called fullerenes. Fullerenes are ball-shaped molecules composed entirely of dozens of carbon atoms. The first fullerene (C_{60}) was discovered in 1985 and consisted of 60 carbon atoms arranged as 12 pentagons and 20 hexagons exactly as in a soccer ball (Kroto *et al.*, 1985). From the very beginning, fullerene-type structures attracted a lot of attention of researchers from all different fields of science not only for the beauty of their design but also for their remarkable properties. Among them, the significant absorbance of visible light that results in the

formation of a long-lived excited triplet state allowed fullerenes to act as PSs. Later, various functionalized fullerene derivatives have been produced that were water soluble and thus compatible with biological applications. Fullerenes have been used for the photoinactivation of viruses (Kassermann and Kempf, 1997), as well as different types of bacteria and fungi (Tegos *et al.*, 2005). Among six fullerene derivatives tested, the *bis*- and *tris*-cationic fullerenes in combination with white light produced a 4–6-log reduction in the numbers of Gram-positive, Gram-negative bacteria and fungi. It was shown that these compounds performed significantly better than a widely used PS, TBO. Despite being rather exotic compounds, the high selectivity and efficacy exhibited by these PSs definitely deserve further investigation.

Among other PSs used for antimicrobial treatment, Rose Bengal, Malachite Green, and Phloxine B are attracting significant interest for application in the food industry. These compounds belong to the class of xanthylium dyes. This class of dye is widely used as fluorescent stains in biology and they are readily available at a reasonable cost (Table 3.2). Phloxine B, in particular, has negligible toxicity and is approved for use in food and cosmetic products as a colorant. Two of these dyes (Rose Bengal and Phloxine B) are weak acids and thus carry a negative charge. Malachite Green, on the other hand, is positively charged under normal conditions. Their efficiency as PSs was investigated for the photodynamic killing of a variety of microorganisms. Malachite Green demonstrated successful photoinactivation ($\sim$3-log reduction) of Gram-negative *Actinobacillus actinomycetemcomitans* upon illumination with a low-power red laser for 5 min (Prates *et al.*, 2007). In another publication (Brovko *et al.*, 2009), Malachite Green was shown to be more active against Gram-positive bacteria (*L. monocytogenes* and *Bacillus* sp.) and did not produce significant photokilling of Gram-negative bacteria (*E. coli* and *Salmonella*). This probably can be explained by the different illumination conditions; red laser was used in the first case and white light from a halogen lamp was used in the latter case.

Both Rose Bengal and Phloxine B, though known to effectively produce the triplet excited state under illumination, carry a negative charge and thus are not the obvious candidates for being effective PSs in the photodynamic killing of bacteria. Nevertheless, both dyes were shown to be able to produce a significant photodynamic killing of various bacteria (Brovko *et al.*, 2009; Decraene *et al.*, 2006; Demidova and Hamblin, 2005a, Rasooly and Weisz, 2002; Schafer *et al.*, 2000). It was shown that these dyes actually did not bind to bacterial cells, as simple washing did remove all dye from the sample. However, concentration levels of 50–500 μg/ml of Rose Bengal were sufficient to produce a $>$6-log reduction in the number of *E. coli* and *L. monocytogenes* cells due to PDT (Table 3.1). Phloxine B was effective as PS only for Gram-positive bacteria—*L. monocytogenes* and *Bacillus* sp.

TABLE 3.4 Photokilling effect of dye-poly (vinyl amine) (PVAm) conjugates

	Mean log reduction in count after 30 min treatment with the dye conjugate				
Conjugate	*E. coli*	*Salmonella* Typhimurium	*Bacillus* sp.	*L. monocytogenes*	*S. cerevisiae*
PVAm-Rose Bengal	>6	2.31	>4	>5	0.08
PVAm-Phloxine B	>6	1.33	>4	>5	−0.46

Rose Bengal and Phloxine B at concentrations of 4.6% and 4.8% (wt/v), respectively (adapted from Brovko *et al.*, 2009).

The conjugation of Rose Bengal and Phloxine B with the cationic polymer poly (vinyl amine) produced much more powerful photosensitizers (Brovko *et al.*, 2009). The photokilling effect of these conjugates was much more pronounced than it was for comparable concentrations of the respective dyes in solution (Table 3.4). This was explained by the enhanced interaction of the positively charged polymer-dye conjugates with the negatively charged bacteria cell wall which probably brought PS closer to the target cell and thus facilitated photodestruction. This theory was consistent with the fact that both of these dyes in solution as well as in the form of conjugates did not produce any significant photokilling effect of yeast cells.

VI. PDT FOR ENVIRONMENTAL CLEANING AND DISINFECTION

The majority of currently accepted applications of PDT are in the medical area. MB has been widely used by several European blood transfusion services for the decontamination of blood plasma. Ready-to-use reagents and automatic systems are commercially available for photodynamic plasma disinfection from Baxter Healthcare and Maco Pharma (UK). It has been shown previously that the PDT of plasma is particularly effective in the inactivation of enveloped viruses such as HIV, influenza, herpes simplex, West Nile virus, and others (Williamson *et al.*, 2003).

Several papers have been published on the photodynamic inactivation of microorganisms in waste water treatment (Acher and Juven, 1977; Gerba *et al.*, 1977a,b, Kussovski *et al.*, 2001; Martin and Perez-Cruet, 1987). Despite the fact that the effectiveness of photodynamic disinfection

and the low cost of the procedure were demonstrated long ago, the procedure was not yet applied in practice for environmental cleaning and disinfection. It may partly be explained by the undesirable presence of photodynamic dyes in the treated water, which required additional steps to remove the residual PS prior to the release of the water. To avoid this problem, recently it was proposed to use immobilized photoactive dyes for water photodisinfection (Bonnett *et al.*, 2006). Zinc(II) phthalocyanide tetrasulfonic acid as a PS was covalently immobilized on a chitosan membrane reinforced with a nylon net. This membrane was placed into the flow photoreactor system with circulating water containing a bacterial pathogen (*E. coli*). The schematics of the apparatus are shown in Fig. 3.9. Effective photokilling of *E. coli* was observed in the system, providing a 2.5-log reduction in the number of live cells within 2 h.

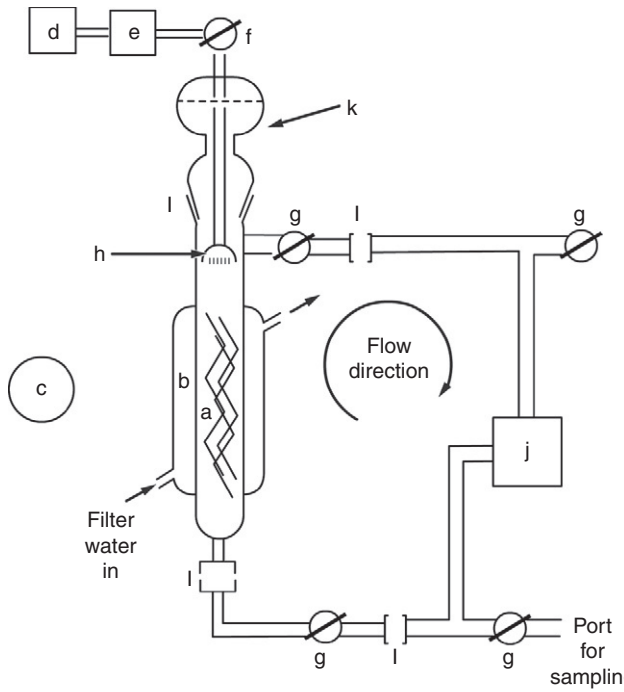


FIGURE 3.9 Circulating water photoreactor system for determination of photomicrobiocidal activity under water flow conditions. a, reinforced membrane used in the study; b, water jacket, continuous flow, infrared filter; c, light source; d, air pump; e, bacterial air filter; f, 3-way tap/pressure release; g, 2-way taps; h, frit for aeration; j, peristaltic pump; k, reservoir; l, ground glass joints for ease of cleaning and sterilization (Bonnett *et al.*, 2006).

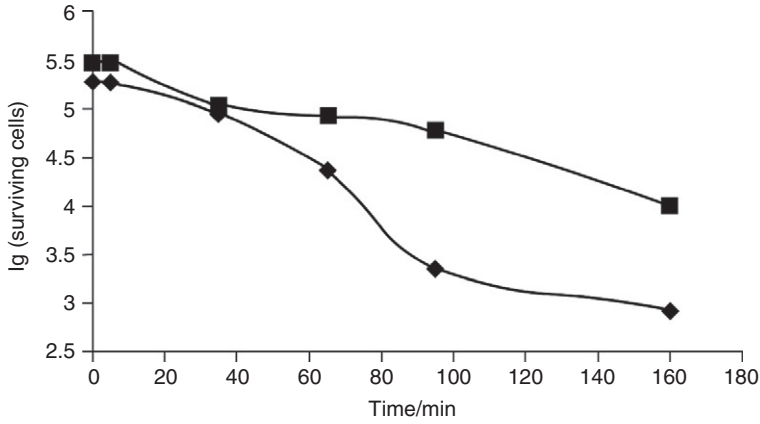


FIGURE 3.10 Photomicrobiocidal activity of the reinforced zinc phthalocyanide/chitosan membrane on *E. coli* suspensions in log survival scale. ◆, freshly prepared membrane; ■, the same membrane after 9 months (Bonnett *et al.*, 2006).

The membrane remained active at a slightly reduced level after 9 months storage in the dark (Fig. 3.10). The proposed approach can be used to lower microbial levels in water flow systems and also might have applications to water detoxification.

The photodynamic approach has been applied for the cleaning and disinfection of artificial surfaces, especially for the destruction and inactivation of biofilms. In the majority of cases, it was proposed for the cleaning of surfaces in hospitals (Decraene *et al.*, 2008a,b) and the disinfection of medical devices such as implants (Sharma *et al.*, 2008). Only a few papers on the application of PDT targeted to the needs of the food industry have been published.

In 2001, Kreitner *et al.* (2001) published the results of their study where food-grade PSs were tested for their efficiency in nonthermal food surface pasteurization as a new possibility for protecting foods from microbial spoilage. The two PSs used in this study were sodium chlorophyllin and heamatoporphyrin—natural constituents of food. Chlorophyllin represented negatively charged PSs, while heamatoporphyrin was neutral or positively charged depending on the environment. To mimic bacteria contaminating the ''dry'' food surfaces, where they have reduced mobility, the cells were placed on agar plates together with an appropriate PS (10 μM). Plates were illuminated for 1 h with a halogen lamp (1000 W) placed at the distance of 25 cm. All of the studied bacteria and yeasts were susceptible to photodynamic inactivation to various degrees (Table 3.5). On average, yeast cells demonstrated a lower susceptibility to photoinactivation, with *R. mucilaginosa* being the most resistant. This low susceptibility of *R. mucilaginosa* to photodynamic inactivation was explained by its ability to

TABLE 3.5 PS-mediated inactivation of tested bacteria and yeasts (Kreitner *et al.*, 2001)

	Log reduction (CFU/ml)	
Microorganism	Heamatoporphyrin	Chlorophyllin
S. aureus	3.9	3.1
B. cereus	3.2	3.1
B. subtilis	4.7	4.2
R. mucilaginosa	1.7	0.3
S. cerevisiae	2.3	2.5
K. javanica	3.3	3.3

TABLE 3.6 Log reduction of *E. coli* (EC), *Salmonella* Typhimurium (ST), *Bacillus subtilis* (BS), *Listeria monocytogenes* (LM), and *Saccharomyces cerevisiae* (SC) after incubation for 30 min with the acriflavin at a concentration of 50 μg/ml on plastic (polystyrene) and stainless steel surfaces; illumination with white light 0.4 mW/cm^2 (Tiwana, 2006)

	Plastic		Stainless steel	
Organism	PS + light	PS only	PS + light	PS only
EC	>2	1	>3	>3
ST	>4	0.01	>3	−0.23
BS	>5	−0.11	>2	>2
LM	>2	0.21	>2	0.49
SC	>1	>1	>2	0.69

synthesize deep pink pigments on wort agar, which may compete with chlorophyllin for the light absorption and thus interfere with the production of cytotoxic oxygen species. Probably, the selection of another PS that absorbs light at a different wavelength would produce better results.

It was shown that the treatment of bacteria on stainless steel surfaces and on polystyrene for 30 min with acriflavin (50 μg/ml) combined with white light illumination resulted in a substantial reduction in live cell numbers for different bacteria and yeast (Table 3.6; Tiwana, 2006). In control samples that were kept in the dark, almost 100% of the applied cells survived, thus confirming the light-induced mechanism of killing. At the studied concentrations, acriflavin is water soluble, so, after treatment, the residual dye can be easily removed by rinsing the surface with water. These data provide evidence that PDT can be used for the cleaning and sanitation of such food-processing and food-handling surfaces.

A similar approach was used to inactivate pathogens on the surface of food packaging material (Luksiene *et al.*, 2009). Yellow packaging trays

were soaked in a suspension of *B. cereus* (10^7 CFU/ml) for 30 min prior to the experiment. Afterward, trays were dried for 30 min for further bacteria adhesion. Then, samples were incubated in the dark with a 3–7.5-mM concentration of ALA to induce the production of endogenous porphyrin-based PSs by bacteria. After treatment with ALA, dried samples were illuminated with a light-emitting diode (LED, 400 nm, 20 mW/cm^2) for 5–20 min. The level of decontamination of the packaging material from adhered *B. cereus* after ALA-based photosensitization reached 4 logs for vegetative cells and 2.7 logs for bacterial spores. These data support the idea that PDT can, in the future, be developed into a completely safe, nonthermal surface sanitation and food preservation technique.

The incorporation of PSs into materials was shown to be effective for the construction of surfaces with ''self-cleaning'' ability when illuminated by white light. TBO and Rose Bengal were incorporated in cellulose acetate film by casting the film from an acetone solution of a cellulose acetate-PS mixture (Decraene *et al.*, 2006). Aliquots of microbial suspensions were placed onto these films and illuminated with a 25-W compact fluorescent lamp. The number of survivors was assessed by a plate counting technique. The obtained data are presented in Table 3.7. Consistent with previous observations, it was shown that Gram-negative bacteria were more resistant to the PDT. However, after 16 h of illumination, 88–100% of the cells present were killed by the contact with the constructed material. One of the possible problems associated with such coating is ''photobleaching'' of PSs which could result from self-destruction of the dyes by the generated singlet oxygen radicals. However, when these coatings were exposed to seven cycles of alternating light and dark periods (16 h light and 8 h of darkness), no reduction in its

TABLE 3.7 Effects on viable counts of contact with a cellulose acetate coating containing toluidine blue and rose bengal and exposed to light from a 25-W fluorescent lamp (adapted from Decraene *et al.*, 2006)

Organism	Light exposure time (h)	% Reduction in viable count	Log_{10} reduction in viable count
Staphylococcus aureus	2	99.6	2.4
Staphylococcus aureus	6	100	6.3
Methicillin-resistant *Staphylococcus aureus*	6	100	6.4
Clostridium difficile	4	100	6.7
Candida albicans	16	88	0.9
Bacteriophage X174	16	91	1.1
Escherichia coli	6	24	0.1
Escherichia coli	16	100	6.3

TABLE 3.8 Photodynamic inactivation of microorganisms on the surface of paper treated with conjugates of Rose Bengal (RB) and Phloxine B (PhB) with poly (vinyl amine) (PVAm) (adapted from Brovko *et al.*, 2009)

Mean log reduction of the number of cells after 30 min illumination				
Dye conjugate	*E. coli*	*Bacillus* sp.	*L. monocytogenes*	*S. cerevisiae*
PVAm-RB	>2	>1	>2	0.7
PVAm-PhB	0.64	>2	>2	<0.01

photo-inducible bactericidal activity was detectable. These findings suggested that photobleaching was not a problem at least in the short term. The constructed light-activated antimicrobial coatings were further tested in hospital environments and were proved to provide a simple, cost-effective means of reducing the microbial load on surfaces in real-life conditions (Decraene *et al.*, 2008a,b).

Paper-based ''self-cleaning'' materials were constructed using conjugates of Rose Bengal and Ploxine B with poly (vinyl amine) (Brovko *et al.*, 2009). Poly (vinyl amine) is used as a strengthening agent in paper manufacturing (Lorencak *et al.*, 2000). Positively charged polymer chains help to keep together negatively charged cellulose fibers. In the described study, regular filter paper (Whatman No. 1) was impregnated with the solution of PS-conjugate and dried before the experiment. Suspensions of a variety of microorganisms were placed onto this paper and illuminated with regular white light (halogen lamp) for 30 min. The numbers of surviving bacteria were assessed by a plate-counting technique and compared with the initial number of cells in the sample. The obtained data are presented in Table 3.8. For all bacteria, a significant reduction in the number of live cells was observed. In the majority of cases, there was no growth visible on the plates. Yeast cells, as can be expected, were slightly more resistant to photodynamic inactivation, but nevertheless, contact with RB-PVAm conjugate-treated paper killed around 95% of *Saccharomyces cerevisiae* cells after 30 min of illumination.

VII. CONCLUSIONS

The vast amount of scientific data accumulated so far strongly suggest that PDT and novel ''self-cleaning'' materials based on the photodynamic effect deserve the very close attention of researchers. In the current age of emerging ''superbugs,'' PDT could offer a very efficient and cost-effective

way to combat the microbial contamination of foods that can lead to disastrous social and economic consequences. This chapter was written with the hope to encourage future research that is needed to bring this new technology to reality.

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