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review

New Approach to Inactivation of Harmful and Pathogenic Microorganisms by Photosensitization*

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Summary

Photosensitization is a treatment involving the administration of a photoactive compound that selectively accumulates in the target cells or microorganisms and is followed by irradiation with visible light. The combination of the two absolutely nontoxic elements, drug and light, in the presence of oxygen results in the selective destruction of target microorganism. It is important to note that truly major advances have been made in photosensitized antimicrobial chemotherapy, in particular disinfection of the blood and blood products, or treating local infections. By no means, prevention of any disease by microbial control of environment, including food manufacturing, is of greatest importance. Thus, development of new antimicrobial methods is necessary. In this context, photosensitization has been shown to be really effective: different microorganisms such as drug-resistant bacteria, yeasts, viruses and parasites can be inactivated by this method. So far, a photosensitization phenomenon can open new and interesting avenues for the development of novel, effective and ecologically friendly antimicrobial treatment, which might be applied to increase food safety.

Key words: photosensitization, inactivation microorganisms

Introduction

Photodynamic therapy is an entirely new treatment modality and its development can be likened to that of the discovery of antibiotics. This is just the beginning, and its possible uses are only limited by the imagination.

J.S. McCaughan, Drugs and Aging, 15 (1999) 49–68.

The field of antimicrobial fight is one of the constant challenge, particularly in view of the rapid evolutionary changes and plethora of new pathogens encountered (*1*). It is obvious that fight against microorganisms can develop in two directions: (*i*) elimination of diseases by inactivation of microbes inside the organisms; and (*ii*) disease prevention by microbial control of the environment.

Unfortunately, pathogenic and harmful microorganisms are widely spread everywhere: in the air, buildings, on different surfaces, plants and food. Moreover, the methods recently applied for inactivation of these microorganisms are not always efficient and ecologically friendly. For instance, novel nonthermal technologies, which increase food microbial control, can alter the structure of proteins and polysaccharides, causing chan-

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ges in the texture, physical appearance and functionality of food (*2*). In addition, the resilience of bacterial spores and the existence of highly resistant microbial subpopulations also limit the efficacy of the emerging nonthermal technologies (*3*).

Consequently, foodborne diseases have been estimated to cause billions of illnesses, millions of hospitalizations and thousands of deaths each year. Hence, the continued occurrence of foodborne diseases indicates that much remains to be done in this area.

At the simplest level, foodborne disease might be described as an interaction of three independent factors: the pathogen, the host, and the environment in which they exist (Fig. 1). For instance, decrease or elimination of pathogen might induce notable decrease in the occurrence of the foodborne diseases.

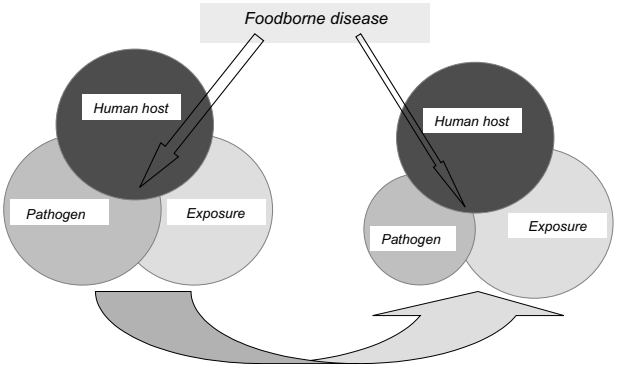

Fig. 1. Foodborne illness might be effectively reduced by the increased microbial control

According to the situation described, it seems that presently existing methods for inactivation of harmful and pathogenic microorganisms in different fields, including medicine, food manufacturing and safety or occupational environment, are not effective. Inevitably, new approach to inactivate harmful microorganisms in cost-effective and environmentally friendly way is necessary. In this context photosensitization might serve as a really promising method. Thus, a question inevitably arises: what is it and how does it work?

In general, photosensitization is a treatment involving the administration of a photoactive compound that selectively accumulates in the target cells or microorganisms and is followed by irradiation with visible light. The combination of two absolutely nontoxic elements, drug and light, in the presence of oxygen results in the selective destruction of target microorganism. According to Dougherty *et al.* (*4*) and Luksiene (*5*) the era of photosensitization was initiated by Raab in 1900. He observed the death of *Paramecium caudatum* after light exposure in the presence of acridine orange (*6*). In the 1930s and later it was shown that bacteria and viruses stained with dyes became photosensitive and eventually lost their viability (*4*). Subsequently, according to Wainwright (*7*), Von Tappeiner and Jesionek described the use of topical eosin and visible light for the treatment of skin tumors due to the fact that eosin easily accumulates in highly proliferating tumor cells. The expanding use of photodynamic cancer treatment is based on the pioneering work of Dougherty (*3,4*) who presented extensive data on the successful application of this novel technique.

The interest in photosensitization as an effective tool to eradicate pathogenic microorganisms can be traced back before the age of chemotherapy (*8,9*). Ehrlich, after intensive experimental work on staining effects of aniline dyes on microbial cells, introduced the idea of the »magic bullet«, as described by Leistner and Gould (*10*). At the turn of the century the famous scientist formulated the principle of selectivity and laid the foundations of modern chemotherapy. Thus, the principle of photosensitized killing of microorganisms followed: if living microorganism accumulates vital stain and can be afterwards selectively detected, it should be possible to destroy the stained microbe after the irradiation.

Major advances have been made in photosensitised antimicrobial chemotherapy. The technique has been shown to be effective *in vitro* against resistant bacteria, yeasts, viruses and parasites (*10*). Currently, the major use of photosensitization is in disinfection of blood and blood products, particularly for viral inactivation, treating locally infected wounds or different oral infections. This method has been proposed as a potential, low-cost approach to the treatment of locally occurring infection and is gaining increasing acceptance.

Three Indispensable Components of Photosensitization

Photosensitization is a result of the combined effect of three nontoxic agents: photosensitizer, light and oxygen. Therefore it is necessary to describe all of them separately.

Photosensitizers (photoactive dyes)

A large number of photosensitizing drugs have been tested *in vitro* and *in vivo* during last 10 years (*5, 11*). A great deal of work has been carried out to evaluate the correlation between antimicrobial efficiency and structure of the compound. As a rule, photosensitizers are usually aromatic molecules that can form long-lived triplet excited states. Table 1 presents photosensitizers and their pre-cursors which are most commonly used against the pathogenic microorganisms (*9*).

Several lines of evidence indicate that physicochemical properties of the photosensitizer have potential impact on the efficacy of photosensitization. Lipophilicity (logP), ionization (pK_a), light-absorption characteristics and the efficiency of singlet oxygen production (Φ_Δ) must be included in a putative photoantimicrobial profile (*1*). Sometimes desirable physicochemical properties of photosensitizer can be improved by chemists.

For instance, chlorin e6 has limited photoactivity and subsequent antimicrobial activity, whereas newly designated ce6-5K, which is composed of a lysine pentamer linked covalently through the N terminus to the C-20 carboxymethyl group of chlorin e6, is much more effective. This compound showed high killing activity against *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum* and *Actino-*

Table 1. Photosensitizers and their precursors

Porphyrins
Hematoporphyrin derivative
Dihematoporphyrin ether/ester
Porfimer sodium
Tetrasodium-meso-tetraphenylporphyrin sulphonate
Metallotetra-azaporphyrin
Deuteroporphyrin
Tetramethylpyridyl porphyrin
Porphyrin precursors
δ-aminolevulinic acid (ALA)
δ-aminolevulinic acid (ALA)-methyl-, propyl-, hexyl-esters
Phthalocyanines
Chloroaluminum tetrasulphophthalocyanine
Zinc(II) phthalocyanine
Silicone naphthalocyanine
Aluminium sulphonated phthalocyanine
Porphycenes
9-acetoxy-2,7,12,17-tetra-*N*-propylporphycene
2-hydroxyethyl-7,12,17-tris(methoxyethyl)porphycene
23-carboxy-24-methoxycarbonylbenzo(2,3)-7,12,17-tris(methoxyethyl)porphycene
Chlorins
Monoaspartyl chlorin e6, diaspartyl chlorin e6
Chlorin e6 sodium, bacteriochlorin a
Benzoporphyrin derivative monoacid ring A
Pheophorbides
Pheophorbide a, bacteriopheophorbide
Others
Fluoresceins (fluorescein sodium, tetrabromfluorescein-eosin)
Anthracenes (anthraquinone, acridine orange, yellow)
Hypericin
Furocoumarine (5-methooxypsoralen, 8-methoxypsoralen)
Chlorophyll derivatives
Purpurins (metallopurpurin, tin etiopurpurin Sn ET2)
Phenothiazines
Methylene blue, violet green
Azure C, thionine, Nile blue A
Hypocrellin
Rose Bengal
Rhodamine 123
Lutetium texapyrin

myces viscosus (*12*). Other important factors that define the killing efficiency are the intracellular localization and binding site of the photosensitizer. Both of them are highly affected by the chemical structure of the photosensitizer (*13*).

Other interesting approach has been suggested that photosensitization could be based on the activation of endogenous synthesis of porphyrin-type photosensitizer by δ-aminolevulinic acid (ALA), which is naturally occurring precursor of haem synthesis in bacteria (*14,15*). It was postulated that the existence of endogenous porphyrins within the cell, with no need to penetrate any cell barriers, would result in total photodestruction of the strains that can produce high amounts of endogenous porphyrins. In fact, only staphylococcal strain is really very sensitive to this treatment (*15*).

Despite the possible synthetic photosensitizers, there are, however, many examples of natural photosensitizers that have evolved over the years either in plants or in fungi. For instance, psoralen derivatives have been used in Asia for millennia for the treatment of various skin disorders (*1*). Currently, several groups are investigating the use of psoralens for the disinfection of blood from viruses (*16*). Similarly, traditional Chinese medicine has made the use of the extract of *Hypocrella bambusae*, which contains hypocrellin, an obvious antiviral candidate (*17*). Moreover, photosensitizers, derived from vital strains, are known to be nontoxic in much higher concentration than those required for effective pathogen killing. To summarize, chemical purity, capability to accumulate in the microorganism, strategically important localization inside the microorganism, high killing efficiency and lack of mutagenicity or genotoxicity are desirable features of an ideal photosensitizer (*6*). In addition, photosensitizers, being readily available and inexpensive, should be attractive in the area of low-cost antimicrobial methods.

Light sources for photosensitization

In general, every visible light source with the suitable spectrum and power density can be used for photosensitization. Initially, photosensitization was performed with the use of conventional gas discharge lamps (*18*). The popular filtered slide projectors are being replaced by incoherent light sources constructed especially for their use: metal halogen lamp, which emits 600–800 nm radiation at high power density (*18*), short-arc xenon lamp, tuneable over a bandwidth between 400 and 1200 nm as well as narrow band-UV lamp in the range of 407–420 nm (*15*).

Traditionally, lasers as coherent light sources were considered to be superior to the conventional light sources, such as incandescent lamps. On the other hand, the usage of lasers also has some essential drawbacks. First of all, they are very expensive. Second, they require specially trained personnel to work with them. As a result, the alternative conventional light sources were developed. For instance, in treatment of surface lesions non-coherent light sources are more suitable, because they can evenly irradiate an entire lesion's field in order to ensure equal light portions for the whole surface (*19*).

Light emitting diode (LED) is one of such nonconventional light sources, which has got promising properties, wide suitability and flexibility that contribute to its rapid development (Fig. 2). Firstly, it is inexpensive, small, light in mass and portable. Its lifetime can reach up to hundred thousands of hours. Besides, the work with LED based devices does not require special staff retraining. In addition, it has a quite big efficiency (about 10 %, which is much larger compared to other conventional sources and lasers, except diode laser) and output power of LED array can be much bigger than that of diode lasers. Eventually, they can be arranged in arrays to irradiate large areas and can be powered by batteries, which make them totally and easily portable (*20*).

Fig. 2. Light emitting diode (LED) samples: microchip, LED in a package and ring array of LEDs

Mechanism of photosensitization, the role of the oxygen

Basically, as mentioned before, photosensitization requires the presence and interaction of 3 absolutely nontoxic components: photosensitizer, light, and oxygen (*21*).

The initiating step of the photosensitizing mechanism is the absorption of a light photon by the sensitizer, causing a promotion of the drug molecule from its ground state to the extremely unstable excited singlet state with a half-life in range of 10^{-6}–10^{-9} s (Fig. 3). The singlet excited photosensitizer either decays back to the ground state, resulting in the fluorescence, or undergoes intersystem crossover to the longer lived (10^{-3} s) tripled excited state. Cell destruction is most efficient when using compounds with a long tripled half-life and a high quantum yield for the triplet excited state. The interaction of the triplet sensitizer with surrounding molecules results in two types of photooxidative reaction.

Type I pathway involves electron or hydrogen atom transfer, producing radical forms of the photosensitizer or the substrate. These intermediates may react with oxygen to form peroxides, superoxide ions, and hydroxyl radicals, which initiate free radical chain reactions. Type II mechanism is mediated by an energy transfer process with ground state oxygen ($^1O_2$) (*22*). Both reactions occur simultaneously and in competition. The *in situ* generation of singlet oxygen via type II pathway appears to play the central role in the cytotoxicity induced by photosensitization because of the highly efficient interaction of the $^1O_2$ species with different biomolecules (*23*). Eventually, the target cell is killed by apoptosis or necrosis (*5,22*).

Mechanism of Microbial Inactivation

As it was mentioned before, early in the last century it was known that certain microorganisms can be killed by the combination of dyes and light *in vitro* (*21*). Since then, there have been several reports about the possibility to kill microorganisms by photoactive dye and light. What is the mechanism of microbial inactivation? Recently we have been able to outline the steps required for the photosensitization-based inactivation of a bacterial cell: (*i*) accumulation of the photosensitizer in the bacteria is the main prerequisite for its photoinactivation; (*ii*) translocation of the photosensitizer into the cytoplasm must be possible; (*iii*) two ways are proposed to explain the lethal damage of bacteria: destruction of either DNA or membrane (Fig. 4).

Breaks in both single- and double-stranded DNA have been detected in both Gram(+) and Gram(–) bacteria after photosensitization with a wide range of different photosensitizers (*24,25*). An important observation is that *D. radiodurans*, having very efficient DNA repair mechanism, can be easily killed by photosensitization as well (*26,27*). Another way to kill the microorganism is to damage its cytoplasmic membrane, which usually results in leakage of cellular contents. The alteration of proteins of cytoplasmic membrane was shown by Valduga *et al.* (*28*). Later new data revealed that there was significant difference in susceptibility to photosensitization between Gram(+) and Gram(–) bacteria. Deeper and more detailed investigations have shown convincingly that neutral or anionic photoactive dyes might efficiently bind and subsequently, after the irradiation, inactivate Gram(+) bacteria. This might be easily explained by the fact that Gram(+) bacteria have the cytoplasmic

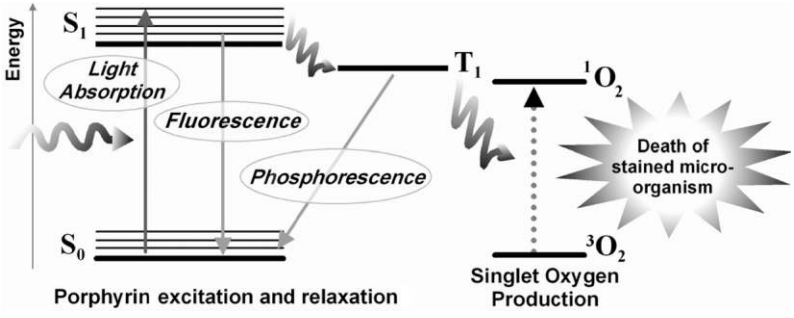

Fig. 3. Scheme of the photosensitization: absorption of light, excited S_1 and T_1 states, transfer of excitation energy to the triplet oxygen $^3O_2$, resulting in the cytotoxic singlet oxygen production

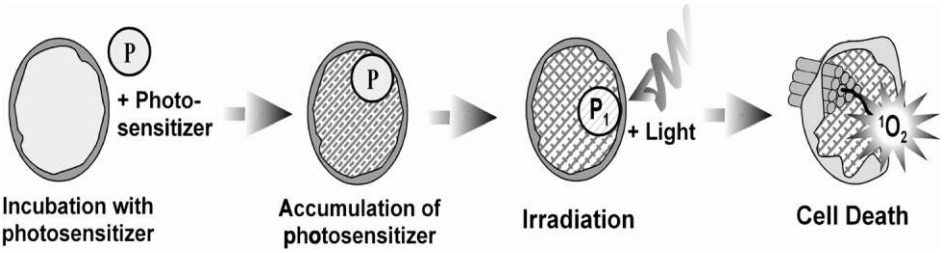

Fig. 4. Mechanism of destructive action of photosensitization in the cell: P – photosensitizer, P_1 – excited state of photosensitizer after absorption of light, $^3O_2$ – triplet oxygen, $^1O_2$ – singlet reactive oxygen

membrane surrounded by relatively porous layer of peptidoglycan and lipoteichoic acid which allows the photosensitizer to cross it (*29*). The cell envelope of Gram(–) bacteria consists of an inner cytoplasmic membrane and an outer membrane which are separated by the peptidoglycan-containing periplasm (*21*). It seems that the outer membrane forms a physical and functional barrier to communicate with the surroundings. The most important fact is that this is efficient and irreversible killing of microorganisms (*30*). So far, a plethora of microbial strains can be inactivated by different photosensitizers after irradiation (Table 2). It is obvious that this phenomenon opens new possibilities destroying series of drug resistant pathogens.

As a rule, in Gram(+) bacteria and yeasts the photosensitizer accumulates in the cell wall. After irradiation with visible light, reactive oxygen species (including radicals) induce rapid disruption of the native structure of the cell wall (Fig. 4). Afterwards, the translocation of the photosensitizer to the inner membrane with several critical targets occurs. It is important that prolonged irradiation induces injuries of cytoplasmic structures, inhibition of DNA and RNA synthesis without any detectable mutagenicity or genotoxicity (Table 3) (*44*).

On the contrary, as described before, Gram(–) bacteria are resistant to the photosensitizing action of neutral or anionic porphyrins (*44*). Recently several research groups have independently observed that cationic porphyrins might efficiently photosensitize and kill Gram(–) bacteria (*45*).

A very attractive feature, peculiar to photosensitization as antimicrobial treatment, is the possibility of the singlet oxygen and other reactive species to chemically destroy a lot of secreted virulence factors. For instance, Komerik *et al*. (*46*) showed that LPS from *E. coli* and proteases of *P. aeruginosa* were inactivated after exposure to red light and toluidine blue O.

It seems that photosensitization might help to overcome the problem of bacterial multidrug resistance. For instance, Gram(+) bacteria such as *Staphylococcus aureus* or *Deinococcus radiodurans* or Gram(–) *Acinetobacter baumannii*, which represent a significant problem in hospitals, are actually very sensitive to this treatment *(27,47)*.

Several photosensitizers have been shown to be able to inactivate the enveloped and nonenveloped viruses. Type I reaction can give rise to hydroxyl radicals ($HO^{\cdot}$), the superoxide anion and hydrogen peroxide, leading to cytotoxic antimicrobial events. Type II processes produce singlet oxygen, which react with molecules involved

Table 2. Microorganisms sensitive to photosensitization

Microorganism	Photosensitizer
Escherichia coli	5-aminolevulinic acid (*14*) Photosens (*31*)
Proteus mirabilis	Photosens (*31*)
Streptococcus spp.	Toluidine blue (*32*) Methylene blue (*32*)
Candida albicans	Methylene blue (*33*)
Helicobacter pylori	Toluidine blue (*34*)
Helicobacter mustelae	Toluidine blue (*34*)
Trypanosoma cruzi	Silicon phthalocyanine (*35*)
Plasmodium falciparum	Silicon phthalocyanine (*36*)
Streptococcus pyogenes	Methylene blue (*33*)
Streptococcus sanguis	Phthalocyanine (*37*)
Staphylococcus aureus	Methylene blue (*33*) Aluminium phthalocyanine (*38*) Photosens (*31*)
Streptococcus mutans	Toluidine blue (*32*)
Porphyromonas gingivalis	Toluidine blue (*39*) Chlorin e6 (*12*)
Actinobacillus actinomycetem-comitans	Toluidine blue (*39*) Chlorin e6 (*12*)
Bacteroides forsythus	Toluidine blue (*39*) Chlorin e6 (*12*)
Campylobacter rectus	Toluidine blue (*39*) Chlorin e6 (*12*)
Eikenella corrodens	Toluidine blue (*39*) Chlorin e6 (*12*)
Porphyromonas spp.	Deuteroporphyrin (*27*)
Pseudomonas aeruginosa	Photosens (*31*)
Corynebacterium minutissimum	Methylene blue (*33*)
Propionibacterium acnes	Methylene blue (*33*)
Bacteriophage T7	Tetraphenyl porphyrins (*40*)
Acanthamoeba palestinensis	Phthalocyanine (*41*)
Saccharomyces cerevisae	Meso-arylglycosylporphyrins (*42*)
Deinococcus radiodurans	5,10,15,20-tetra(4-*N*-methyl-pyridyl)porphine (*27*)
Acinetobacter baumannii	Tetra(4-methyl pyridyl)-porphyrin (*27*)
Trichophyton rubrum	Deuteroporphyrin mono-methylester (*43*)
Enterococcus hirae	5-aminolevulinic acid (*13*)

Table 3. Photosensitization-induced damages and cytotoxic events in the microorganisms (*1*)

Site of action	Action	Result	Consequence	Cytotoxic event
Water	Hydrogen abstraction	Formation of hydroxyl radical ($HO^{\bullet}$)	Formation of hydrogen peroxide, superoxide (O_2)	Further oxidative processes
Cell wall/membrane unsaturated lipids/steroids	Peroxidation	Peroxidation	Hydroperoxide formation	Increased ion permeability (Na+/K+ leakage)
Viral protein coat	Oxidation of Tyr/Met/His residues	Peptide cross-linking Protein degradation	Enzyme inactivation	Loss of repair facility; lysis Loss of viral infectivity
Respiratory chain	Redox reactions			Inhibition of respiration
Cytoplasmic enzymes/ viral enzymes (*e.g.* reverse transcriptase)	Oxidation or cross-linking (as above)			Inhibition of ribosome assembly; Inhibition of replication /infectivity
Nucleic acid residues (typically guanosine)	Oxidation of base or sugar	8-hydroxy-guanosine	Nucleotide degradation; sugar degradation /cleavage	Base substitution; strand cleavage; mutation; inhibition of replication

in the viral envelope. It is more likely that positively charged photosensitizers cause nucleic acid damage (oxidation of guanosine residues), whereas anionic photosensitizers act against the viral envelope. Aminolipids and peptides in the viral envelope are potential targets, leading to the inactivation of membrane enzymes and receptors (*1,48*), whereas lipid peroxidation is detrimental to membrane integrity, leading to loss of fluidity and increased membrane permeability (Table 3). Several reports have concluded that some yeasts, for instance *Saccharomyces cerevisiae,* might be killed *in vitro* by photosensitization (*49*).

Really difficult problem is efficient inactivation of several pathogenic and harmful microfungi. So far only few reports from our laboratory have been published reflecting this problem (*30,48,50,51*). According to our data, series of microfungi, like strains *Rhyzopus oryzae, Aspergillus flavus, Aspergillus fumigatus, Aureobasidium pullans, Fusarium avenaceum, Trichotecium roseum, Acremonium strictum, Ulocladium chartarum* and *Alternaria alternata* might be totally killed by hematoporphyrin dimethyl ether and visible light.

Conclusions

Due to the wide variety of pathogens encountered, the field of antimicrobial fight must be emphasized as one of constant challenge. Multi-antibiotic resistance of pathogens, especially bacteria, is a rapidly growing and alarming phenomenon. Hence, the discovery of new drugs and novel, cost–effective, nonmutagenic and human friendly technologies to inactivate harmful and pathogenic microorganisms seems an imperative. In this context, photosensitization as really effective technique against a range of microorganisms should encourage its use in a wider arena. Photosensitization of bacteria has repetitively been shown to be independent of the antibiotic resistance spectrum, it induces loss of viral infectivity, it is not mutagenic or genotoxic. In our opinion, this phenomenon opens a new and interesting avenue for the development of effective, human and ecologically friendly antimicrobial treatment. Its proper application for the treatment of food, packaging and processing equipments might be really useful to increase microbial food control and subsequently decrease foodborne diseases.

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Novi pristup inaktivaciji štetnih i patogenih organizama fotosenzibilizacijom

Sažetak

Fotosenzibilizacija je postupak dodavanja fotoaktivnog sastojka određenoj stanici ili mikroorganizmu gdje se selektivno akumulira. Nakon toga slijedi iradijacija vidljivim snopom svjetla. Kombinacijom dvaju netoksičnih elemenata, lijeka i svjetla, u prisutnosti kisika selektivno se uništava određeni organizam. Bitan je napredak postignut u fotosenzibiliziranoj antimikrobnoj kemoterapiji, osobito u dezinfekciji krvi i krvnih proizvoda pri obradi lokalnih infekcija. Vrlo je važna zaštita od bilo koje bolesti mikrobnom kontrolom okoliša, a i proizvodnje hrane. Fotosenzibilizacija bi mogla biti vrlo učinkovita u inaktivaciji različitih mikroorganizama kao što su bakterije otporne na lijekove, kvasci, virusi i paraziti. Fotosenzibilizacija otvara nove mogućnosti za razvoj učinkovitih i ekološki neškodljivih antimikrobnih postupaka koji poboljšavaju sigurnost hrane.