Photodynamic treatment for surface sanitation

Lubov Brovko*, Nadya A. Romanova, Christina Leslie, Helene Ollivier, Mansel W. Griffiths
Canadian Research Institute for Food Safety, University of Guelph, Guelph, ON N1G 2W1, Canada

ABSTRACT

The bactericidal effect of visible light illumination on bacteria treated with non-toxic photosensitizers (PS) has been shown previously, its effectiveness depended on both cell type and nature of photosensitizer used. The photosensitizer (PS)-mediated bactericidal effect of light against different types of microorganisms including vegetative bacteria (both in planktonic form and in biofilms), bacterial spores, yeasts, viruses was investigated for both cells in liquid media, and on surface. Bactericidal effect was monitored for different photosensitizer such as TBO and derivatives of rosamine at different concentrations. The possibility of using photodynamic treatment for surface sanitation was investigated.

Key words: Photodynamic treatment, bacteria, biofilms, spores, viruses, surface sanitation

1. INTRODUCTION

The method of photodynamic destruction of bacteria relies on illumination of miroorganisms treated with non-toxic photosensitizers (PS) by low-power visible light. 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 method has been sucsessfully used for treatment of certain types of cancer (PhotoDynamic Therapy – PDT) during last decade [1]. Antimicrobial Photodynamic Treatment (APDT) research has increased in the last years due to concerns with emerging antibiotic resistant bacteria and urgent need for new approaches to fight infections, but is still in its early stages of development [2-4]. Some possible future clinical application of APDT are being investigated including infections in wounds and burns, infections in body cavities such as mouth, ear, sinus, stomach and surface infections of cornea and skin. However, the same approach can be also used for sanitation of surfaces in food industry and environment as well as for surface decontamination of some food ingredients or environmental samples. In addition, there is considerable interest in self-decontaminating materials to be used in protective clothing or other equipment for the military and first responders in the case of use of biowarfare agents [5-7].

Recent research has indicated that bacteria can acquire resistance to sanitizers commonly used in food industry and, as a result of such adaptation, cross-resistance to antibiotics has been observed [8-9]. Emergence of multi-antibiotic-resistant pathogens is a risk to human and animal health and to the safety of environment and food products. Besides, commonly used sanitizers very often are not effective against bacterial spores and biofilms as well as against viruses. That is why development of novel cost-effective strategies for surface decontamination and sanitation that will result in minimizing risk of infections, bacterial loads of ready-to-eat foods, improving quality of food as well as decrease possible economic losses due to spoilage and recalls is necessary.

The bactericidal effect of visible light illumination on bacteria treated with non-toxic PS has been shown, its effectiveness depends on both cell type and the nature of the photosensitizer used. Illumination of bacteria treated with non-toxic PS by low-power light for 5-60 min resulted, in some cases, in a 5-7 log cycle reduction in bacterial counts, indicating the promise of this approach [3]. Some advantages of this technique include the possibility of targeted delivery of bactericidal action both in terms of species and localization using specific PS, as well as focusing light in the desired area, or use of incidental light for passive "self-decontamination". Development of resistance to PS has not been reported which makes photodynamic treatment a novel alternative for antimicrobial treatment. We propose to use this approach for sanitation of surfaces in the food industry and environment as well as for decontamination of some food ingredients.

* lbrovko@uoguelph.ca; phone 1-519-824-4120 ext. 58301; fax: 1-519-763-0952

2. MATERIALS AND METHODS

Bacterial cultures and bacteriophage. The following bacterial strains were used*:* *Salmonella typhimurium* DT108 (multiresistant strain, BioPhage Inc., Montreal); *Listeria monocytogenes* LM 222 and *Bacillus cereus* (CRIFS collection); bacteriophage BP-6 (BioPhage Inc.). All bacteria were cultivated at 30 or 37°C on the shaker (120 RPM) for 24 hr using Luria Bertani (LB) broth, BHI broth and Nutrient broth for *Salmonella*, *Listeria* and *Bacillus*, respectively. Enumeration of bacterial cells was performed using plate count technique. Aliquotes of bacterial suspension were serially diluted 10-fold in broth and 100 μl of each dilution was plated in duplicates on agar medium, incubated at 37°C for 24 h. Number of formed colonies was counted and weighted by the dilution factor. Presuming that each colony has been formed from one bacterial cell this number represented bacterial population in the original sample as colony forming units (CFU) per ml. *Bacillus cereus* spores were produced by growing cells on nutrient agar supplemented with 0.03% w/v of $MnSO_4$ for 5 days. Spores were harvested from the surface of agar and purified by multiple centrifugation (4000 RPM) and re-suspension in sterile distilled water. Level of sporulation was >95% as determined by light microscopy. For spore enumeration, their germination was first initiated by heat shock (10 min at 80°C) in nutrient broth followed by plating on nutrient agar.

Photosensitizers used in the study were the following: toluidine Blue-O (TBO) (Sigma-Aldrich), tetramethyl rosamine-O (TMR-O), tetramethylrosamine-S (TMR-S), and tetramethylrosamine-Se (gift from M.Detty, SUNY, Buffalo) [10,11]. Phosphate Buffer Saline pH 7.4 (PBS) was used as a diluent for all photosensitizers.

Photodynamic treatment of planktonic bacterial cells and spores. One ml of overnight bacterial culture was spinned down (600 rpm, 6 min), resuspended in 1 ml of PBS. One hundred microliters of this bacterial suspension was added to the each well of 96-well polysterene flat bottom, non-treated microtiter plate. For each concnetration of photosensitzer 100 μl of photosensitizer solution was added to the bacterial suspension in triplicate. For the control samples 100 μl of PBS was used. The plate was then placed under Illumatool (Montreal Biotech Inc.) for illumination for 30 min. Light source was at the distance of 6.5 cm from the surface of the plate and provided uniformal white light illumination with average intensity of 0.45±0.04 mW/cm^2. After illumination bacterial population in each well was determined using plate count technique described earlier. The identical plate was kept in the dark and used as a dark control.

Preparation and Photodynamic treatment of biofilms. Overnight culture of bacteria was diluted 10-fold in fresh broth and 250 μl of suspension was added to each well of 96-well plate. Plate was incubated at 30 or 37°C for four days. Broth in the plate was removed daily and 220 μl of fresh broth added. On the fifth day broth was removed and formed biofilm was washed once with sterile distilled water. To enumerate cells in biofilm the walls and edges of each well were scraped thoroughly for 10 s and 100 μl of bacterial suspension was used for plating.

For photodynamic treatment of biofilm 220 μl of photosensitizer solution was added in each well in triplicate. PBS was used as a control. The plate was then illuminated in Illumatool (Montreal Biotech, Montreal) for 30 min. The identical plate was kept in the dark and used as a dark control. Number of cells was determined in each well.

Photodynamic treatment of spores. To eliminate any remaining vegetative cells in spore preparation, spore suspension was incubated in water bath at 80°C for 10 min. This treatment also initiated spore germination. One hundred microloters of spore suspension was placed in each well of 96-well plate and PS at different concentrations were added to the wells in triplicates. PBS served as a control. Plate was illuminated for 30 min, and number of spores in each well was determined. Identical plate kept in the dark was used as a control.

Photodynamic treatment of bacteriophage. Salmonella *tiphimurium* DT108 bacteriophage BP-6 was used as a model virus. Number of bacteriophage particels in the sample was determined by soft agar overlay method as plaque forming units per ml (PFU/ml) using Salmonella *tiphimurium* DT108 as a host [12]. For photodynamic treatment 100 μl of phage suspension was added to each well of 96-well plate. Equal volume of PS solutions with different concentration was added to each well using PBS as a control. Plate was illuminated for 30 min, and number of phage particles was determined in each well. Identical plate kept in the dark was used as a dark control.

Photodynamic treatment of surface. One hundred microliters of overnight culture of Salmonella *tiphimurium* DT108 was placed into the three empty Petri dish and was left open in biosafety cabinet for 10 min to let it dry. In two dishes the spot was after overlaid with 100 μl of photosesitizer solution and the third with 100 μl of PBS used as a control. One PS-treated dish and control dish were illuminated for 30 min, and the other PS-treated dish was left in the dark. After illumination 10 ml of melted nutrient soft agar (45°C) was added to each plate and plates were incubated at 37°C for 24 h. Bacterial growth in the plates was examined visually.

3. RESULTS

Photodynamic treatment of planktonic bacterial cells

Suspension of planktonic bacterial cells of *Salmonella* typhimurium, *Listeria monocytogenes* and *Bacillus cereus* were treated by different photosensitizers at concentration ranging from 3 to 5000 μg/ml. Samples were illuminated for 30 min by white light source, number of cells that survived treatment was determined and compared with the control samples kept in dark. Results are presented in Table 1.

Table 1. Killing effect of photodynamic treatment observed for planktonic bacterial cells

PS, μg/ml	Log-reduction of planktonic bacterial cells after photodynamic treatment		
	S. tiphimurium	*L. monocytogenes*	*B. cereus*
TBO			
5	2.08	1.66	0.55
50	5.43	3.89	1.77
500	1.48	0	0.51
5000	0.29	1.17	0.47
TMR-O			
3		0.76	0.47
TMR-S			
0.5			0.51
5	>4	2.1	1.42
50	>4	>8	2.05
500	>4	>8	>4
5000	>4	>8	>4
TMR-Se			
5	0.57	0.07	0.83
50	0.74	0.31	0.67
500	>8	0.69	0.89
5000	>8	1.07	1.22

Four photosensitizers with different light absorption - λ_{max} 552, 571, 582 and 630 nm for TMR-O, TMR-S, TMR-Se [11] and TBO, respectively - were tested in the experiment. The observed killing effect was dependent on both type of cell and the photsensitizer used. The thio analogue of tetramethylrosamine (TMR-S) was shown to be the most efficient for planktonic cells. Treatment of *S.typhimurium*, *L.monocytogenes* and *B.cereus* with this photosensitizer at concentration above 0.5 mg/ml resulted in total elimination of live bacterial cells from the samples. For tetramethylrosamine derivatives the observed killing effect was increasing with concentration of photosensitizer used. For TBO optimal concentration around 50 μg/ml was shown for all types of cells. Incubation of bacterial cells with the same photosensitizers in the dark did not cause any significant decline in cell numbers.

Photodynamic treatment of bacteria in biofilms

Single-strain *Salmonella* and *Listeria* biofilms were prepared and treated with photosensitizers both with illumination and in the dark. The numbers of live cells in all samples were determined and compared with control samples. The results are presented in Table 2.

Table 2. Killing effect of photodynamic treatment observed for bacterial biofilms

PS, μg/ml	Log-reduction of bacterial cells in biofilms after photodynamic treatment	
	S. tiphimurium	*L. monocytogenes*
TBO		
5	1.92	1.25
50	0.94	0
TMR-O		
3	1.04	0
TMR-S		
5	1.08	3.34
50	2.18	5.22
TMR-Se		
5	0.28	
50	1.24	0.81
500	2.05	2.0
5000	2.61	

The observed killing effect of photodynamic treatment of bacterial biofilms was significantly lower than for respective planktonic cells. The highest effect was achieved for thio-tetramethylrosamine derivative (TMR-S), it increased with photosensitizer concentration and reached 5-log reduction in cell numbers for *Listeria monocytogenes* treated with 50 μg/ml of TMR-S. There was no total elimination of live cells in biofilms even after treatment with the highest concentration of PS used (5 mg/ml).

Sporicidal effect of photodynamic treatment was tested for spores of *Bacillus cereus*. They were treated with different photosensitizers followed by illumination. The numbers of live spores were determined and compared with control samples kept in dark. The obtained results are presented in Table 3.

Table 3. Killing effect of photodynamic treatment observed for spores of *Bacillus cereus*

PS, μg/ml	Log-reduction of *Bacillus cereus* spores after photodynamic treatment
TBO	
5	0.34
50	0.39
500	0.34
5000	0
TMR-O	
3	0.14
TMR-S	
5	0.54
50	0.37
TMR-Se	
5	0.17
50	0.19
500	1.45
5000	2.63

TBO was shown to have no sporicidal photodynamic effect. However, for tetramethylrosamine derivatives more than 2-log reduction in spores' number was observed for *B.cereus* spores treated with TMR-Se at concentration 5 mg/ml.

Virucidal effect of photodynamic treatment was tested using bacterial virus – bacteriophage - as a model. Results are presented in Table 4.

Table 4. Killing effect of photodynamic treatment observed for bacteriophage BP-6

PS, μg/ml	Log-reduction of bacteriophage numbers after photdynamic treatment
TBO	
50	6.5
TMR-O	
3	4.25
TMR-S	
50	>9
TMR-Se	
50	>9

All tested photosensitizers were shown to have strong virucidal effect at used concentrations. Photodynamic treatment of bacteriophage with 50 μg/ml of TMR-S or TMR-Se resulted in total elimination of live phage particles from the sample.

Efficiency of photodynamic treatment for surface sanitation was tested for *Salmonella typhimurium* cells deposited and dried on the surface of polystyrene Petri dish. The number of cells used in the original sample was producing uniformal bacterial lawn after drying and overlaying with nutrient soft agar (Figure 1, left Petri dish). Treatment of cells with photosensitizer in the dark did not cause the significant decrease in numbers of surviving cell and produced the similar uniformal lawn (Figure 1, Petri dish in the middle). Photodynamic treratment of dried cells on the surface resulted in significant decrease of surviving cells producing separate colonies after growth under soft nutrient agar (Figure 1, right Petri dish).

Figure 1. Image of *Salmonella typhimurium* growth in Petri dishes after depositing on the surface of empty dish, drying and overlaying with nutrient soft agar: Petri dish on the left– control, Petri dish in the middle – bacterial cells treated with 50 μg/ml of TMR-S and kept in dark; Petri dish on the right bacterial cells treated with 50 μg/ml of TMR-S and illuminated with white light for 30 min.

For all photosensitizers used in the study the similar killing effect of photodynamic treatment was observed for bacterial cells on the surface at concentrations in the range 25-50 μg/ml.

4. DISCUSSION AND CONCLUSIONS

The obtained results show that photodynamic treatment may be a good alternative to chemical surface santation techniques currently used. Novel photosensitizers based on tetramethylrosamine analogues were proved to be effective against both Gram-positive and Gram-negatiove bacteria in planktonic form as well as in biofilms and on the surface. However, for photodynamic treatment of biofilms to be effective higher concentration of photosensitizers were used. Only seleno analogue of tetramethylrosamine was effective in photodynamic treatment of spores. All used photosensitizers showed high virucidal effect making the proposed approach useful for treatment of different kind of surface contamination.

To develop surface photo-sanitation technique compatable with requirements of food industry more basic research is necessary to choose the most appropriate photosensitizer that will be effective against wide variety of different food-related bacteria in different forms and viruses and at the same time will not interfere with food quality. The nature of the surface also can also have an impact on the results of photodynamic sanitation and its effect should be investigated.

REFERENCES

1. Huang, Z. 2005, “A review of progress in clinical photodynamic therapy”, Technology in cancer research & treatment v.4 (3): 283-293
2. Wainwright, M. 1998, “Photodynamic antimicrobial chemotherapy”, Journal of antimicrobial chemotherapy, v. 42(1): 13-28
3. NA Romanova, LY Brovko, L.Moore, E.Pometun, AP Savitsky, NN Ugarova, MW Griffiths, 2003, “Assessment of photodynamic destruction of *Escherichia coli* O157:H7 and *Listeria monocytogenes* by using ATP-bioluminescence”. Appl. Environ. Microbiol., v.69 (11): 6393-6398.
4. Demidova, TN, Hamblin, MR 2004, “Photodynamic therapy targeted to pathogens”. Int. J Immunopathology and pharmacology, v. 17(3): 245-254
5. Goyan RL, P Lin, T Mengistu, EF Dickson. 2004 "Characterization and Study of Biological and Chemical Protection Properties of Additive-Containing Electrospun Pellethane Membranes". IFAI 4th International Conference on Safety & Protective Fabrics - Conference Proceedings. p. 138 – 150
6. Russell AJ, JA Berberich, GF Drevon, RR Koepsel. 2003 "Biomaterials for Remediation of Chemical and Biological Warfare Agents". Ann. Rev. Biomedical Engineering, v.5:1 - 27.
7. Sauvet G, Dupond, S, Kazmierski, K, Chojnowski J. 2000 "Biocidal Polymers Active by Contact. V. Synthesis of Polysiloxanes with Biocidal Activity". J. Appl. Polymer Science, 75:1005 – 1012
8. Chapman J.C. 2003, “Disinfectant resistance mechanisms, cross-resistance, and co-resistance”, International Biodeterioration and degradation, v.51(4):271-276.
9. Romanova, N., Favrin, S. & Griffiths, M.W. 2002 Sensitivity of *Listeria monocytogenes* to sanitizers used in the meat processing industry. Appl. Environ. Microbiol., v.68:6405-6409.
10. Ohulchanskyy TY, Donnelly DJ, Detty MR, Prasad PN 2004, “Heteroatom substitution induces changes in excited-state photophysics and singlet oxygen generation in chalkogenooxanthylium dyes: effect of sulphur and selenium substitutions”, J. Phys. Chem., v.108:8668-8672
11. Detty MR, Prasad PN, Donnelly DJ, Ohulchanskyy T, Gibson SL, Hilf R (2004) “Synthesis, properties, and photodynamic properties in vitro of heavy-chalcogen analogues of tetramethylrosamine”, Bioorganic and Medicinal chemistry, v.12:2537-2544.
12. Sambrook J, Fritsch EF, Maniatis T. 1989, Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring harbour, NY, pp. 285-293.