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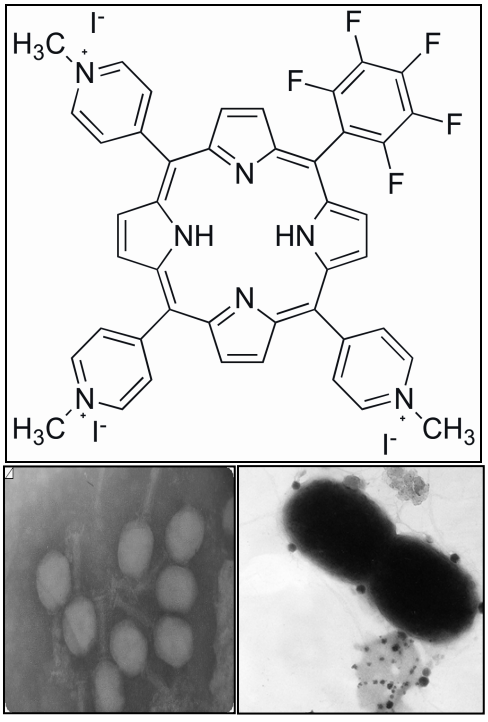
Departamento de Biologia

Liliana Andreia dos Santos Costa

Fotoinativação de vírus através de porfirinas

Photoinactivation of viruses by porphyrins



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Photoinactivation of viruses by porphyrins

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Professora Doutora Maria do Amparo Ferreira Faustino, Professora Auxiliar do Departamento de Química da Universidade de Aveiro

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Dedico este trabalho a todos aqueles que tornaram a sua realização possível e que me apoiaram incondicionalmente em todos os momentos, ao longo destes quatro anos.

o júri

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palavras-chave

Inativação fotodinâmica, porfirinas catiónicas, bacteriófagos, vírus de mamíferos, mecanismos de fotoinativação, espécies reativas de oxigénio, alvos virais, resistência microbiana, viabilidade viral

resumo

A inativação fotodinâmica tem sido usada com sucesso na inativação de microorganismos. Diversos aspetos da inativação fotodinâmica foram já estudados para diferentes microrganismos, contudo, existe ainda pouca informação disponível no que diz respeito à inativação de bacteriófagos por processos fotodinâmicos. Este trabalho pretendeu elucidar e avaliar vários aspetos da fotoinativação de vírus, em particular de bacteriófagos, incluindo (i) o efeito de diversos parâmetros de luz utilizados na fotoinativação de bacteriófagos; (ii) a eficiência da inativação fotodinâmica de diferentes tipos de bacteriófagos (fagos do tipo DNA e RNA); (iii) o principal mecanismo através do qual a inativação fotodinâmica tem lugar; (iv) o efeito da fotoinativação nas proteínas do bacteriófago; e (v) o possível desenvolvimento de resistência e recuperação da viabilidade após vários tratamentos fotodinâmicos consecutivos. Para avaliar o efeito dos diferentes parâmetros de luz, suspensões fágicas com 10^7 UFP mL^{-1} foram irradiadas com diferentes fontes e doses de luz, intensidades luminosas e tempos de irradiação (30,90 e 270 min) na presença de 0,5; 1,0 e 5,0 μM dos derivados porfirínicos catiónicos Tri-Py^+-Me-PF e Tetra-Py^+-Me. A eficiência da fotoinativação de diferentes fagos do tipo DNA e RNA, foi avaliada através da irradiação da suspensão fágica com luz branca (40 W m^{-2}) durante 270 min na presença de 0,5 e 5,0 μM do derivado porfirínico Tri-Py^+-Me-PF, respetivamente para os fagos do tipo RNA e DNA. O mecanismo através do qual a fotoinativação de fagos de DNA (fago do tipo T4) e de RNA (fago Qβ) tem lugar foi avaliado por exposição da suspensão fágica à luz branca com uma potência de 40 W m^{-2}, na presença de fotossensibilizador (Tri-Py^+-Me-PF e Tetra-Py^+-Me) e inibidores, quer do oxigénio singuleto (azida de sódio e L-histidina) quer de radicais livres (D-manitol e L-cisteína). Os danos nas proteínas do fago do tipo T4, induzidos pelas espécies reativas de oxigénio geradas por 5,0 μM Tri-Py^+-Me-PF, foram avaliados pelo método convencional de SDS-PAGE e por espectroscopia de infravermelho. O possível desenvolvimento de resistência e recuperação da viabilidade após a inativação fotodinâmica dos bacteriófagos foi avaliado após dez ciclos consecutivos de tratamento fotodinâmico incompletos (120 min sob irradiação de luz branca a uma potência de 40 W m^{-2}) na presença de 5,0 μM do derivado porfirínico Tri-Py^+-Me-PF. Os resultados deste trabalho mostraram que (i) quando uma quantidade de energia (dose de luz) determinada foi aplicada numa suspensão fágica, a partir de uma mesma fonte irradiação, a fotoinactivação do fago foi tanto mais eficiente quanto mais baixa foi a potência luminosa aplicada; (ii) os bacteriófagos foram eficientemente inativados até ao limite de deteção (redução de 6-7 log); (ii) os fagos do tipo RNA foram inativados mais facilmente do que os fagos do tipo DNA (tempos de exposição mais curtos e com concentração de fotossensibilizador dez vezes menor do que a usada para inativar os fagos do tipo DNA); (iii) o mecanismo do tipo II (*via* produção de oxigénio singuleto) foi o principal mecanismo através do qual a fotoinativação dos bacteriófagos teve lugar; (iv) foi possível detectar danos no perfil proteico após tratamento fotodinâmico e a espectroscopia de infravermelho apresentou-se como uma metodologia promissora de *screening* para avaliação dos danos induzidos pela inativação fotodinâmica em proteínas; e (v) após dez ciclos consecutivos de tratamento fotodinâmico, o fago do tipo T4 não revelou nenhum tipo de resistência ao tratamento fotodinâmico nem recuperou a sua viabilidade. Como conclusão, a inativação fotodinâmica microbiana é uma tecnologia bastante eficaz para a fotoinativação de bacteriófagos do tipo DNA e RNA sem invólucro, a qual pode ser considerada como uma alternativa ao tratamento convencional com agentes antivíricos, mesmo com intensidades luminosas baixas, sem o risco associado de desenvolvimento de mecanismos de resistência.

keywords

Photodynamic inactivation, cationic porphyrins, bacteriophages, mammalian viruses, mechanisms of photoinactivation, reactive oxygen species, viral targets of photoinactivation, resistance, viability

abstract

Microbial photodynamic inactivation (PDI) has been successfully used to inactivate microorganisms. PDI has already been studied under different conditions for different microorganisms; however, there is still scarce information about bacteriophage inactivation by photodynamic procedures. The goal of this study was to elucidate and evaluate several aspects of viral PDI which include (i) the effect of different light sources, doses and intensities on phage inactivation; (ii) the photoinactivation efficiency on different types of bacteriophages (DNA- and RNA-type phages), (iii) the main mechanism by which phage photosensitization takes place, (iv) the effect of PDI on phage proteins; and (v) the possibility of resistance development and viability recovery after consecutive phototreatments. To evaluate the efficiency of photoinactivation, T4-like phage suspensions of 10^7 PFU mL^{-1} were exposed to different light sources(fluorescent PAR lamps, solar light and halogen lamp), and fluence rates (40 W m^{-2}, 600 W m^{-2} and 1690 W m^{-2}) during 30, 90 and 270 min in the presence of 0.5, 1.0 and 5.0 μM of the cationic porphyrin derivatives Tri-Py^+-Me-PF and Tetra-Py^+-Me. DNA- and RNA-type phages were exposed to white light (40 W m^{-2}) during 270 min in the presence of Tri-Py^+-Me-PF at the concentrations of 0.5 and 5.0 μM, respectively for RNA- and DNA-type phages. The mechanism of phage inactivation was evaluated for DNA- (T4-like) and RNA-type (Qβ) phages, in the presence of photosensitizer (Tri-Py^+-Me-PF and Tetra-Py^+-Me) and singlet oxygen quenchers (sodium azide and L-histidine) and free radicals scavengers (D-mannitol and L-cysteine). The damages on T4-like phage proteins, induced by the ROS generated by Tri-Py^+-Me-PF, were assessed by the conventional SDS-PAGE analysis and by IR spectroscopy. Ten consecutive and incomplete (120 min of irradiation at 40 W m^{-2}) cycles of T4-like phage photosensitization by 5.0 μM Tri-Py^+-Me-PF were also performed in order to determine the possible development of resistance and viability recovery after phage PDI. From this study it can be concluded that (i) considering the same light source and a fixed light dose, applied at different fluence rates, phage photoinactivation was significantly higher when low fluence rates were used at long irradiation times; (ii) the phages were efficiently inactivated to the detection limit (reductions of 6-7 log); (ii) RNA-type phages were much more easily inactivated than the DNA-type ones (sooner and with ten times less porphyrin concentration than that used for DNA-type phages); (iii) type II mechanism (production of singlet oxygen) was the main mechanism by which phage photosensitization took place; (iv) IR spectroscopy represents a promising and fast-screening methodology when the damages induced by photosensitization on phage proteins are to be studied; and (v) after ten consecutive photodynamic cycles, T4-like phage did not exhibit any resistance to PDI nor recovered its viability. In conclusion, viral PDI is a very efficient technology for the inactivation of non-enveloped DNA- and RNA-type phages, which may be used as an alternative to the conventional antiviral treatments, even at low light fluence rates, without the problem of viral resistance.

List of Acronyms and Abbreviations

AIDS	Acquired immune deficiency syndrome
$AlPcS_4$	Aluminum phthalocyanine tetrasulfonate
ANOVA	Analysis of variance
AS	Phage of *Aeromonas salmonicida*
AZT	Azidothymidine
BVDV	Bovine viral diarrhoea virus
DC	Dark control
DMSO	Dimethyl sulfoxide
DMTU	Dimethylthiourea
DNA	Deoxyribonucleic acid
DPBF	1,3-diphenylisobenzofuran
ds	Double stranded
EMCV	Encephalomyocarditis virus
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papillomatosis virus
HSV	Herpes simplex virus
$HO^{\cdot}$	Hydroxyl radical
$HOO^{\cdot}$	Hydroperoxide radical
H_2O	Water
H_2O_2	Hydrogen peroxide
IR	Infrared
LC	Light control
LED	Light emitting diode
MB	Methylene blue
NMR	Nuclear magnetic resonance
NM	Not mentioned
NQ	Not quantified
OD	Optical density
$O_2^{\cdot -}$	Superoxide radical
PA	Phage of *Pseudomonas aeruginosa*
PACT	Photodynamic antimicrobial chemotherapy
PAR	Photosynthetically active radiation

PBS	Phosphate buffered saline
Pc_4	Silicon phthalocyanine
PCR	Polymerase chain reaction
PDI	Photodynamic inactivation
PDT	Photodynamic therapy
PFU	Plate forming unit
PS	Photosensitizer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SARS	Severe acute respiratory syndrome
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFV	Semliki Forest virus
SHV	Suid herpes virus
SOD	Superoxide dismutase
ss	Single stranded
SSB	Single strand break
TBO	Toluidine blue O
Tetra-Py^+-Me	5,10,15,20-Tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide
Tri-Py^+-Me-PF	5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin tri-iodide
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet
VA	Phage of *Vibrio anguillarum*
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus
$^1O_2$	Singlet oxygen
$^3O_2$	Molecular oxygen
^1PS	Ground state photosensitizer
$^3PS^*$	Triplet excited state photosensitizer

TABLE OF CONTENTS

THESIS OUTLINE

The present work is focused on the potential use of a photodynamic inactivation (PDI) procedure to inactivate viruses, which are known to pose a threat to public health. As the traditional viral culture methods are time-consuming, labour-intensive processes, some viruses are not cultivated in cell lines and molecular methods do not allow determining viral viability, bacteriophages were used as surrogates of mammalian viruses to evaluate the efficiency of viral PDI. In this work, only non-enveloped model bacteriophages were used but, as enveloped viruses are significantly more sensitive to photodynamic inactivation than non-enveloped ones, a PDI protocol that is effective to inactivate non-enveloped phages will also be effective against non-enveloped and enveloped mammalian viruses.

In order to get a clear insight of the process of viral photoinactivation, several goals were established:

- Evaluation of the effect of light parameters (light sources, doses and intensities) on the photodynamic inactivation of bacteriophages.
- Evaluation of the different susceptibilities to photosensitization of DNA- and RNA-type bacteriophages;
- Evaluation of the main mechanism by which phage photosensitization takes place;
- Evaluation of the damages induced by photosensitization on phage proteins and;
- Evaluation of resistance development and viral viability recovery after consecutive episodes of photosensitization.

A careful bibliographic revision of the state of the art of viral photodynamic inactivation was done (Chapter 1), experimental studies were performed (Chapters 2-6) and a final discussion of all the results was undertaken (Chapter 7) in order to achieve the main goals of this study.

The phage photoinactivation procedures were performed in the presence of the 5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py^{+}-Me-PF), a promising and efficient photosensitizer synthesized at the Department of Chemistry of the University of Aveiro, which was generally compared with the well-known photosensitizer 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^{+}-Me) (Figure 1).

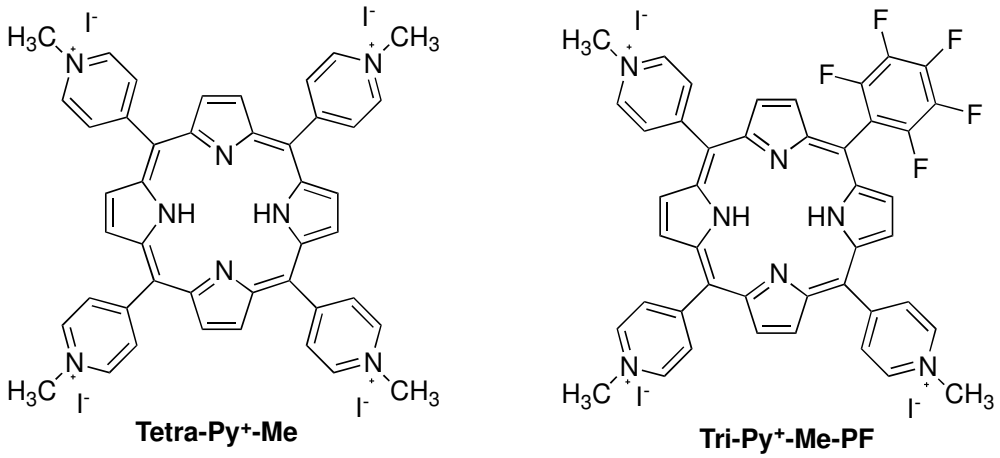

Figure 1. Structure of the PS used for bacteriophages photodynamic inactivation.

In Chapter 1, an introduction to PDI was done and it was also summarized the main approaches developed until now for the photodynamic inactivation of bacteriophages and mammalian viruses. Since bacteriophages are frequently used as surrogates of mammalian viruses it is important to discuss and compare the past and present state of the art of mammalian viruses PDI with phage photoinactivation. This chapter intends to give a special focus to the inactivation of different nucleic acid-type bacteriophages and also to the most relevant mechanisms of photosensitization, viral molecular targets, development of viral resistance and other factors affecting the viral inactivation process.

Since the photosensitizer used to inactivate the viruses need a light source in order to be activated to exert their toxic effect, the factors associated with the light conditions required for an efficient PDI need to be assessed. However, the effect of different light sources, fluence rates and light doses in PDI efficiency was not yet well evaluated for viral inactivation. This chapter includes a study about the effect of different light sources, doses and intensities in the rate of a DNA-type phage photoinactivation (Chapter 2).

The next step of this work was to make a comparative study of the different susceptibilities of several DNA- and RNA-type phages to photosensitization. For this purpose, different non-enveloped DNA- and RNA-type phages were submitted to the same PDI protocol and the efficiency of their photoinactivation was subsequently evaluated (Chapter 3).

After assessing the efficacy of DNA- and RNA-type phages it is important to study the main mechanism involved on DNA- and RNA-type bacteriophages photosensitization. In the presence of molecular oxygen and excited photosensitizers, free radical species (type I mechanism) and singlet oxygen (type II mechanism) are generated, which are known to cause damages to important biomolecules. By the use of different singlet oxygen quenchers and free radicals scavengers, the

main mechanism of DNA- and RNA-type bacteriophages photosensitization was evaluated (Chapter 4).

Reactive oxygen species (ROS) like singlet oxygen and free radicals are extremely toxic and can damage important viral structures, such as the envelope lipids and proteins (in the particular case of enveloped viruses), capsid and core proteins and the nucleic acids. From all these viral targets, the outer structures (proteins) are told to be the most important ones due to their significant destruction after PDI, which is associated with viral inactivation. For this reason, the damages on T4-like phage proteins after photosensitization with Tri-Py^{+}-Me-PF and white light irradiation were evaluated by IR spectroscopy, in comparison with the conventional SDS-PAGE analysis (Chapter 5).

Due to the worldwide increased use of antiviral agents, the risk of viral resistance is gaining a new importance. In this way, viral PDI may represent a good alternative for the purpose of viral inactivation in a less expensive and in an environmental-friendly way. However, the possible development of viral resistance after consecutive episodes of viral photosensitization was not yet evaluated for viruses. The possible development of photoresistance to PDI was evaluated for the DNA phage T4-like after ten cycles of sub-lethal treatments. The possibility of the viruses to recover their viability after repeated phototreatments was also evaluated (Chapter 6).

In the final chapter of this thesis all the experimental results were discussed together and some conclusions about the photoinactivation of viruses were taken (Chapter 7).

CHAPTER 1

INTRODUCTION

Photodynamic inactivation of mammalian viruses and bacteriophages

Abstract

Photodynamic inactivation (PDI) has been used to inactivate microorganisms through the use of photosensitizers. The inactivation of mammalian viruses and bacteriophages by photosensitization has been applied with success since the first decades of the last century. Due to the fact that mammalian viruses are known to pose a threat to public health and that bacteriophages are frequently used as models of mammalian viruses, it is important to know and understand the mechanisms and photodynamic procedures involved in their photoinactivation. The aim of this review is to (i) summarize the main approaches developed until now for the photodynamic inactivation of bacteriophages and mammalian viruses and, (ii) discuss and compare the present state of the art of mammalian viruses PDI with phage photoinactivation, with special focus on the most relevant mechanisms, molecular targets and factors affecting the viral inactivation process.

Keywords: bacteriophages; mammalian viruses; photodynamic therapy; photosensitizer; viral photoinactivation process.

List of Acronyms and Abbreviations

$AlPcS_4$	Aluminum phthalocyanine tetrasulfonate
AZT	Azidothymidine
BVDV	Bovine viral diarrhoea virus
DMTU	Dimethylthiourea
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papillomatosis virus
HSV	Herpes simplex virus
LED	Light emitting diode
MB	Methylene blue
Pc_4	Silicon phthalocyanine

PDI	Photodynamic inactivation
PDT	Photodynamic therapy
PS	Photosensitizer
ROS	Reactive oxygen species
SARS	Sudden acute respiratory syndrome
SFV	Semliki Forest virus
SHV	Suid herpes virus
SOD	Superoxide dismutase
SSB	Single strand breaks
TBO	Toluidine blue O
Tri-Py^{+}-Me-PF	5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin tri-iodide
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus
$^{1}O_2$	Singlet oxygen
$^{3}O_2$	Molecular oxygen
^{1}PS	Ground state photosensitizer
$^{3}PS^{*}$	Triplet state photosensitizer

1. Introduction

Humans are exposed to pathogenic viruses through various routes and the development of viral-induced diseases is a common occurrence.

Although the transmission of viral diseases has been reduced by the development of good water supplies and hygienic-based procedures for a whole range of human activities (Jori and Brown, 2004), pathogenic viruses are still the causative agents of many diseases in humans and other species. The most usual human diseases caused by viruses include the common cold (coronaviruses), influenza (influenza viruses), chickenpox (varicella zoster virus), cold sores (herpes simplex virus), gastroenteritis and diarrhoea (caliciviruses, rotaviruses and adenoviruses) (van der Poel et al, 2000; Blerkom, 2009). Pathogenic viruses are also implicated in serious diseases, such as Ebola (Ebola virus), AIDS (immunodeficiency viruses), avian influenza and sudden acute respiratory syndrome (SARS) (SARS-coronavirus), and they are also an established cause of cancer (papillomavirus, hepatitis B and C viruses, Epstein-Barr virus, Kaposi's sarcoma-associated herpes virus, human T-lymphotropic virus, and Merkel cell polyomavirus) (Pulitzer et al, 2009).

The enhanced implication of viruses in severe infectious diseases and the increasing knowledge about the complex mechanisms of viral pathogenesis have greatly contributed to the rapid development of antiviral drugs. Consequently, the use of antivirals has largely increased in the last years and resistance to antiviral drugs is now well documented for several pathogenic viruses (Sullivan et al, 1993; Smee et al, 1995; Kimberlin and Whitley, 1996; Jabs et al, 1998; Pillay and Zambon, 1998; Smee et al, 2002).

Moreover, as viruses are genetically flexible, they may mutate quickly and mutations come as no surprises, leading to the development of resistance to conventional antiviral drugs. Consequently, the emergence of antiviral drug resistance can become a great problem, such the resistance observed for bacteria relative to antibiotics. So, alternative methods unlikely to cause resistance are required. Photodynamic inactivation (PDI) of viruses represents a promising and inexpensive potential alternative to meet that need.

The sensitivity of viruses to photodynamic procedures was reported in the 1930s (Schultz and Krueger, 1928; Perdrau and Todd, 1933) but only within the last 30 years, with the development of new active molecules, namely photosensitizers (PS), and an increment of light technologies (lasers, LED, portability, *etc.*), have photodynamic techniques for the inactivation of viruses received growing attention (Käsermann and Kempf, 1998). Most of the clinical applications of PDI for treatment of infections have so far been directed to viral lesions (Hamblin and Hasan, 2004). Clinical PDI was first applied to the treatment of herpes infection in the early 1970s (Felber et al, 1973), particularly for herpes genitalis. Since then, a great variety of viruses has been effectively inactivated by photodynamic treatment using *in vitro* conditions (Almeida et al, 2011) but, considering the clinical use of viral PDI, the procedures are limited to the treatment of papillomatosis, caused by human papillomatosis virus (HPV), like laryngeal papillomatosis (Mullooly et al, 1990) and epidermodysplasia verruciformis (Karrer et al, 1999) and, in a small scale, to the treatment of viral complications in AIDS patients (Lavie et al, 1995; Smetana et al, 1997). However, considerable progress has been made in the viral photodynamic disinfection of blood products. The major threat of viral contamination in blood and blood products comes from the immunodeficiency viruses (HIV) (Sloand et al, 1995), hepatitis viruses (Mannucci, 1992; Klein, 1994; Sloand et al, 1995), cytomegalovirus (Klein, 1994), human parvovirus B19 (Azzi et al, 1993) and human T-cell lymphotropic virus type I and type II (Klein, 1994). HIV has been inactivated *in vitro* following a photodynamic procedure (Asanaka et al, 1989; Dixon et al, 1990; Lambrecht et al, 1991; Levere et al, 1991; Matthews et al, 1992; Lenard et al, 1993; Neurath et al, 1993; Debnath et al, 1994; North et al, 1994; Bachmann et al, 1995; Song et al, 1997; Vzorov et al, 2002; Vanyur et al, 2003; Dairou et al, 2004; Marchesan et al, 2005). The photoinactivation of hepatitis viruses in blood products has also been successfully tested against the hepatitis C virus (HCV) (North et al, 1992; Müller-Breitkreutz and Mohr, 1998; Vanyur et al, 2003; Cheng et al, 2010), hepatitis B virus (HBV) (Lin and Hu, 2008) and hepatitis A virus (HAV) (Casteel et al, 2004). Inactivation of cytomegalovirus (O'Brien et al, 1992), human parvovirus B19 (Mohr et al, 1997) and human T-cell lymphotropic virus (Sieber et al, 1987) in blood products was also efficiently achieved after photodynamic treatment.

The availability of a simple and quantitative assay to follow the viral photoinactivation process is important. Traditional viral quantification techniques, such as *in vitro* viral cultures, are time-consuming and labor-intensive processes. Molecular quantitative methods such as nucleic acid amplification procedures, including real time PCR, are rapid and sensitive but detect only viral nucleic acid and do not determine infectivity. When the virucidal properties of different photosensitizing compounds are initially evaluated, bacteriophages can be useful as surrogates of mammalian viruses. The reasons for their use

are: (i) the detection methods are much simpler, faster and cheaper than those of mammalian viruses, avoiding the advanced facilities and equipment needed for propagating human pathogens; (ii) they are non-pathogenic to humans; (iii) they can be grown to higher titers than most mammalian viruses and, therefore, enhancing the sensitivity of the assay; (iv) the results of bacteriophages assays are available within several hours post-inoculation, instead of the days or weeks required by mammalian viruses infectivity-based assays; (v) they are at least as resistant as the mammalian viruses to environmental factors and to water treatment (Leclerc et al, 2000).

It has been shown that enveloped viruses are significantly more sensitive to photodynamic destruction than non-enveloped viruses (Rywkin et al, 1994; Käsermann and Kempf, 1997). As most of the bacteriophages are non-enveloped, they are more difficult to suffer photoinactivation than the enveloped viruses. In general, this property makes them good indicators to evaluate the efficiency of viral PDI. A PDI protocol that is effective to inactivate a non-enveloped phage will most likely be effective against enveloped mammalian viruses.

Several bacteriophages were used in photoinactivation studies as surrogates for mammalian viruses, *e.g.*, MS2 (Casteel et al, 2004), M13 (DiMascio et al, 1989; Abe et al, 1997), PM2 (Specht, 1994), Qβ (Schneider et al, 1993; Jockush et al, 1996; Lee et al, 1997), PRD1 (Hotze et al, 2009), λ (Kastury and Platz et al, 1992; Martin et al, 2005), ϕ6 (Wagner et al, 1998), R17 (Wagner et al, 1998), *Serratia* phage *kappa* (Brendel, 1970), T5 (Yamamoto, 1957), T3 (Witmer and Fraser, 1971), T7 (Gábor et al, 2001; Egyeki et al, 2003; Hotze et al, 2009) and T4-like (Kadish et al, 1964; Costa et al, 2008; Costa et al, 2010; Costa et al, 2011), and the results show that they are effectively photoinactivated.

2. Antimicrobial PDI

PDI is a simple and controllable method for the inactivation of microorganisms based on the production of reactive oxygen species (ROS) (free radicals and singlet oxygen). This technology requires the combined action of oxygen, light and a photosensitizer (PS), which absorbs and uses the energy from light to produce those ROS (DeRosa and Crutchley, 2002). Therefore, the photodynamic effects depend on multiple variables including: the structural features of the PS, the concentrations of PS and molecular oxygen, and the properties of the light used (*e.g.*, wavelength, type, dose and fluence rate) (DeRosa and Crutchley, 2002; Capella and Capella, 2003; Castano et al, 2004; Costa et al, 2008; Prates et al, 2009; Costa et al, 2010). Changes in any of these parameters will affect the rate of microbial photoinactivation (Welsh and Adams, 1954; Huang et al, 2004; Costa et al, 2008; Costa et al, 2010).

The majority of the PS used in PDI is derived from tetrapyrrolic macrocycles known as porphyrins. These chromophores and their analogs, such as chlorins and bacteriochlorins, are involved in very important biological functions, such as respiration (heme group) and photosynthesis (chlorophyll and bacteriochlorophyll (Figure 1). Based on these macrocycles, the scientific community was able to develop a number of synthetic analogs, such as *meso*-tetraarylporphyrins, phthalocyanines, texaphyrins, porphycenes and saphyrins, which proved to have very promising features for being used as PS (Figure 2)

(Almeida et al, 2011). Also, non-tetrapyrrolic derivatives, such as the naturally occurring hypericin, or synthetic dyes like toluidine blue O (TBO), rose bengal, eosin, methylene blue (MB) and fullerenes, were considered in many PDI studies (Figure 3) (Castano et al, 2004).

In order to be efficient, photosensitizing agents used for viral PDI must bind specifically to vital viral components, such as lipid envelope (when present), the protein coat or to the nucleic acids (Jockush et al, 1996).

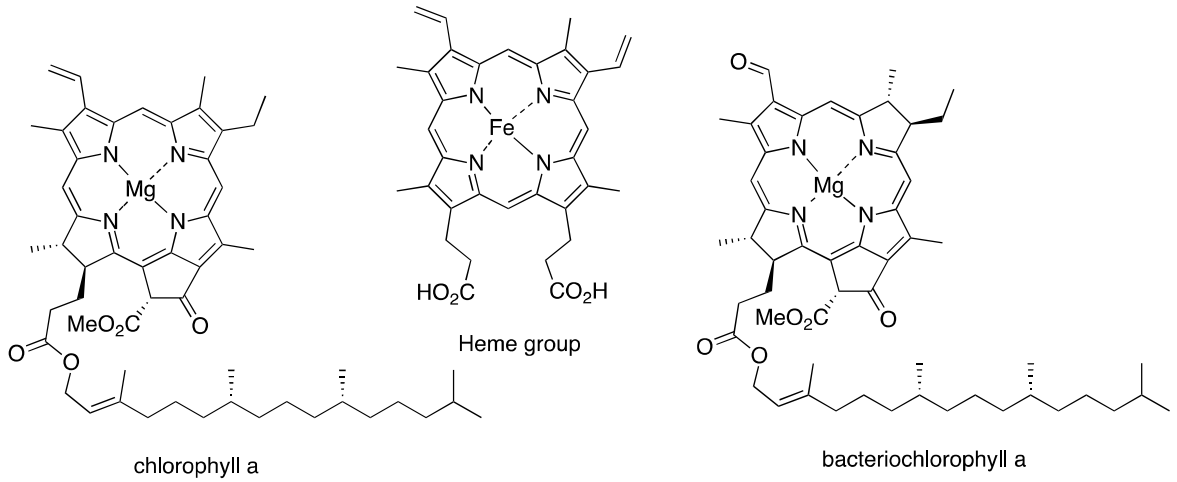

Figure 1. Structure of some tetrapyrrolic macrocycles with natural occurrence.

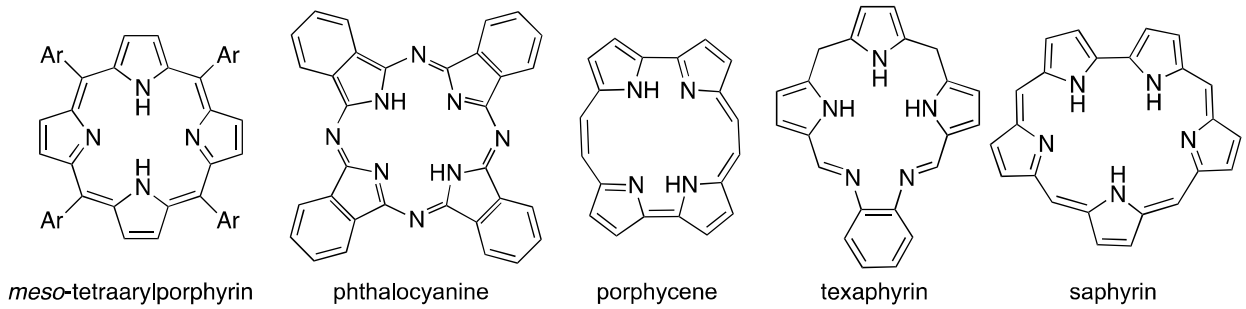

Figure 2. Skeletons of some synthetic pyrrolic macrocycles used as photosensitizers.

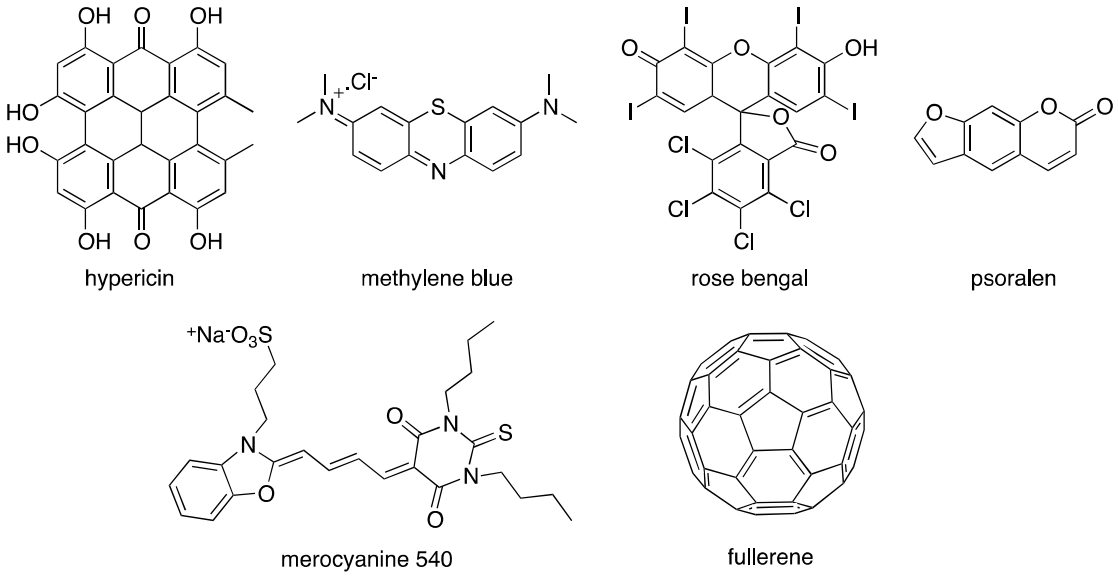

Figure 3. Structure of some non-tetrapyrrolic photosensitizers.

The efficiency of mammalian viruses and bacteriophages PDI has been described for porphyrin derivatives, chlorin derivatives, chlorophyll derivatives, phthalocyanine derivatives, hypericin, methylene blue, rose bengal, merocyanine 540, proflavine, and fullerene derivatives (Table 1).

Table 1. Some PS used for mammalian viruses and bacteriophages PDI

Photosensitizer	Microorganism	PDI	Reference
Mammalian viruses			
Hematoporphyrin derivative	HSV-1	7 log	Schnipper et al, 1980
	HSV-1	< 0.8 log	Vzorov et al, 2002
Uroporphyrin	Adenovirus	7 log	Schagen et al, 1999
Natural metalloporphyrin derivatives	HIV-1	< 0.8 log	Vzorov et al, 2002
Chlorophyll derivatives	VSV	~ 6 log	Lim et al, 2002
7-despropionate-7-hydroxypropylmesopyropheophorbide a	BVDV EMCV	~ 5 log ~ 0.2 log	Sagristá et al, 2009
Benzoporphyrin derivative monoacid ring A	HIV-1	> 4 log	North et al, 1994
Glycoconjugated *meso*-tetraarylporphyrin derivatives	HSV-1 HSV-2	6 log 6 log	Tomé et al, 2005
Metallo tetrasulfonated *meso*-tetraarylporphyrin derivatives	HIV-1	≤ 2 log	Vzorov et al, 2002

Tetrasulfonated *meso*-tetraarylporphyrin derivatives	HIV-1	≤ 2 log	Vzorov et al, 2002
	HAV	~ 4 log	Casteel et al, 2004
meso-Tetrakis(1-methylpyridinium-4-yl)porphyrin	HAV	~ 4 log	Casteel et al, 2004
meso-Tetrakis(1-butylpyridinium-4-yl)porphyrin	HAV	> 3.8 log	Casteel et al, 2004
meso-Tetrakis(1-octylpyridinium-4-yl)porphyrin	HAV	> 3.9 log	Casteel et al, 2004
Cationic β-vinyl substituted *meso*-tetraphenylporphyrin derivatives	HSV-1	< 3 log	Silva et al, 2005
Aluminum dibenzodisulfophthalocyanine	HIV-1	3.7 log	Rywkin et al, 1994
Aluminum phthalocyanine tetrasulfonate	HIV-1	> 5 log	Rywkin et al, 1994
	VSV	4.2 log	Moor et al, 1997
	Adenovirus	4 log	Schagen et al, 1999
Silicon phthalocyanine derivative	VSV	4 log	Moor et al, 1997
Cationic phthalocyanines	HIV-1	> 5 log	Rywkin et al, 1994
	HSV-1	≥ 5 log	Smetana et al, 1998
Hypericin	HIV-1	NQ	Lenard et al, 1993
	VSV	4-5 log	
	Influenza virus	NQ	
	Sendai virus	NQ	
Methylene blue	VSV	4.7 log	Abe and Wagner, 1995
	HSV-1	5 log	Müller-Breitkreutz et al, 1995
	SHV-1	2.5 log	Müller-Breitkreutz et al, 1995
	HCV	< 2 log	Müller-Breitkreutz and Mohr, 1998
	HIV-1	< 2 log	Müller-Breitkreutz and Mohr, 1998
	Adenovirus	7 log	Schagen et al, 1999
	Dengue virus	5-6.4 log	Huang et al, 2004
	Enterovirus 71	~ 8 log	Wong et al, 2010
	Vaccinia virus	5 log	Turner and Kaplan, 1968
Phenothiazine derivatives	VSV	> 4.4 log	Wagner et al, 1998
Rose bengal	Vaccinia virus	5 log	Turner and Kaplan, 1968
	HIV-1	NQ	Lenard et al,

	VSV	4-5 log	1993
	Influenza virus	NQ	
	Sendai virus	NQ	
	Adenovirus	7 log	Schagen et al, 1999
Buckminsterfullerene	SFV	7 log	Käsermann and Kempf, 1997
	VSV	7 log	
Merocyanine 540	HSV-1	5-6 log	O'Brien et al, 1992
Bacteriophages			
Glycoconjugated *meso*-tetraarylporphyrins	T7 phage	< 3 log	Gábor et al, 2001
	T7 phage	< 3.5 log	Egyeki et al, 2003
Tetrasulfonated *meso*-tetraarylporphyrin derivatives	MS2 phage	> 3.8 log	Casteel et al, 2004
meso-Tetrakis(1-methylpyridinium-4-yl)porphyrin	λ phage	< 7 log	Kastury and Platz, 1992
	MS2 phage	> 4.1 log	Casteel et al, 2004
	T4 phage	7 log	Costa et al, 2008; Costa et al, 2010
	T7 phage	< 4 log	Zupán et al, 2008
5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin	T4 phage	7 log	Costa et al, 2008; Costa et al, 2010; Costa et al, 2011
5-(4-methoxicarbonylphenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin	T4 phage	7 log	Costa et al, 2008
5-(4-carboxyphenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin	T4 phage	3.9 log	Costa et al, 2008
5,10-bis(4-carboxyphenyl)-15,20-bis(1-methylpyridinium-4-yl)porphyrin	T4 phage	1.4 log	Costa et al, 2008
5,15-bis(4-carboxyphenyl)-10,20-bis(1-methylpyridinium-4-yl)porphyrin	T4 phage	1.2 log	Costa et al, 2008
5,10,15-tris(1-methylpyridinium-4-yl)-20-phenylporphyrin	T7 phage	1.7 log	Zupán et al, 2008
Methylene blue	*Serratia* phage kappa	> 4 log	Brendel, 1970
	M13 phage	2.2 log	Abe and Wagner, 1995; Abe et al, 1997
	f2 phage	5 log	Lee et al, 1997
	Qβ phage	7-8 log	Lee et al, 1997
	Qβ phage	7-8 log	Schneider et al, 1998
Phenothiazine derivatives	R17 phage	4-7 log	Wagner et al, 1998
	ϕ6	4-6.5 log	

Rose bengal	PRD1 phage T7 phage	~ 3.5 log* ~ 4.5 log*	Hotze et al, 2009
Riboflavin	λ phage	< 4 log	Martin et al, 2005
Proflavine	*Serratia* phage kappa T3 phage	4 log 7-11 log	Brendel, 1970 Witmer and Fraser, 1971
Polyhydroxylated fullerene	MS2 phage	~ 4 log	Badireddy et al, 2007
	PRD1 phage T7 phage MS2 phage	~ 2.5 log* ~ 3.5 log* ~ 5 log*	Hotze et al, 2009

* log (N/N0)

Besides this, viral PDI has also been described for phthalocyanine derivatives (Abe and Wagner, 1995), MB (Yamamoto, 1957; Specht, 1994; Floyd et al, 2004; Marotti et al, 2009), TBO (Yamamoto, 1957; Wallis and Melnick, 1965; Specht, 1994), neutral red (Wallis and Melnick, 1965), proflavine (Wallis and Melnick, 1965), azure B (Specht, 1994) and merocyanine 540 (Sieber et al, 1987; Lytle et al, 1991; O'Brien et al, 1992).

3. Mechanisms of Photodynamic Inactivation

The mechanisms of PDI are based on the ability of the PS to absorb energy from light and transfer that energy to molecular oxygen. In the dark, the electronic configuration of a PS exists in the so-called ground state. The absorption, by the PS, of a photon at an appropriate wavelength initially leads to the production of an unstable, electronically-excited state of the PS molecule (the lifetime of this state ranges from 10^{-9} to 10^{-6} s) (Via and Magno, 2001). The excited PS molecule can then decay to the ground state by emission of light (radiative pathway - fluorescence) or by intersystem crossing, affording the excited triplet state which has a longer lifetime (10^{-3} to 10 s) (Via and Magno, 2001). At this point, the PS can reach the ground state either by spin inversion followed by phosphorescence emission, or by a non-radiative process. Due to the longer lifetime of the PS triplet state, this excited state can also react in one of two ways (Figure 2): by initiating photochemical reactions that can directly generate reactive oxygen species (ROS) (type I pathway), or indirectly by energy transfer to molecular oxygen (type II pathway), leading to the formation of singlet oxygen. These events afford toxic species which are responsible for the irreparable oxidative damages induced to important biological targets (Schmidt-Erfurth and Hasan, 2000; Via and Magno, 2001; DeRosa and Crutchley, 2002; Jori and Brown, 2004).

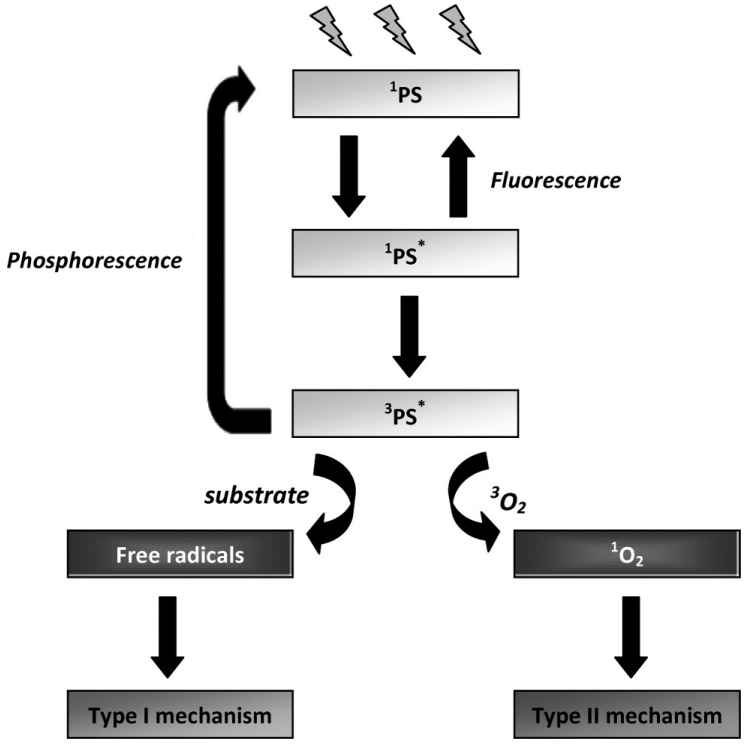

Figure 4. Schematic representation of the photosensitization process (adapted from Wainwright, 1998).

3.1. Type I and Type II Mechanisms

Type I mechanism involves hydrogen-atom abstraction or electron-transfer between the excited PS and a substrate, yielding free radicals [Equations (1) and (2)]. These radicals can react with oxygen to form active oxygen species, such as the superoxide radical anion [Equation (3)]. Superoxide is not particularly reactive in biological systems but, when protonated, can lead to the production of hydrogen peroxide and oxygen [Equations (4) and (5)] or highly reactive hydroxyl radicals [Equations (6)–(8)] (Bonnett, 2000). Type II photooxidation is considerably less complex mechanistically than type I and in general there are far fewer products (Girotti, 2001). In this pathway, the excited triplet state PS (^{3}PS*) can transfer the excess energy to molecular oxygen ($^3O_2$) and relax to its ground state (^{1}PS) creating an excited singlet molecular oxygen ($^1O_2$) [Equation (9)] (DeRosa and Crutchley, 2002). $^1O_2$ is highly electrophilic and can interact with numerous enzymes, leading to the inhibition of protein synthesis and molecular alteration of DNA strands, which alters the transcription of the genetic material during its replication (mutagenic effect) and, in this way, leading to microbial death [Equation (10)] (Bonnett, 2000; Calin and Parasca, 2009). Like nucleic acids and proteins, unsaturated lipids are also prominent targets of $^1O_2$ and free radical attack. Lipid peroxidation-ensuing reactions can alter surrounding proteins, nucleic acids and other molecules, in addition to the lipids themselves (Bonnett, 2000). Therefore, it is likely that damage of different kinds caused to the viral envelope is important in the process of microbial inactivation (Käsermann and Kempf, 1998).

$$\text{Substrate}H_2 + PS \rightarrow PSH^{\bullet} + \text{Substrate}H^{\bullet} \quad (1)$$

$$PS^* + \text{Substrate} \rightarrow PS^{\bullet-} + \text{Substrate}^{\bullet+} \text{ or } PS^* + \text{Substrate} \rightarrow PS^{\bullet+} + \text{Substrate}^{\bullet-} \quad (2)$$

$$PS^{\bullet-} + {}^3O_2 \rightarrow PS + O_2^{\bullet-} \quad (3)$$

$$O_2^{\bullet-} + H^+ \rightleftharpoons HOO^{\bullet} \quad (4)$$

$$2\ HOO^{\bullet} \rightarrow H_2O_2 + O_2 \quad (5)$$

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + OH^- + Fe^{3+} \quad (6)$$

$$\text{Biomolecule}H + HO^{\bullet} \rightarrow \text{Biomolecule}^{\bullet} + H_2O \quad (7)$$

$$\text{Biomolecule}^{\bullet} + {}^3O_2 \rightarrow \text{Biomolecule-}OO^{\bullet} \rightarrow \text{products} \quad (8)$$

$$PS^* + {}^3O_2 \rightarrow PS + {}^1O_2 \quad (9)$$

$$\text{Biomolecules} + {}^1O_2 \rightarrow \text{oxidative products} \quad (10)$$

Both type I and type II mechanisms can occur simultaneously or exclusively, and the ratio between these processes depends on the PS used and on the concentrations of substrate and oxygen (Via and Magno, 2001). The competition between organic substrates and molecular oxygen for the $^3PS^*$ determines whether the reaction pathway is type I or type II and the predominant mechanism can be changed during the course of the PDI process (Min and Boff, 2002).

3.2. Evaluation of the Specific Involvement of Type I and Type II Mechanisms

An important goal in the investigation of viral PDI is to identify the type of mechanism involved (type I or type II) in the presence of a selected PS (Maisch et al, 2005). The simple detection of a reactive species does not necessarily explain the mechanism by which a specific PS induces the toxic effect. It is generally easier to draw a negative conclusion, *i.e.*, if singlet oxygen is absent, it cannot be the reactive species responsible for the photodynamic effect (Ochsner, 1997). The simplest approach for determining whether singlet oxygen (type II mechanism) or free radicals (type I mechanism) is involved in the photodynamic process is to study the inhibitory effects of various scavengers, *i.e.*, compounds that can intercept these ROS at high rates and in a putatively selective manner (Girotti, 2001; Min and Boff, 2002; Wondrak et al, 2005).

3.2.1. Type I Mechanism Scavengers

A first line of defence against ROS is, of course, the protection against their formation. However, the interception of the damaging species once formed, to prevent it from further deleterious reactions, is also a deactivation strategy of defence. In general, free radical scavengers neutralize the radical species by donating one of their own electrons. The scavenging agents themselves are not particularly toxic before and after the electron donation (Sies, 1997).

Three different types of scavenging are possible, which include the transfer of the radical character with the formation of a reactive scavenger-derived radical; trapping of free radicals with the formation of a stable or inert free radical trap; and molecules which mimic quenching enzyme activities. In general, scavenger molecules either prevent free radicals from being formed or remove them before they can damage vital molecular components (Sies, 1997).

Several free radical scavengers have been used to evaluate the specific involvement of type I mechanism during mammalian viruses and bacteriophages PDI with different PS (Table 2).

Table 2. Free radical scavengers used in mammalian viruses and bacteriophages PDI.

PS	Scavenger	Microorganism	Scavenger protection	Reference
Mammalian viruses				
Aluminum phthalocyanine tetrasulfonate	Reduced glutathione	VSV	little/no effect	Rywkin et al, 1992
	Mannitol	VSV	little/no effect	
	Glycerol	VSV	little/no effect	
	SOD	VSV	little/no effect	
Polyhydroxylated fullerene	Glutathione (2.0 mM)	SFV	no effect	Käsermann and Kempf, 1997
		VSV	no effect	
	Hydroquinone (2.0 mM)	SFV	no effect	Käsermann and Kempf, 1997
		VSV	no effect	
Merocyanine 540	Glutathione (10 and 30 mmol L^{-1})	HSV-1	30-50%	O'Brien et al, 1992
	Cysteamine (10 and 30 mmol L^{-1})		60-70%	
	SOD (1.5 to 29 U mL^{-1})		no effect	
Methylene blue	Mannitol (100 mM)	HSV-1	24%	Müller-Breitkreutz et al, 1995
	Glycerol (10 mM)		24%	
	SOD (300 U mL^{-1})		24%	
	Catalase (30 U mL^{-1})		24%	
Bacteriophages				
5,10,15-(4-β-D-glucosylphenyl)-20-phenylporphyrin	DMTU (0.1-5.0 mM)	T7 phage	44%	Gábor et al, 2001
5,10.15,20-Tetrakis(4-β-D-glucosylphenyl) porphyrin	DMTU (0.1-5.0 mM)	T7 phage	79%	Gábor et al, 2001
5,10,15-(4-β-D-galactosylphenyl)-20-(pentafluorophenyl)-porphyrin	DMTU (0.1-5.0 mM)	T7 phage	89%	Egyeki et al, 2003
5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin	D-mannitol (100 mM)	T4-like phage	20%	Costa et al, *submitted*
		Qβ	no effect	
	L-cysteine (100 mM)	T4 phage	9%	Costa et al, *submitted*
5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin	D-mannitol (100 mM)	T4 phage	no effect	Costa et al, *submitted*
Proflavine	L-cysteine (0.025 M)	T3 phage	75-80%	Witmer and Fraser, 1971

Polyhydroxylated fullerene	SOD	MS2 phage	no effect	Badireddy et al, 2007

3.2.1.1. Free Radicals in PDI of Mammalian Viruses

Free radical species had, in general, little or no effect on the photoinactivation of the studied mammalian viruses (Table 2). In fact, it can be observed that the rate of inactivation of HSV (O'Brien et al, 1992; Rywkin et al, 1992; Müller-Breitkreutz et al, 1995), influenza virus (Lenard and Vanderoef, 1993), Semliki Forest virus (SFV) and VSV (Käsermann and Kempf, 1997) in the presence of different PS and scavengers like glutathione, mannitol, glycerol, superoxide dismutase (SOD), catalase and hydroquinone was not significantly affected. Although this data suggest that free radicals are not major players in the viral inactivation process, the participation of type I reaction pathways cannot be ruled out, as was shown by the considerable level of protection afforded by glutathione and cysteamine when merocyanine 540 was used as PS for inactivation of HSV-1 (O'Brien et al, 1992).

3.2.1.2. Free Radicals in PDI of Bacteriophages

The photoinactivation rate of some bacteriophages can be reduced in the presence of free radical scavengers, suggesting a contribution of radical species in the inactivation process (Table 2). In particular, it was reported that the inhibition of T7 phage photoinactivation in the presence of glycoconjugated *meso*-tetraarylporphyrins varied according to the structure of the PS and the concentration of dimethylthiourea (DMTU) (Gábor et al, 2001; Egyeki et al, 2003). In fact, T7 phage PDI by *meso*-tetrakis(4-β-D-glucosylphenyl)porphyrin (Gábor et al, 2001) and 5,10,15-(4-β-D-galactosylphenyl)-20-(pentafluorophenyl)porphyrin (Egyeki et al, 2003) seemed to be mainly mediated by free radical species, as revealed by the protection effect of free radical scavenger DMTU, contrary to T7 phage photosensitization by 5,10,15-(4-β-D-glucosylphenyl)-20-phenylporphyrin, which revealed a significantly smaller contribution from type I mechanism. The highest inhibition was reached at about 1.0 mM of DMTU; further increase in scavenger concentration did not decrease the slope of photoinduced inactivation of phages. However, in spite of inhibiting the efficacy of the PS, DMTU did not completely inhibit T7 phage PDI (Gábor et al, 2001; Egyeki et al, 2003). Similar results were reported for T3 phage in the presence of L-cysteine as the scavenger and proflavine as the PS. However, the photoinactivation rate of MS2 by a polydroxylated fullerene was not affected by the presence of SOD, suggesting a negligible contribution of radical species, such as the superoxide radical anion (Badireddy et al, 2007). T4-like phage PDI was also little or not affected by the presence of free radical scavengers L-cysteine and D-mannitol in the presence of porphyrin derivatives, leading to the conclusion that free radical species are not major participants in phage PDI (Costa et al, *submitted*).

3.2.2. Type II Mechanism Quenchers

In general, the action of chemical singlet oxygen quenchers involves the reaction of singlet oxygen with the quenching agent, producing an oxidized product. Another possibility is the deactivation of singlet oxygen to ground state ($^3O_2$) by physical quenching, achieved by either energy or charge transfer, without consumption of oxygen or product formation (Bisby et al, 1999; Min and Boff, 2002). Residues of histidine, tryptophan and tyrosine in proteins are considered to be major natural quenchers of singlet oxygen (Baker and Kanofsky, 1992).

Several singlet oxygen quenchers have been used to evaluate the specific involvement of type II mechanism during viral PDI with different PS (Table 3).

Table 3. Singlet oxygen quenchers used on mammalian viruses and bacteriophage PDI.

PS	Quencher	Microorganism	Quencher protection	Reference
Mammalian viruses				
Aluminum phthalocyanine tetrasulfonate	Sodium azide	VSV	significant effect	Rywkin et al, 1992
	Tryptophan	VSV	significant effect	Rywkin et al, 1992
Rose bengal	β-carotene	Influenza virus	significant effect	Lenard and Vanderoef, 1993
	Sodium azide	Influenza virus	significant effect	Lenard and Vanderoef, 1993
Hypericin	Sodium azide	HIV	significant effect	Degar et al, 1992
Methylene blue	Imidazole (5.0 and 10 mM)	HSV-1	55-75%	Müller-Breitkreutz et al, 1995
Bacteriophages				
5,10,15-(4-β-D-galactosylphenyl)-20-(pentafluorophenyl)porphyrin	Sodium azide (0.1-5.0 mM)	T7 phage	38%	Egyeki et al, 2003
5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin	Sodium azide (100 mM)	T4 phage Qβ	80% 39%	Costa et al, *submitted*
	L-histidine (50 mM)	T4 phage	74%	Costa et al, *submitted*
meso-tetrakis(1-methylpyridinium-4-yl)porphyrin	Sodium azide (100 mM)	T4 phage	90%	Costa et al, *submitted*
	L-histidine (100 mM)	T4 phage	78%	Costa et al, *submitted*

5,10,15,20-Tetrakis(4-β-D-glucosylphenyl)porphyrin	1,3-diphenylisobenzofuran (0.1-5.0 mM)	T7 phage	42%	Gábor et al, 2001
5,10,15-(4-β-D-gluco- sylphenyl)-20-phenylporphyrin	1,3-diphenylisobenzofuran (0.1-5.0 mM)	T7 phage	74%	Gábor et al, 2001
Polyhydroxylated fullerene	β-carotene	T7 phage PRD1 phage	69% 56%	Hotze et al, 2009
	β-carotene (26 μM)	MS2 phage	50-60%	Badireddy et al, 2007
Rose bengal	Sodium azide (3.5-35 mM)	M13 phage	31%	Abe et al, 1997

3.2.2.1. Singlet Oxygen in PDI of Mammalian Viruses

Singlet oxygen seems to be the most important mediator of the virucidal activity (Table 3) on mammalian viruses. The rate of viral photoinactivation is significantly inhibited by oxygen removal or by addition of singlet oxygen quenchers, such as β-carotene, imidazole, L-histidine or sodium azide (O'Brien et al, 1992; Rywkin et al, 1992; Lenard and Vanderoef, 1993; Müller-Breitkreutz et al, 1995; Costa et al, *submitted*). Hypericin may induce photochemical alterations on HIV major capsid protein p24, which are inhibited by sodium azide, suggesting that the damage results from singlet oxygen (Degar et al, 1992). When merocyanine 540 (O'Brien et al, 1992), phthalocyanine derivatives (Rywkin et al, 1992) or rose bengal (Lenard and Vanderoef, 1993) were used as PS, the results suggest that $^1O_2$ is the main cytotoxic species involved in VSV photoinactivation, while type I reactants such as hydroxyl radicals are less important.

3.2.2.2. Singlet Oxygen in PDI of Bacteriophages

Considering the PDI of bacteriophages in the presence of singlet oxygen quenchers, the results (Table 3) suggest that, in most of the studied cases, singlet oxygen is an important mediator of the toxic effect induced by PDI. However, the participation of free radicals cannot be ruled out. For instance, the inactivation of M13 bacteriophage by MB was inhibited from 1.72 log to 0.54 log by sodium azide in a quencher dose-dependent mode, up to a concentration of 3.5 mM. However, photoinactivation occurred even in the presence of sodium azide, suggesting that both type I and type II mechanisms may be involved in the M13 photoinactivation process. In the presence of quencher concentrations ranging from 3.5 to 35 mM, sodium azide protective effect was not observed, as evidenced by increasing rates of M13 phage photoinactivation, reaching a plateau thereafter (Abe et al, 1997). Also, the effect of singlet oxygen quenchers and of hydrogen peroxide indicated singlet oxygen as the main factor responsible for the loss of biological activity of bacteriophage M13 by rose bengal (DiMascio et al, 1989).

The efficiency of 5,10,15-(4-β-D-galactosylphenyl)-20-(pentafluorophenyl)porphyrin to photoinactivate T7 phage decreased in 38% in the presence of sodium azide (Egyeki et al, 2003). This result, and the ones obtained in the presence of DMTU (Table 2), proved that for this PS, both mechanisms play a role in T7 phage photoinactivation, with type I being the predominant one. Similar results were obtained by Gábor et al (2001) in the presence of glycoconjugated *meso*-tetraarylporphyrin derivatives as PS and using 1,3-diphenylisobenzofuran as the singlet oxygen quencher. When T7 phage was phototreated with 5,10,15,20-tetrakis(4-β-D-glucosylphenyl)porphyrin, the rate of inactivation decreased 42% in the presence of 1,3-diphenylisobenzofuran. When 5,10,15-(4-β-D-glucosylphenyl)-20-phenylporphyrin was used, the rate of protection substantially increased (74%). It can then be concluded that the type of PDI mechanism depends on the PS structure, with the symmetric derivative exerting its toxic effect mainly *via* the generation of free radicals, whether the asymmetric derivative proceeds mainly by singlet production (Gábor et al, 2001). However, in the study of Egyeki et al (2003) using the same asymmetric 5,10,15-(4-β-D-galactosylphenyl)-20-(pentafluorophenyl)porphyrin as PS, and the same phage, the toxic effect occurred mainly via free radical generation. Besides this, the contribution of type I and type II processes was PS concentration-dependent and the sum of the photoinactivation rate measured in the presence of scavengers was smaller than the one measured without the scavengers. This result may imply a synergism between singlet oxygen and hydroxyl radical-mediated damages or it can also be supposed that the efficiency of neither scavenger is 100% (Gábor et al, 2001; Egyeki et al, 2003).

A recent study showed that irradiation of polyhydroxylated fullerene suspensions (40 μM) in the presence of β-carotene reduced the photoinactivation rate of PRD1 and T7 phages, demonstrating singlet oxygen involvement (Hotze et al, 2009). Also, when the T4-like phage was irradiated in the presence of porphyrin derivatives and singlet oxygen quenchers sodium azide and L-histidine, the rate of phage inactivation was considerably reduced, suggesting that singlet oxygen may be an important mediator of the virucidal activity of these PS (Costa et al, *submitted*). However, from the data obtained, other inactivation mechanisms cannot be excluded (Hotze et al, 2009; Costa et al, *submitted*).

Although some data about the importance of type I and II mechanisms in PDI of bacteriophages are discrepant, in general, it seems that the type II pathway is more important than the type I mechanism in phage PDI. On the other hand, there are only a few studies focusing on the simultaneous effect of singlet oxygen and free radicals scavengers under the same protocol of viral PDI (Rywkin et al, 1992; Müller-Breitkreutz et al, 1995; Gábor et al, 2001; Egyeki et al, 2003; Badireddy et al, 2007; Costa et al, *submitted*).

4. Molecular Targets of Viral PDI

The short-lived ROS generated by photodynamic mechanisms are responsible for the damage induced to critical molecular targets (Wainwright, 2003). Different viral targets, such as the envelope lipids and proteins, capsid and core proteins and the nucleic acid can be attacked by singlet oxygen and/or

other ROS (hydrogen peroxide, superoxide and hydroxyl radicals) to achieve the loss of infectivity (Müller-Breitkreutz et al, 1995). For a better understanding of the photoinactivation process, the knowledge of how the molecular targets are affected by PDI assumes a great importance (Garcia et al, 2009). For this reason, a detailed photophysical and photochemical study of the interactions between the toxic species generated by the PS and key biomolecules such as lipids, proteins and nucleic acids is essential for the knowledge and prediction of photosensitization process efficiency (Miranda, 2001). However, the studies performed show that the primary target of PDI depends on the chemical structure of the PS, the targeted virus and the mechanism of photoinactivation (Gábor et al, 2001).

4.1. Nucleic Acids

Depending upon the viruses, the nucleic acid can be either DNA or RNA (single or double stranded). The size of the nucleic acid also varies depending on the viruses. Several studies have shown that both DNA and RNA mammalian viruses and phages are efficiently inactivated by PDI. There is now considerable information that PS like MB can bind to and penetrate viral membranes, whereupon they intercalate with nucleic acids. Upon activation by light, the generated ROS can cause the destruction of the nucleic acids, particularly at guanine residues, preventing viral replication (Wainwright, 2000). However, there is a difference in target selectivity depending on the mechanism involved: sugar moieties are usually attacked by radicals (generated *via* type I process) and guanine residues are the targets of singlet oxygen (generated *via* type II process) (Wainwright, 1998).

4.1.1. DNA Damage

From the four DNA bases, guanine is the most susceptible component to suffer a type I photosensitization reaction, due to the fact that it exhibits the lowest oxidation potential among DNA bases and it is the only base that can be oxidized by singlet oxygen (type II process) (McBride et al, 1992).

The treatment of viruses with MB and other heterocyclic dyes resulted in the damage of viral DNA (Kadish et al, 1967; Schnipper et al, 1980; Specht, 1994; Schagen et al, 1999) either by base modification or base loss, single strand breaks (SSB), or cross-links of DNA with proteins (Schnipper et al, 1980; OhUigin et al, 1987; Abe and Wagner, 1995; Bachmann et al, 1995; Zupán et al, 2008). It is known that cationic porphyrins can bind to nucleic acids via intercalation into base pairs or self-stacking, inducing lesions upon photoinactivation due to the easy oxidation of guanine residues (Mettath et al, 1999; Kubát et al, 2000; Caminos and Durantini, 2008).

The binding of cationic porphyrins to DNA is presumably due to the electrostatic interaction between the positively-charged substituents in the porphyrin macrocycle and the negatively charged phosphate oxygen atoms of DNA (Caminos and Durantini, 2009). However, porphyrin binding to DNA is not a prerequisite for an efficient photosensitization, since free porphyrins are more effective in virus

inactivation than the DNA-bound species (Zupán et al, 2008). This observation, which is in conflict with the generally accepted idea that the porphyrin molecule must be in close vicinity with the site of photosensitized damage, may be explained by the lower quantum yield of singlet oxygen by the bound porphyrin when compared with the free one (Zupán et al, 2008).

4.1.1.1. Damages in the DNA of Mammalian Viruses

Viral DNA is thought to be a critical target structure for PDI by MB and light (Wallis and Melnick, 1965). DNA isolated from adenovirus treated with 1.3 μM MB exhibited a smear in Southern blot analysis, indicative of random DNA fragmentation (Schagen et al, 1999). MB plus light treatment of HSV-1 gives rise to DNA damage and blocks DNA replication (Müller-Breitkreutz and Mohr, 1997).

4.1.1.2. Damage in the DNA of Bacteriophages

An internal component of T4 phage has been suggested as an important target because MB needs to cross the outer barrier made by its protein capsids in order to produce a significant effect (Kadish et al, 1967). In fact, some of the irradiated phages could still inject functional genetic material but have lost their ability to form plaques, suggesting that their DNA was damaged. Protein synthesis was also severely impaired (Kadish et al, 1967). Treatment of M13 phage with MB and aluminum phthalocyanine tetrasulfonate ($AlPcS_4$) caused strand breaks and piperidine-labile bonds in DNA, which is correlated with the loss of infectivity. This is in agreement with the proposal that lesions of the viral genome might be responsible for the lethality induced by sensitization (Abe and Wagner, 1995). DNA strand cleavage was found to be MB concentration and light dose dependent. Viral inactivation and DNA damage were found to be oxygen-dependent processes. However, DNA damage was not correlated with the loss of PM2 phage infectivity, as observed in transfection studies which measured the infectivity of the extracted viral DNA, indicating that DNA from MB-treated phage was just as capable of generating progeny virus as the untreated controls (Specht, 1994). The observed DNA damage is not correlated with loss of phage infectivity and may not be the prime target of viral PDI, because 100% of closed circular DNA was recovered from the MB phototreated PM2 phage (Specht, 1994). Concerning the effects of PDI on isolated viral DNA, treatment of M13mp2 DNA with increasing concentrations of MB, in the presence of light, yielded increasing amounts of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodguo), a prevalent adduct produced by singlet oxygen and perhaps by oxygen free radicals. At 100 μM MB, 1 residue of 8-oxodguo was produced for every 40 residues of deoxyguanosine in DNA. Thus, treatment of M13mp2 DNA with MB plus light resulted in putative alterations at deoxyguanosine residues that impede the progression of DNA synthesis *in vitro* (McBride et al, 1992).

4.1.2. RNA Damage

RNA has been suggested to be a key factor in viral PDI with many PS, but direct evidence of a correlation between RNA damage and infectivity loss has not been reported yet, as is the case of VSV when treated with phthalocyanine derivatives (Abe and Wagner, 1995). In RNA, as for DNA (Castano et al, 2004), guanine is suggested as the major target for oxidation by photosensitizing agents and light.

4.1.2.1. Damage in the RNA of Mammalian Viruses

VSV genome was damaged by 30 μg mL^{-1} of a chlorophyll derivative and red light illumination which caused a decrease of as much as 85% in RNA polymerase activity, which can be due to damage in the viral RNA polymerase complex, and 98% inhibition of viral RNA synthesis in 6 hours (Lim et al, 2002). According to Moor et al (1997), the RNA and/or the RNA polymerase complex of VSV might be a major target for its photoinactivation by $AlPcS_4$ and MB. MB and phthalocyanine derivatives inactivated VSV and inhibited fusion of the virus envelope with Vero cells. The degree of inhibition was small compared to the extent of virus inactivation, suggesting that non-membrane targets, like the viral RNA, might be involved in VSV photoinactivation. However, there is no report of a correlation between RNA damage and loss of infectivity (Abe and Wagner, 1995). Photoinactivation of HIV-1 by MB and light lead to destruction of its RNA (Bachmann et al, 1995).

4.1.2.2. Damage in the RNA of Bacteriophages

Following MB plus light exposure, the Qβ RNA genome exhibited sufficient lethal lesions to account for phage inactivation (Schneider et al, 1999). However, the protein component of the phage also exerted some effect in viral PDI (Schneider et al, 1999). In a comparison of RNA photoinactivation using MB and rose bengal as the PS, Schneider et al (1993) suggested a causal relationship between 8-oxodguo formation in RNA and R17 and Qβ bacteriophage inactivation. However, no direct relationship between photodynamically induced RNA damage and viral inactivation was described (Schneider et al, 1993). 8-oxoguanosine formation or oxidative damage of Qβ RNA alone does not directly account for the lethal event of the virus. Directly treating extracted phage RNA with MB and light caused a loss of activity in the infectious RNA assay but there was a much greater loss of activity if the phage RNA was treated with MB and light in the phage *per se*. The results demonstrated that Qβ RNA infectious activity is significantly more affected by photoinactivation in its protein-associated virion state as compared with its purified isolated polymer state (Schneider et al, 1999; Floyd et al, 2004). Inactivation of purified RNA by MB and light, in the absence of proteins, most likely occurs due to oxidative damage to the RNA at the site at which MB is bound and might involve oxidized bases such as 8-oxodguo or strand breaks (Schneider et al, 1999).

In spite of the reduced number of reports focusing on the damage induced by PDI in the nucleic acids of mammalian viruses and bacteriophages, it can be concluded that both DNA and RNA are

potential targets of viral PDI. However, there are no studies specifically focusing on the damages induced to DNA and RNA of both mammalian viruses and bacteriophages under the same PDI protocol.

4.2. Outer Structures

Enveloped viruses are inactivated more rapidly than non-enveloped viruses because the destruction of the envelope structure is generally accompanied by loss of virus infectivity (Lytle et al, 1991; North et al, 1992; Smetana et al, 1994; Ben-Hur and Horowitz, 1996; Käsermann and Kempf, 1998). The damages caused by photodynamic reactions on unsaturated lipids present in their envelopes and/or on major envelope proteins, which act as PS binding-sites, modify their structure and avoid cell infection and virus replication (Müller-Breitkreutz et al, 1995; Käsermann and Kempf, 1997). However, some studies showed that non-enveloped viruses can also be efficiently inactivated by the toxic action of PS (Welsh and Adams, 1954; Yamamoto, 1957; Kadish et al, 1967; Lytle et al, 1991; Kastury and Platz, 1992; Abe and Wagner, 1995; Jockush, 1996; Lee et al, 1997; Schneider et al, 1999; Gábor et al, 2001; Egyeki et al, 2003; Costa et al, 2008; Zupán et al, 2008; Costa et al, 2010).

The higher susceptibility to PDI of enveloped viruses, relatively to non-enveloped viruses, indicates that the viral envelope may be a more important target than nucleic acids for photosensitization. It also indicates that the unsaturated lipids present in the envelope, as well as the major envelope proteins, are important PDI targets. However, as far as it is known, no studies focus on the degradation of viral envelope lipids after PDI or even on other viral internal lipids. There are, however, many studies about the effects of PDI on viral envelope proteins as well as on other core proteins.

The statement that enveloped viruses are more easily inactivated than non-enveloped ones is only based in indirect studies which compare the inactivation results of enveloped and non-enveloped viruses. The enveloped viruses used in PDI protocols (O'Brien et al, 1992; Lenard et al, 1993; Abe and Wagner, 1995; Moor et al, 1997; Smetana et al, 1998; Lim et al, 2002; Vzorov et al, 2002) were only assayed for their protein alterations and no additional experimental work was done concerning their lipids. However, the results of PDI obtained by Lytle et al (1991) with the enveloped ϕ6 phage, although indirectly, are in good accordance with what is reported in the literature about the major contribution from lipids for the viral photoinactivation process.

Relative to proteins degradation by PDI, the results of different studies showed that the main damage is the formation of protein cross-links, followed by other types of damage, which include loss of proteins, alterations in protein molecular conformation, mass and charge, and alterations in protein band intensity (Table 4).

When proteins are irradiated with UV or visible light in the presence of a PS, photooxidation of sensitive amino acid residues such as cysteine, histidine, tyrosine, methionine and tryptophan, and covalent cross-linking of peptide chains can be observed, leading to the formation of molecular aggregates (Girotti, 1976; Verweij and Steveninck, 1982), disrupting their normal folding conformation, thus forcing them into other conformations that affect their normal functioning (Macdonald and

Dougherty, 2001). In fact, the formation of cross-linked/aggregated material appears to be a major consequence of photosensitized-mediated protein oxidation (Davies, 2003), and it has been demonstrated that the formation of protein cross-links is not a primary photodynamic event, but a secondary reaction between the photooxidation products of sensitive amino acid residues and other groups in the protein (Verweij and Steveninck, 1982).

The PS *per se* can induce alterations in the folding of some enzymes, leading to the exposure of some amino acid residues normally shielded in the protein, and to the shielding of others usually exposed in the molecule. These protein modifications lead to changes in properties such as solubility, proteolytic susceptibility, absorbance, and fluorescence emission of several of their amino acids. These alterations are mainly mediated by hydrogen peroxide and hydroxyl radical generation, although singlet oxygen mediated reactions could also occur (Afonso et al, 1999). The amino acids located in the surface of the protein are photooxidized at a much faster rate than the residues buried in the interior of the molecule. If a protein is completely unfolded, susceptible amino acids may also be attacked and photodegraded (Jori et al, 1970; Ochsner, 1997).

Table 4. Degradation of viral outer structures after mammalian viruses and bacteriophages PDI.

Virus	Type of damage	PS	Reference
Enveloped-mammalian viruses			
HSV-1	Viral envelope (reduced ability to adhere to and penetrate host cells)	Merocyanine 540	O'Brien et al, 1992
	Viral envelope (prevention of viral adsorption and host penetration)	Phthalocyanine derivatives	Malik et al, 1993
	Glycoprotein D; loss of proteins; dimerization; protein cross-links; alterations in protein molecular mass and charge	Phthalocyanine derivatives	Smetana et al, 1998
HSV-2	Viral envelope (prevention of viral adsorption and host penetration)	Phthalocyanine derivatives	Malik et al, 1993
HSV	Protein cross-links	Phthalocyanine derivatives	Malik et al, 1996
VZV	Viral envelope (prevention of viral adsorption and host penetration)	Phthalocyanine derivatives	Malik et al, 1993
HIV	Major capsid protein p24	Hypericin	Degar et al, 1992
HIV-1	Loss of infectivity; loss of fusion function; membrane proteins cross-links	Hypericin	Lenard et al, 1993
	Loss of infectivity; loss of fusion function; membrane proteins cross-links	Rose bengal	Lenard et al, 1993
	p24 and gp120 proteins; proteins cross-links	Methylene blue	Bachmann et al, 1995

	Inhibition of cell fusion activity of Env proteins	Natural and sulfonated Tetraarylporphyrins	Vzorov et al, 2002
VSV	Loss of infectivity; loss of fusion function; cross-linking of G and M proteins	Hypericin	Lenard et al, 1993
	Loss of infectivity; loss of fusion function; cross-linking of G and M proteins	Rose bengal	Lenard et al, 1993
	Inhibition of fusion of the envelope to Vero cells; G protein	Methylene blue	Abe and Wagner, 1995
	Inhibition of fusion of the envelope to Vero cells; G protein	Aluminum phthalocyanine tetrasulfonate	Abe and Wagner, 1995
	G and M proteins; protein cross-links	Phthalocyanine derivatives	Moor et al, 1997
	G, M, L and N proteins; protein cross-links	Chlorophyll derivatives	Lim et al, 2002
Influenza virus	Loss of infectivity; loss of fusion function; cross-linking of G and M proteins	Hypericin	Lenard et al, 1993
	Loss of infectivity; loss of fusion function; cross-linking of G and M proteins	Rose bengal	Lenard et al, 1993
	Loss of infectivity; HA fusion protein; protein cross-links	Rose bengal	Lenard and Vanderoef, 1993
Sendai virus	Loss of infectivity; loss of fusion function; cross-linking of G and M proteins	Hypericin	Lenard et al, 1993
	Loss of infectivity; loss of fusion function; cross-linking of G and M proteins	Rose bengal	Lenard et al, 1993
Vaccinia virus	Histidine residues in virus proteins	Rose bengal	Turner and Kaplan, 1968
Human cytomegalovirus	Viral envelope (reduced ability to adhere to and penetrate host cells)	Merocyanine 540	O'Brien et al, 1992
Sindbis virus	Viral envelope (reduced ability to adhere to and penetrate host cells)	Merocyanine 540	Sieber et al, 1987
	Viral capsid protein	Hypericin	Yip et al, 1996
Friend erythroleukemia virus	Viral envelope (reduced ability to adhere to and penetrate host cells)	Merocyanine 540	Sieber et al, 1989
Non-enveloped mammalian viruses			
Adenovirus	not damaged	Phthalocyanine derivatives	Malik et al, 1993
Enterovirus 71	Appearance/disappearance of protein bands; increase of the protein bands' intensity	Methylene blue	Wong et al, 2010
Non-enveloped bacteriophages			
T7 phage	Protein capsid; loosening of the protein-DNA interaction	Glycoconjugated *meso*-Tetraarylporphyrins	Gábor et al, 2001
	Capsid and core proteins; loosening of protein-DNA interaction	Glycoconjugated *meso*-Tetraarylporphyrins	Egyeki et al, 2003
	Capsid proteins; protein cross-links	*meso*-Tetrakis(1-methylpyridinium-4-yl)porphyrin	Zupán et al, 2008
	Capsid proteins; protein cross-links	Polyhydroxylated fullerene	Hotze et al, 2009

M13 phage	Coat protein	Methylene blue	Abe and Wagner, 1995
	Coat protein	Aluminum phthalocyanine tetrasulfunate	Abe and Wagner, 1995
PRD1 phage	Capsid proteins; protein cross-links; phospholipids (less affected)	Polyhydroxylated fullerene	Hotze et al, 2009
Qβ phage	Coat and maturation (A) proteins; formation of protein carbonyls; RNA-protein cross-links	Methylene blue	Schneider et al, 1998
	RNA-protein cross-links	Methylene blue	Floyd et al, 2004
MS2 phage	A protein	Polyhydroxylated fullerene	Hotze et al, 2009

4.2.1. Damage on Mammalian Viral Outer Structures

It has been shown that enveloped viruses can be inactivated due to protein damage (Lenard et al, 1993; Malik et al, 1993; Moor et al, 1997; Smetana et al, 1998). However, while the same treatment is reported to be ineffective against some non-enveloped viruses (Malik et al, 1993; Smetana et al, 1998), the results from Wong et al (2010) showed that even a non-enveloped virus can be efficiently inactivated due to the damage induced by PDI to its viral proteins (Table 4).

The proteins in the viral envelope of HSV-1 were considered to be major targets of merocyanine 540 photosensitization (O'Brien et al, 1992). Some phtahlocyanine derivatives have been shown to induce cross-links in HSV protein that might be responsible for the observed loss of infectivity (Malik et al, 1996). Protein analysis by SDS-PAGE, after treatment with phthalocyanine derivatives, revealed irreversible changes in the HSV-1 envelope proteins, which were reflected by the loss of many proteins, the appearance of cross-linked material on the top of the gel and by alterations in the molecular mass and molecular charge of the proteins. These alterations contribute, in all likelihood to HSV-1 inactivation (Smetana et al, 1998).

In VSV treated with 3.75–30 μL mL^{-1} of chlorophyll derivatives and light, the M protein band was not detected, which was accompanied by a decrease in the intensity of the G protein band (Lim et al, 2002). Large complexes of proteins were also detected on the top of the gel, indicating that viral PDI cross-linked the proteins (Lim et al, 2002). Using a fusion assay and protein analysis, it was shown that MB and $AlPcS_4$ caused a decrease in the intensity of the G-protein (which is known to play a crucial role in binding VSV to the host cell) band and a slight decrease in the intensity of M protein (matrix protein) band and protein cross-links. However, the observed damage in viral proteins could not account for VSV PDI (Moor et al, 1997). VSV was inactivated by MB and phthalocyanine derivatives, which inhibited the fusion of the virus envelope to Vero cells. However, the degree of this inhibition was small compared to the extent of virus inactivation (43% inhibition *vs.* 4.7 log or 99.998% inactivation, for MB) (Abe and Wagner, 1995). Abe and Wagner (1995) also found few changes in the relative abundance of VSV G protein after MB and AlPcS4 phototreatment, and they also observed additional protein bands on SDS-PAGE analysis (Abe and Wagner, 1995). It was found, by Western blot analysis, that HIV-1 p24 and

gp120 proteins were altered in size, possibly due to cross-linking, after MB phototreatment (Bachmann et al, 1995). However, using the same PS, $AlpcS_4$ and MB, no changes in protein patterns after SDS-PAGE of the viral proteins were observed, under conditions that caused complete VSV inactivation (Melki et al, 1994).

The results from Vzorov et al (2002) indicated that the porphyrins inhibited the cell fusion activity of HIV Env proteins (a biological function that is important for viral entry as well as induction of viral cytopathic effects) when expressed from recombinant vectors. These results showed that the viral Env protein is an important target of these compounds (Vzorov et al, 2002).

PDI of influenza virus by rose bengal altered the HA fusion protein and led to protein cross-links (Lenard and Vanderoef, 1993).

Photoinactivation of vaccinia virus with rose bengal significantly altered the concentration and oxidized histidine in vaccinia virus protein, suggesting that inactivation was attributed to alterations in viral proteins, as opposed to nucleic acids (Turner and Kaplan, 1968).

Treatment of influenza and Sindbis viruses by hypericin (Lenard et al, 1993), lead to an extensive cross-linking of the envelope proteins, which may have impaired the capacity of the viruses to adhere to and penetrate the host cells.

The protein profile of the non-enveloped enterovirus 71 was considerably altered after a low dose PDI and a MB concentration ≥ 0.5 μM, as revealed by a smearing and the disappearance of several protein bands (Wong et al, 2010). However, enterovirus 71 PDI was also due to damages in the viral genome (Wong et al, 2010).

4.2.2. Damages on Bacteriophage Outer Structures

In spite of the limited available data for enveloped bacteriophages, substantially higher photoinactivation rates compared with other non-enveloped phages were described (Lytle et al, 1991). The photoinactivation by merocyanine 540 of four bacteriophages, two non-enveloped phages without lipids (phi X174 and T7), a non-enveloped phage with lipids (PRD1), and an enveloped phage with an external lipoprotein envelope (phi 6) was studied by Lytle et al (1991). The survival curves of the different viruses clearly demonstrated different levels of sensitivity to photoinactivation by this PS, with phi 6 being the most sensitive, followed by T7 (21-fold less sensitive). While both PRD1 and phi 6 have lipid components, only phi 6 was photoinactivated by the PS. Thus, the internal lipid components of PRD1 were not sufficient to allow photoinactivation by merocyanine 540. A higher inactivation rate with a fullerene derivative was also observed by Hotze et al (2009) for a phage without lipids (T7 phage) than for PRD1 phage. The dissimilarities in phage composition resulted from differential resistance to singlet oxygen by the outer structures, since PRD1 has a double capsid with an internal lipid membrane, whereas T7 has a single proteinaceous capsid lacking lipids, and both phages contain double stranded DNA with similar GC content (48% for T7 and 51% for PRD1) (Hotze et al, 2009). Phage proteins were significantly affected by photosensitization (30–92%) when compared to the relatively smaller effect on

nucleic acids in both PRD1 and T7, and lipids in PRD1 phage (≤ 13%), as assessed by FTIR spectra analysis (Hotze et al, 2009). The higher T7 phage inactivation is consistent with greater damage to its proteinaceous capsid. Besides this, SDS-PAGE analysis further evidenced that oxidative cross-linking of capsid proteins induced by exogenous singlet oxygen is the likely cause of phage inactivation (Hotze et al, 2009). The high propensity for MS2 phage inactivation by this PS (compared to PRD1 and T7 phages) possibly arises from damage to its A protein, which is necessary for infecting its host *Escherichia coli* since it contains highly reactive amino acids such as methionine, cysteine, histidine, and tyrosine and not to damages to the nucleic acid (Hotze et al, 2009). Glycosylated substituted porphyrins led to structural changes at the protein capsid and/or loosening of the protein-DNA interaction, which can be responsible for T7 phage inactivation (Gábor et al, 2001). Besides of the alteration of the DNA structure, the phototreatment pointed to significant alterations in the protein structure and/or in the DNA-protein interaction, which may be the cause of photodynamic inactivation (Egyeki et al, 2003; Zupán et al, 2008). The alterations in the DNA secondary structure might also be the result of photochemical damage in phage capsid proteins and consequent disruption of the phage particle. Photomodification of core proteins can also lead to phage inactivation, even if the primary structure of the DNA part is preserved, since these proteins play an important role in the early events of infection and DNA penetration (Egyeki et al, 2003). The damage of T7 nucleoprotein is a complex process and clearly both phage DNA and protein capsid are affected by photoreactions (Zupán et al, 2008). Irradiation of Qβ bacteriophage in the presence of increasing concentrations of MB resulted in exponentially increasing amounts of viral RNA-protein cross-linkage products, and this is probably the most important event in viral inactivation (Floyd et al, 2004). The RNA genome of Qβ bacteriophage contained sufficiently lethal lesions following MB plus light exposure to account for the resulting phage inactivation. Nevertheless, the data also indicate that the protein component of the phage somehow contributes to the inactivation of the phage (Schneider et al, 1999). The protein component of Qβ phage is involved in the process of photoinactivation because the formation of protein carbonyls and RNA-protein cross-links were efficiently formed by MB plus light exposure (Schneider et al, 1998). The close correlation of cross-link formation with phage inactivation and the expectation that even one such cross-link in a phage genome would be lethal makes the RNA-protein cross-link lesion a strong candidate for the primary inactivating lesion of Qβ phage exposed to MB and light (Schneider et al, 1999).

Little alteration of M13 phage proteins on SDS-PAGE after MB and $AlPcS_4$ photoinactivation was observed by Abe and Wagner (1995). The results of Zupán et al (2004), suggested that the tetracationic porphyrin *meso*-tetrakis(1-methylpyridinium-4-yl)porphyrin did not interact with capsid proteins and did not disturb protein-DNA interaction, even if it has a strong stabilization effect on the intraphage DNA.

5. Resistance to PDI and Recovery of Viability

The development of increasing numbers of antiviral agents over the past decades, in the same way as with antibiotics, has provided the clinician with therapeutic options previously unavailable. With the increasing utilization of antiviral drugs, however, has come an enhanced appreciation of the development of antiviral resistance (Malik et al, 1982; Kimberlin and Whitley, 1996; Pillay, 1998; Yoshikawa, 2002; Jori and Brown, 2004; Maisch et al, 2004). Drug resistance is costly to the health service, to the patient who fails to gain maximum therapeutic benefit, and for the community in which resistant viruses may be spread (Pillay and Zambon, 1998).

There is now an urgent need for the development of novel, convenient and inexpensive measures for combating antimicrobial-untreatable infections and limiting the development of additional antimicrobial resistant microorganisms. Photodynamic technology may provide one approach to meet this need, both in terms of therapy and in terms of sterilization, by a mechanism that is markedly different from that typical of most antimicrobials (Reddi et al, 2002; Jori and Brown, 2004; Jori and Coppellotti, 2007).

As mentioned before, photosensitization involves the generation of singlet oxygen and free radical species, which cause molecular damage. Whether microorganisms could develop resistance to these active oxygen species is still questionable (Minnock et al, 2000) and, consequently, the development of microbial resistance to photosensitization is still under debate. Until now, the development of microbial resistance to PDI is not known and is thought very improbable to be developed. In general, the development of resistance to PDI by microbial strains should be considered as an unlikely event since this process is typically multi-target, with ROS causing damage to many microbial components, which is at a variance with the mechanism of action of most antimicrobial drugs (Maisch et al, 2004; Demidova and Hamblin, 2005; Jori et al, 2006). In contrast to most common antimicrobials, the number of molecular alterations required to ensure survival would be too great and the microorganism would require multi-site mutations to become highly resistant, an event with significantly lower probability than single-site mutations, which is often sufficient for conferring resistance to small-molecule inhibitors (Wainwright, 2009; Cheng et al, 2010). This particular property of microbial PDI is important regarding the repeated treatment of chronic and/or recurrent infections (Maisch et al, 2004).

Microbial PDI, when compared to standard treatments which may require application for several weeks to achieve an effective killing of the microorganism, shortly after initiation of light exposure, exhibits serious and irreversible damage of microorganisms (Costa et al, 2008; Costa et al, 2011). This damage does not allow the creation or operation of any kind of anti-drug or mutagenic mechanism. Microbial PDI is therefore very effective and, up until now, no photosensitization-resistant mutants have been found (Costa et al, 2011).

5.1. Resistance of Mammalian Viruses and Recovery of Viability after Photosensitization

Data from North et al (1994) show that HIV azidothymidine (AZT)-resistant strains were as susceptible as the AZT-sensitive ones to photosensitization with a benzoporphyrin derivative. This

finding comes as no surprise since the mechanisms of action of AZT (inhibition of reverse transcription) and light-activated benzoporphyrin derivative are different. Thus, mutations in the virus that occur at the reverse transcriptase level will not affect photodynamic destruction (North et al, 1994).

Studies focusing on the possible development of viral resistance are extremely scarce and little is known about the recovery of viral viability after consecutive photodynamic treatments.

5.2. Bacteriophage Resistance and Viability Recovery after Photosensitization

Concerning bacteriophages, there is only one study focusing on the possible development of viral resistance after photosensitization (Costa et al, 2011). After 10 consecutive cycles of photodynamic treatment,

the T4-like phage, in the presence of the tricationic porphyrin 5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin (Tri-Py^+-Me-PF) at 5.0 μM under white light irradiation, exhibited no changes in the rate of photoinactivation during the course of the experiments, meaning that no resistance was observed. If phage resistance would occur, important reductions on phage photoinactivation efficiency would be detected between experiments. Besides that, T4-like phage did not recover its viability after exposure to Tri-Py^+-Me-PF during 120 min of irradiation (Costa et al, 2011). In a preliminary study by Perdrau and Todd (1933), all attempts at reactivating the inactivated *Staphylococcus* phage by MB were unsuccessful.

6. Factors Affecting Viral PDI

6.1. Effect of the Number of Charges, Symmetry, Size of Meso Substituent Groups and Photosensitizer Concentration

It has been shown that the location and binding site of the PS, which is highly dependent on the structure and intramolecular charge distribution, is an important factor in microbial PDI (Merchat et al, 1996; Minnock et al, 2000).

In terms of molecular structure, molecular charge is important in determining antimicrobial activity. Positively charged PS are generally more efficient and can act at lower concentrations than neutral and anionic PS molecules (Demidova and Hamblin, 2005). The positive charges on the PS molecule appear to promote a tight electrostatic interaction between the positively charged PS and the negatively charged sites at the viral capsids and envelopes, orientating the PS toward sites which are critical for the stability and metabolism of a particular microorganism (Merchat et al, 1996; Dowd et al, 1998; Casteel et al, 2004). This kind of association increases the efficiency of the photoinactivation process.

Cationic PS photodamage can be induced in nucleic acid or viral outer structures by PS binding or by PS localized in its vicinity (Zupán et al, 2004). For instance, it is more likely that positively charged

PS will be effective in causing nucleic acid damage than will neutral or anionic congeners, which mainly act against the outer side of the microorganism (Wainwright, 2004).

The symmetry and the size of the chain of *meso* substituent groups also affect the photodynamic effect. PS with opposite charged groups are more symmetrical than PS with adjacent charged groups. The adjacent positive charges in the PS macrocycle should result in a molecular distortion due to electrostatic repulsion (Kessel et al, 2003). The toxicity of a PS can be modulated by the introduction of selected substituents on the macrocycle periphery. In this way, the physicochemical properties of a synthetic PS can be manipulated in order to enhance its interactions with the structural features of the viruses, such as viral capsids, and to minimize the interactions with plasma membranes or mammalian cell membranes (Casteel et al, 2004).

The amphiphilic nature of a PS is another important feature affecting PDI efficiency and can be modulated by the introduction of adequate functionalities in the macrocycle periphery, such as different numbers of positive charges, an asymmetrical charge distribution, or introduction of aromatic hydrocarbon side chains (Banfi et al, 2006; Almeida et al, 2011).

PS concentration is also an important parameter that must be taken into account since viral PDI was shown to be strongly influenced by PS concentration. Increasing the PS concentration reduces the time needed to achieve complete viral inactivation, thus increasing the efficiency of a particular PDI protocol (Costa et al, 2008).

6.1.1. Mammalian Viruses PDI

Complete inactivation of VSV (4.2 log) can be obtained by treating it with 1.0 μM of the anionic phthalocyanine derivative $AlPcS_4$ and 5 min illumination with red light. For the neutral phthalocyanine derivative (Pc_4), complete inactivation (4 log) was achieved using a much lower amount of PS (4.5 nM) in combination with 10 min illumination (Moor et al, 1997). The inactivation of VSV in PBS showed a linear relationship with illumination time (Moor et al, 1997). Inactivation of the fusion activity of VSV, influenza and Sendai viruses was reached with nanomolar concentrations of hypericin and rose bengal and was absolutely dependent upon light and increased with increasing time of illumination (Lenard et al, 1993). HAV in PBS or plasma was completely inactivated within 10 min (>3.7 log) by the cationic symmetric porphyrin *meso*-tetrakis(1-methylpyridinium-4-yl)porphyrin. In contrast, inactivation of HAV to 3.6 log with the anionic symmetric porphyrin *meso*-tetrakis(4-sulfonatephenyl)porphyrin required 90 min (Casteel et al, 2004). The rate and extent of inactivation appeared to vary with the nature of the *meso* substituent groups (Casteel et al, 2004). HIV and VSV lost infectivity upon illumination with hypericin and rose bengal in a concentration-dependent manner (Lenard et l, 1993).

6.1.2. Bacteriophage PDI

MS2 phage inactivation has been observed with neutral porphyrin derivatives. However, this required higher irradiation periods (30 min) than for the cationic ones (1 min) (Casteel et al, 2004). Neutral glycosylated substituted porphyrins can also significantly photoinactivate the T7 phage (Gábor et al, 2001; Egyeki et al, 2003). The T4-like phage PDI was achieved by exposing the phage in the presence of six cationic porphyrins at different concentrations (0.5, 1.0 and 5.0 μM) to white light for 270 min. The results showed that phage photoinactivation varied according with the PS concentration, with higher concentrations being the most efficient ones (Costa et al, 2008). The T4-like phage PDI also varied with the number of porphyrin charges, with tri- and tetracationic porphyrin derivatives being more effective in viral inactivation that the dicationic ones, which inactivated the phage below the limit of detection. Tetra- and tricationic porphyrin derivatives (*meso*-tetrakis(1-methylpyridinium-4-yl)porphyrin and 5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin, respectively) lead to complete T4-like phage inactivation (~7 log) after 270 min of irradiation with 40 W m^{-2} (Costa et al, 2008). This tetracationic porphyrin showed similar results in another study (7 log of reduction) for lambda phage inactivation, when irradiated with light of 658 nm (Kastury and Platz, 1992). Increasing porphyrin concentration at a fixed light dose leads to increased viral inactivation (Kastury and Platz, 1992). A concentration-dependent effect was also detected with a porphyrin derivative (Egyeki et al, 2003), but over 2.0 μM of PS the process was saturated. A further increase in porphyrin concentration did not lead to a higher inactivation rate of T7 phage. Aggregation and/or photobleaching of PS are likely explanations (Egyeki et al, 2003). Cationic *meso*-tetrakis(1-alkylpyridinium-4-yl)porphyrin derivatives with different alkyl substituent groups were tested for MS2 phage inactivation but, with the exception of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin, showed toxicity even in the absence of light (Casteel et al, 2004).

In a study conducted by Gábor et al (2001), the porphyrin derivative with symmetrical glycosylated groups was found to be twice as effective as the asymmetrical one on the inactivation process of T7 phage. According to Costa and colleagues (2008), the rate of T4-like phage inactivation was also dependent on the lipophilic character of the *meso*-substituent groups. The presence of a lipophilic aryl group in one of the *meso* positions of the porphyrin core appears to have an important role in phage inactivation, affecting the rate and efficiency of T4-like phage (Costa et al, 2008). Casteel et al (2004) have also observed differences in the photoinactivation rate of MS2 phage when they used PS with different alkyl substituent groups and concluded that the rate and extent of inactivation appeared to vary with the nature of the *meso* substituent groups.

6.2. Effect of Different Light Sources and Fluence Rate on Antimicrobial PDT

PDT requires a source of light to activate the PS by exposing it to visible or near-visible light at a specific wavelength (Konopka and Goslinski, 2007). The light source for PDT must also exhibit suitable spectral characteristics coinciding preferentially with the maximum absorption wavelength range of the PS, applied in order to generate enough ROS to produce an efficient toxic effect (Robertson et al, 2009).

In parallel with the advances in chemistry (related with the discovery and synthesis of new and more efficient PS) there has also been much activity in developing new light sources, better suited for the photosensitization process. Briefly, these include user-friendly lasers frequently based on solid state laser diodes, as well as inexpensive light emitting diodes (LED) and filtered broad-band lamps (Brancaleon and Moseley, 2002).

PS activation has been achieved *via* a variety of light sources, such as arc plasma discharge lamps, metal halogen lamps, slide projector illumination assemblies, and a variety of lasers. For treatment of larger areas, non-coherent light sources, such as tungsten filament, quartz halogen, xenon arc, metal halide, and phosphor-coated sodium lamps, are in use. Recently, non-laser light sources, such as LED, have also been applied in PDT. These light sources are much less expensive and small, lightweight and highly flexible, its lifetime can reach up to one hundred thousands hours, and can be manufactured to wavelengths that activate commercially available PS (Veenhuizen and Stewart, 1995; Allison et al, 2004; Juzeniene et al, 2004; Kübler, 2005; Konopka and Goslinski, 2007).

At first glance, the available literature on fluence rate effects for PDT seems contradictory. Some studies indicate less damage at low fluence rate, others indicate more killing at lower, compared to higher, fluence rates for the same total fluence and some indicate no influence of fluence rate at all (Juzeniene et al, 2004; Kübler, 2005; Konopka and Goslinski, 2007). A reduction in the fluence rate lowers the rate of oxygen consumption, thereby extending the radius over which singlet oxygen may be formed and consequently increasing the phototoxic effect (Lukšiene, 2005). Qin et al (2008) showed that an increase in the fluence rate increases microbial damage, although, it seems to have an upper limit of photons to observe this effect. Since each PS molecule can only absorb one photon at a time, when the number of light photons bypasses the number of PS molecules, the PS will no longer be able to absorb the photons "in excess" and the rate of PDI will not increase. In fact, if the number of photons is higher than this limit, the antimicrobial effect will decrease because the dye in suspension will not absorb all the excess light (Qin et al, 2008). Schindl et al (2001) referred that the biological effect of light depends on the fluence, irrespective of the time over which this dose is delivered. Maclean et al (2008) also indicate that the inactivating light may be applied at high irradiance over a short time or at lower irradiance over a longer time. A numerical model, assuming that the rate of photodynamic damage occurring at time t is proportional to the fluence rate at that time and the local concentrations of PS and oxygen can be established. However, according to this model, relatively low fluence rates can be nearly as effective as high fluence rate sources if applied over the same period of time (Langmack et al, 2001).

There is also a direct correlation between the phototoxic effect and the PS concentration and light fluence. With a lowering of the PS concentration, more light has to be applied to achieve identical effects, and *vice versa*. Lower doses of PS require higher activating light fluences, and higher fluence requires a longer duration of light application (Schmidt-Erfurth and Hasan, 2000).

6.2.1. Effect of Light on Mammalian Viruses PDI

The effects of dengue virus inactivation were increased with the increase of MB concentration, the enhancement of power density of the light source and the extension of illumination time, as well as the decrease of illumination distance. This enabled the narrow bandwidth light system to kill or inactivate the enveloped virus at much greater distance in much shorter time (Huang et al, 2004). VSV in the presence of MB was rapidly inactivated by red (provided by LED incident light at 272 W cm^{-2}) or green-yellow light (provided by low-pressure sodium lamps at a fluence rate of 165 W cm^{-2}) but slower by white light (provided by a bank of fluorescent tubes at a fluence rate of 42 W cm^{-2}) (Mohr et al, 1997), showing that higher power densities produce a high rate of viral inactivation than low fluence rates. Wagner et al (1993) also showed that red light of 9 W m^{-2}, given at a total dose of 1.8×10^4 and 3.2×10^4 J m^{-2}, inactivated MB-treated VSV by 6 and ≥ 7 log, respectively. VSV inactivation was linearly dependent on the fluence rate of red light illumination (Wagner et al, 2002).

6.2.2. Effect of Light on Bacteriophage PDI

In terms of what is known about phage PDI, only one study focusing on the effect of different light sources and power densities (Costa et al, 2010) exists. In this study, cationic porphyrin derivatives (*meso*-tetrakis(1-methylpyridinium-4-yl)porphyrin and 5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin), when irradiated with different sources of light (fluorescent PAR lamps, sun light and halogen lamp) with fluence rates ranging from 40 W m^{-2} to 1690 W m^{-2}, efficiently photoinactivated non-enveloped phages. All light sources tested lead to reductions of about 7 log for the somatic T4-like phage. However, the rate and the extent of inactivation were dependent on the light source, namely when low fluence rates were used (40 W m^{-2}) and on the energy dose, being considerably more effective when light was delivered at a lower fluence rate. However, depending on the light source used, different irradiation periods were required to inactivate T4-like phage to the limits of detection. The results also showed that the efficacy of T4-like phage inactivation, using the same fluence rate, was dependent on the light source used, in particular when the light is delivered at a low fluence rate. M13 phage was phototreated with 5.0 μM MB and was inactivated in an irradiation dose-dependent manner (Abe et al, 1997). Kastury and Platz (1992) showed that increasing the concentration of a PS at a fixed light dose leads to increased viral inactivation as does an increase in the total light exposure at a fixed PS concentration. The inactivation rate of T1 bacteriophage increased with increasing fluence rate, indicating that the distance of the sample from the light source is a variable which must be controlled (Welsh and Adams, 1954). At higher PS concentrations, the inactivation rate reaches a maximum and then decreases, because the filtering effect of the dye decreases the effective fluence rate (Welsh and Adams, 1954). In a simple model purposed by Lee et al (1997), the phage survival ratio can also be considered as a decreasing exponential fraction of the light fluence (assuming that the fluence is uniform throughout the system).

7. Conclusion

The efficiency of different types of PS in viral PDI has been proved for different types of mammalian viruses and bacteriophages, whether they are enveloped or non-enveloped, for either DNA or RNA viruses. Even though enveloped viruses are more easily inactivated than non-enveloped ones, several studies confirm that non-enveloped mammalian viruses and phages can be efficiently inactivated by PDI. The type of viral nucleic acid has not been described as an important factor affecting viral photoinactivation but, as far as it is known, no studies specifically focus on the photoinactivation behaviour of DNA and RNA viruses. However, RNA phage MS2 was highly susceptible to photoinactivation when compared with DNA phages under the same conditions of photosensitization.

The type of mechanisms involved in the process of viral photosensitization was already elucidated and singlet oxygen and free radical species were identified as important contributors for an effective viral PDI. However, the contribution of singlet oxygen seems to be more pronounced in mammalian viruses and bacteriophages PDI. There are, however, few studies simultaneously comparing the contribution of both types of mechanisms (type I and type II) involved in viral PDI. The primary targets for the photoinactivation of viruses, whether treating mammalian viruses or phages, are the outer structures. Although there are several studies about the specific effects of PDI on viral proteins, for different types of mammalian viruses and phages, there are no studies concerning the specific effects of PDI on viral lipids. However, it has been clearly shown that enveloped viruses are more easily inactivated than their non-enveloped counterparts, which imply that the lipids present on viral envelopes are important targets of viral PDI.

PS are effective in inactivating the phages to the limits of detection in a way that they do not recover viability, avoiding the development of viral resistance. Nothing is known yet for the particular case of mammalian viruses but, as the viral targets are the same for mammalian viruses and phages, it is also expected that no resistance will be developed in the case of mammalian viruses. Besides that, viral PDI is equally effective whether the mammalian virus is sensitive or resistant to conventional antiviral agents. Taking into account all these advantages, PDI for viral inactivation can be regarded as a promising alternative therapy to conventional antiviral treatments, namely for the disinfection of blood and blood products, preventing viral contamination and for the treatment of wound and burn infections. Viral PDI has a fast mode of action and has also the additional benefits of being more economical and an environmental friendly technology, which might be successfully used also in the environmental field for wastewater, drinking water and fish-farming water disinfection.

Different PS concentrations and different light sources and fluence rates were tested, showing that they are important PDI parameters that must inevitably be taken into account when a viral photosensitization protocol has to be elaborated. The inactivation of mammalian viruses and phages can be attained at micromolar-level PS concentrations and different light sources are equally effective, depending on the final dose at which the viruses are exposed to. Besides that, PS can also be modulated

by the addition of different *meso* substituent groups and positive charges in order to facilitate their interactions with the viruses, making them more efficient for mammalian viruses and phages PDI.

The similarity of the results obtained for mammalian viruses and bacteriophages show that they exhibit a similar behaviour when submitted to viral photoinactivation techniques: (i) the PS used for viral PDI were equally effective in the photoinactivation of mammalian viruses and bacteriophages; (ii) the mechanism of mammalian viruses and bacteriophage photosensitization involves the production of singlet oxygen (type II mechanism) with a slight contribution of free radical species (type I mechanism); (iii) singlet oxygen and free radicals were shown to affect viral nucleic acids and also the proteins and lipids present in the mammalian viruses and bacteriophages outer surfaces, with the latter being considerably more affected by PDI; and (iv) the rate and extent of mammalian viruses and phages PDI is also affected by the same factors, like the PS concentration and number of positive charges, the nature and position of *meso* substituent groups, the fluence rate and energy dose. Consequently, it is important to persist in the development of more PDI phage studies to clarify some aspects of viral PDI, such as influence of viral nucleic acid type (DNA or RNA) in the photoinactivation efficiency and the possibility of viral resistance development and viability recovery after photosensitization. It will also be important to study the synergistic effect between viral PDI and antiviral classical methodologies using bacteriophages as models of mammalian viruses' photoinactivation.

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CHAPTER 2

Sewage bacteriophage inactivation by cationic porphyrins: influence of light parameters

Abstract

Photodynamic therapy has been used to inactivate microorganisms through the use of targeted photosensitizers. Although the photoinactivation of microorganisms has already been studied under different conditions, a systematic evaluation of irradiation characteristics is still limited. The goal of this study was to test how the light dose, fluence rate and irradiation source affect the viral photoinactivation of a T4-like sewage bacteriophage. The experiments were carried out using white PAR light delivered by fluorescent PAR lamps (40 W m^{-2}), sun light (600 W m^{-2}) and a halogen lamp (40–1690 W m^{-2}). Phage suspensions and two cationic photosensitizers (Tetra-Py^{+}-Me and Tri-Py^{+}-Me-PF) at concentrations of 0.5, 1.0 and 5.0 μM were used. The results showed that the efficacy of the bacteriophage photoinactivation is correlated not only with the sensitizer and its concentration but also with the light source, energy dose and fluence rate applied. Both photosensitizers at 5.0 μM were able to inactivate the T4-like phage to the limit of detection for each light source and fluence rate. However, depending on the light parameters, different irradiation times are required. The efficiency of photoinactivation is dependent on the spectral emission distribution of the light sources used. Considering the same light source and a fixed light dose applied at different fluence rates, phage inactivation was significantly higher when low fluence rates were used. In this way, the light source, fluence rate and total light dose play an important role in the effectiveness of the antimicrobial photodynamic therapy and should always be considered when establishing an optimal antimicrobial protocol.

Introduction

As human population densities increase, it becomes more and more difficult to provide supplies of high-quality potable water from surface and ground water stocks, and the removal of harmful microorganisms, such as bacteria, viruses and protozoa, assumes greater significance. Although the transmission of microbial diseases has been reduced by the development of good water supplies and hygienic procedures for a whole range of human activities, it is still important to find novel, convenient, environmentally-friendly and inexpensive methods to avoid microbial contamination. Currently, photodynamic therapy is receiving considerable interest as a potential antimicrobial treatment. Photosensitizers, namely porphyrin derivatives, are promising chemical disinfectants for the inactivation of pathogens as they are effective in the presence of light and oxygen, without the formation of potentially toxic products (Jemli et al, 2002; Magaraggia et al, 2006; Carvalho et al, 2009).

Photodynamic antimicrobial phototherapy (PACT) has been proven to be a powerful method for inactivating viruses, such as murine retroviral vectors, human immunodeficiency viruses (HIV-1 and -2) (Schagen et al, 1999; Vzorov et al, 2002), hepatitis viruses (A and B) (Wagner et al, 2002; Casteel et al, 2004), vesicular stomatitis virus (VSV) (Horowitz et al, 1991), herpes simplex viruses (Silva et al, 2005; Tomé et al, 2005; Tomé et al, 2007), human papillomavirus (Wainwright, 2004) and influenza A (Lenard and Vanderoef, 1993). The effect of PACT on bacterial viruses (bacteriophages or simply phages), frequently used as indicators of enteric viruses and public health risk, has already been tested with success not only on collection phages (Abe et al, 1997; Lee et al, 1997; Wagner et al, 1998; Egyeki et al, 2003; Zupán et al, 2004; Embleton et al, 2005) but also on sewage bacteriophages (Costa et al, 2008; Almeida et al, 2009; Carvalho et al, 2009).

Several studies indicate that the physicochemical properties of the sensitizer, namely the light-absorption characteristics and the efficiency of singlet oxygen and free radicals production, have an impact on the efficacy of photosensitization (Reddi et al, 2002; Lukšiene, 2005; Lukšiene and Zukauskas, 2009). Photosensitization occurs when the photosensitizer (PS) is able to absorb and transfer the energy of the incident light to molecular oxygen leading to the formation of toxic species. However, it is recognized that in addition to the PS properties, the characteristics of the incident light must be considered and controlled to increase the efficiency of PACT (Prates et al, 2009). In the literature, light parameters are usually discussed in terms of light dose (also identified as fluence) (Kasturi and Platz, 1992; Jemli et al, 2002; Costa et al, 2008; Alves et al, 2009; Prates et al, 2009) but, as the restriction of the reciprocity rule to rather narrow limits for most photobiological reactions, the dose–time relationship (fluence rate) should also be taken into account (Schindl et al, 2001).

A very wide selection of light sources is available, ranging from thermal radiators, which are heated until they start glowing and thus emit light (for instance, incandescent lamps) to luminescent radiators, which utilize the electronic excitation of atoms to emit light (for instance, gas discharge lamps, light emitting diodes (LED) and lasers) (Schmidt, 2005; Lukšiene and Zukauskas, 2009).

Recently we have shown that porphyrins with three or four positive charges are able to inactivate environmental non-enveloped viruses, allowing them to be used as a new, cheap and accessible technology for wastewater treatment (Costa et al, 2008). In order to establish the best conditions for an efficient photoinactivation of somatic bacteriophages, we have studied how phage inactivation, in the presence of efficient PS, is affected by: (i) light source, (ii) light dose and (iii) fluence rate. For this purpose, we selected the T4-like sewage bacteriophage, used as indicator of the presence of enteric viruses, and the photosensitizers 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^+-Me) and 5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py^+-Me-PF). Tri-Py^+-Me-PF is a tricationic porphyrin recently described by our group as a promising PS for the inactivation of several types of microorganisms and Tetra-Py^+-Me is the most extensively studied PS in bacterial and viral photoinactivation processes (Costa et al, 2008; Carvalho et al, 2009; Oliveira et al, 2009).

Material and Methods

Porphyrin synthesis

Porphyrins 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^+-Me) and 5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py^+-Me-PF) (Figure 1) used in this work were prepared in two steps as previously described (Maestrin et al, 2004; Tomé et al, 2004). First, the neutral porphyrins were synthesized by Rothemund and crossed Rothemund reactions, using pyrrole and the appropriate benzaldehydes (pyridine-4-carbaldehyde and pentafluorophenylbenzaldehyde) at reflux in acetic acid and nitrobenzene. Then, the resulting porphyrins were purified by column chromatography (silica) and the pyridyl groups were quaternized by reaction with methyl iodide. Porphyrins were purified by crystallization from chloroform-methanolpetroleum ether and their purities were confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy. The spectroscopic data was in accordance with the literature (Maestrin et al, 2004; Tomé et al, 2004). Stock solutions (500 μM) of each porphyrin in dimethyl sulfoxide were prepared by dissolving the adequate amount of the desired porphyrin in a known volume. The absorption spectral features of the PS were the following: [porphyrin] *l*max nm (log e); [Tetra-Py^+-Me] in DMSO 425 (5.43), 516 (4.29), 549 (3.77), 588 (3.84), 642 (3.30); [Tri-Py^+-Me-PF] in DMSO 422 (5.48), 485 (3.85), 513 (4.30), 545 (3.70), 640 (3.14).

Phage selection and identification

A wastewater sample from a secondary-treated sewage plant of the city of Aveiro (Portugal) was used to select the somatic bacteriophages of *Escherichia coli* C (ATCC 13706) (Costa et al, 2008). DNA extraction and purification of phage suspension was done using standard techniques (Sambrook et al, 1989). The phage was identified as a T4-like phage that has 82% of homology with the Enterobacteria phage RB43 (Costa et al, 2008). The nucleotide sequence of the phage has been deposited in the GenBank database under accession n◦ EU026274.

Bacteriophage host viability test

As bacteria are sensitive to the photosensitizers, the viability of the viral host was evaluated in order to prove that the phage inactivation was only due to the photoinactivation by the photosensitizer and not due to bacterial host inactivation by the porphyrin derivatives. So, accordingly to the procedure described by Costa et al (2008) additional samples were collected in each sampling time after irradiation, with white light delivered by the set fluorescent PAR lamps with a fluence rate of 40 W m^{-2}. Then the samples were washed by ultra-centrifugation at 28.000 *g* for 90 min, at room temperature, to remove the photosensitizer (washed experiments). The photosensitizer-free pellet of phages was re-suspended in PBS buffer, serially diluted and pour plated by the double layer technique, using the agar double layer technique (Adams, 1959) and the aforementioned strain of *E. coli* as host (Costa et al, 2008). The results obtained were compared with those resulting from direct spread (non-washed experiments) after irradiation. This bacteriophage host viability test was done at the beginning of the work and only for the

most effective porphyrin (Tri-Py^{+}-Me-PF) at the highest concentration (5.0 μM). In the other experiments this step was not done, but the Petri dishes were incubated in dark conditions.

Light sources

The photodynamic effect of the cationic PS was evaluated by exposing the sewage somatic bacteriophage in the presence of PS to a set of fluorescent PAR lamps, halogen lamp and sun. The first light source is constituted by 13 fluorescent lamps OSRAM 21 of 18 W each one, PAR radiation (380–700 nm) with a fluence rate of 40 W m^{-2}. The second light source used is comprised of an illumination system (LC-122 LumaCare, London) equipped with a halogen 250 W quartz-type lamp and coupled to an interchangeable fibre optic probe (400–800 nm). This illumination system was used to irradiate the microcosm's setup with white light (400–800 nm) at fluence rates from 40 W m^{-2} to 1690 W m^{-2}. The desired fluence rate was obtained by variation of the distance of the fibre tip to the beakers. The experiments with solar irradiation were carried out outside the laboratory. Samples were exposed to solar PAR light on sunny summer days, with an average fluence rate of 600 W m^{-2}. The microcosm was covered with a glass Petri plate to filter the ultraviolet radiation. Only the PAR radiation of the solar spectrum was used in order to avoid viral inactivation by UV radiation during solar exposure and thus to allow the comparison of the results with those obtained with the two artificial lights. UV radiation would inactivate the viruses and, consequently, would increase the rate of phage inactivation during the exposition to the photosensitizers. All the fluence rates were measured with a radiometer LI-COR Model LI-250.

Experiments with different light sources

The influence of different light sources of white light on phage inactivation by the two cationic porphyrins at different concentrations was evaluated through quantification of the number of bacteriophages in laboratory conditions. A phage suspension was diluted in phosphate buffer (PBS) until 5 x 10^{7} PFU mL^{-1} and aseptically distributed in 600 mL acid-washed and sterilised glass beakers (20 mL per each of five beakers). The photosensitizer from a stock solution (500 μM in DMSO) was added to three beakers to achieve final concentrations of 0.5, 1.0 and 5.0 μM and the other two were used as dark and light controls. In the light control no porphyrin was added but the beaker was exposed to the same irradiation protocol. In the dark control, the photosensitizer at the highest concentration (5.0 μM) was added to the beaker and it was covered with aluminium foil. The test beakers and the light and dark controls were submitted to a pre-irradiation period in the dark during 10 min under 100 rpm stirring at 20–25 ºC. After the pre-irradiation period, the five beakers, under stirring (100 rpm) and in a thermostatized bath at 20–25 ºC, were exposed in parallel to white light radiation generated by the different light sources. Sub-samples of 1.0 mL were taken at regular intervals and analysed, in duplicate, for bacteriophage number. Since the irradiation sources had different fluence rates, it was also compared the efficacy of sewage T4-like bacteriophage inactivation using the same fluence rate delivered by different light sources. These experiments were carried out using the most efficient photosensitizer Tri-

Py^+-Me-PF at the highest concentration (5.0 μM) irradiated at 40 and 600 W m^{-2}. The Petri plates were kept in the dark immediately after spreading and during the incubation to avoid the inactivation of the bacterial host by the photosensitizer. Viral density (PFU mL^{-1}) was determined at each time point as the mean of the two duplicates in the most convenient dilution series. Viral survival at each time was calculated by dividing the mean number of viruses surviving at each time by the initial number (at time zero). Viral reduction was expressed as a log value. For each experimental condition (light source, photosensitizer and concentration) two independent experiments were carried out with two replicates each.

Experiments at different fluence rates with a fixed total light dose

To evaluate the influence of the light fluence rate on the bacteriophage inactivation, phage suspensions, incubated with the most effective porphyrin (Tri-Py^+-Me-PF), were exposed to the same energy dose delivered with four different light fluence rates ranging from 150 to 1200 W m^{-2}. An illumination system (LC-122 LumaCare, London) equipped with a halogen 250 W quartz-type lamp and coupled to an interchangeable fibre optic probe (400–800 nm) was used. The fibre tip was placed at the adequate distance from the beaker in order to deliver a fluence rate of 150, 300, 600 and 1200 W m^{-2}. The total light dose of 216 J cm^{-2} corresponded to irradiation periods of 30 min at 1200 W m^{-2}, 60 min at 600 W m^{-2}, 120 min at 300 W m^{-2} and 240 min at 150 W m^{-2}. Sub-samples of 1.0 mL were collected at established intervals corresponding to 54, 108, 144 and 216 J cm^{-2} light doses.

The rate of phage inactivation was evaluated through the quantification of the number of bacteriophages, using the above described protocol. Light and dark control experiments were also carried out simultaneously. For each irradiation period two independent experiments were carried out with two replicates each.

Statistical analysis

All experiments were done in duplicate. Statistical analysis was performed using SPSSWIN 14.0. The significance of difference in phage inactivation between the two photosensitizers' values was assessed using one-way ANOVA. The differences in phage inactivation during the incubation period were also evaluated using one-way ANOVA. Only the data with normal distribution (assessed by Kolmogorov-Smirnov test) and with homogeneity of variances (assessed by Levene test) were used. A value of $p < 0.05$ was considered significant.

Results

The phototoxic action on T4-like sewage bacteriophage by the two cationic porphyrin derivatives (Figure 1) was initially assessed by exposing the test assembly to different photosensitizer concentrations (0.5, 1.0 and 5.0 μM) and to white light from different light sources and fluence rates.

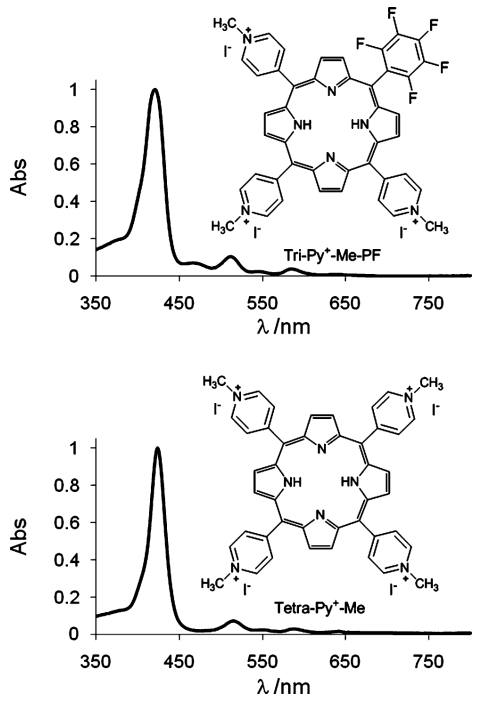

Figure 1. Structure and UV-Vis spectra (in DMSO) of the two cationic porphyrins used in this study for the photoinactivation of T4-like bacteriophage.

Bacteriophage host viability

The bacteriophage host viability test showed that the pattern of phage inactivation was not affected by the presence of photosensitizer during the incubation period (18 h) in the dark. The pattern of phage inactivation was similar in washed and in non-washed samples (Costa et al, 2008). This means that the photosensitizer does not affect the bacteria during incubation of the Petri plates in the dark and, therefore, the washing step by ultra-centrifugation, a time consuming procedure, was not carried out. However, in all the experiments, the Petri plates were kept in the dark during the incubation period.

Bacteriophage viability

Without light (dark control), the photosensitizers at the highest concentration (5.0 μM) did not exhibit activity against the phage for the exposure time (270 min). A similar trend was observed for the phage in the absence of the PS for all the irradiation periods with white light (light control) (Figures 2 to 6). It is important to note that the light used in the experiments did not affect viral viability. Inactivation was observed only when the phage was incubated with the photosensitizer and irradiated with the appropriate light dose.

Influence of light sources on bacteriophage inactivation

The efficiency of the sewage bacteriophage inactivation for the two cationic porphyrin derivatives, irradiated with selected light sources, was different. Although the two cationic porphyrins at 5.0 μM, when activated by all the light sources, were able to inactivate the sewage T4-like phage to the limit of detection, the phage photoinactivation with a halogen lamp occurred faster than with solar irradiation or with fluorescent lamps (Figures 2 to 4).

In fact, when the illumination system (halogen lamp) was used at a fluence rate of 1690 W m^{-2}, the Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me porphyrins, at a concentration of 5.0 μM, were able to inactivate the sewage T4-like phage by more than 7 log (Figure 2) after 25 and 45 min (data not shown), respectively. Under these light conditions, for a concentration of 1.0 mM, both photosensitizers produced a moderate phage inactivation after 30 min of exposure to light (2.2 and 1.3 log for Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me). For the lowest concentration of photosensitizers (0.5 μM) the inactivation varied between reductions of 0.8 to 1.3 log after 30 min of irradiation (Figure 2). With these light conditions (1690 W m^{-2}) the pattern of phage inactivation was similar for both porphyrins (ANOVA, $p > 0.05$) for all the concentration tested (Figure 2).

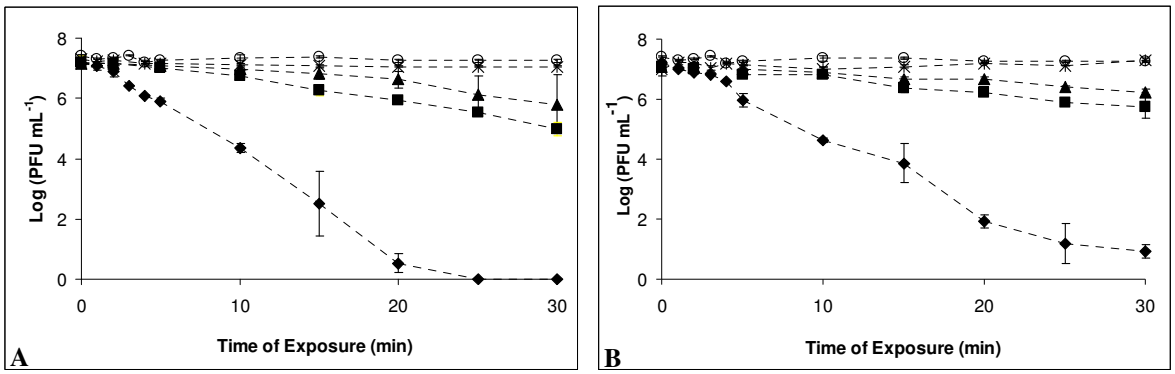

Figure 2. Variation of the numbers of the sewage bacteriophage after irradiation with the illumination system (halogen lamp) at 1690 W m^{-2} in the presence of Tri-Py^{+}-Me-PF (A) and Tetra-Py^{+}-Me (B), respectively (-○- light control, -✳- dark control, -▲- 0.5 μM, -■- 1.0 μM, -♦- 5.0 μM). Error bars represent standard deviations.

Under solar light (~ 600 W m^{-2}), T4-like phage was also efficiently photoinactivated (> 99.9999% of inactivation) with reductions of 7.2 log for Tri-Py^{+}-Me-PF (5.0 μM) after 90 min and 7.0 log for Tetra-Py^{+}-Me (5.0 μM) after 180 min of irradiation (data not shown). With 1.0 μM of sensitizer, the inactivation ranged from 3.1 to 0.8 log after 90 min of exposure for Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me, respectively. For concentrations of 0.5 μM, the reductions observed were 1.5 and 0.1 log (after 90 min), for Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me, respectively (Figure 3). With this light source, the pattern of phage inactivation was similar for Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me, but only for the highest concentration (5.0 μM) (ANOVA, $p > 0.05$). For the other concentrations (0.5 and 1.0 μM) the pattern of phage inactivation was different for both porphyrins (ANOVA, $p < 0.05$) (Figure 3).

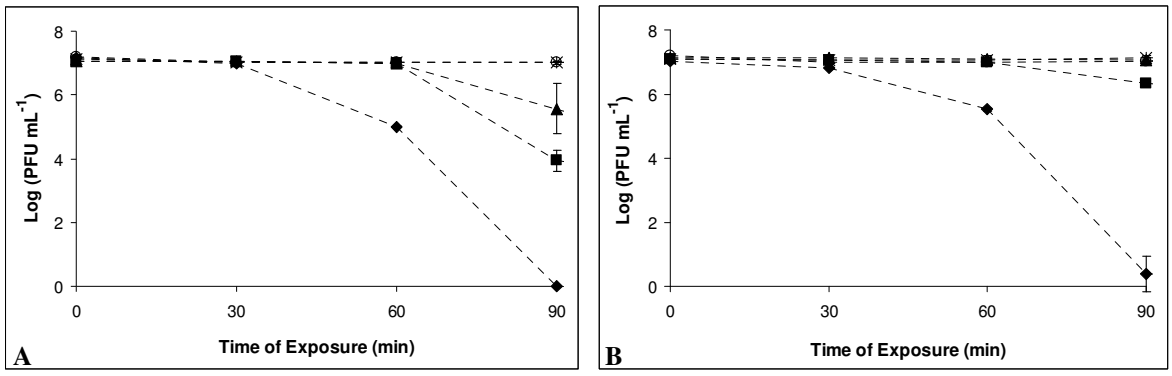

Figure 3. Variation of the numbers of the sewage bacteriophage after irradiation with solar light (600 W m^{-2}) in the presence of Tri-Py^+-Me-PF (A) and Tetra-Py^+-Me (B), respectively (-○- light control, -✳- dark control, -▲- 0.5 μM, -■- 1.0 μM, -♦- 5.0 μM). Error bars represent standard deviations.

Porphyrins irradiated with a set of 13 fluorescent lamps (40 W m^{-2}), at 5.0 μM, also inactivated the T4-like phage to the limit of detection with reductions of 7.0 log (after 180 min of irradiation) and 7.2 log (after 270 min of irradiation) for Tri-Py^+-Me-PF and Tetra-Py^+-Me, respectively. Again, for the lower concentrations (0.5 and 1.0 μM) the inactivation was moderate and ranged from reductions of 3.6 and 1.5 log for 1.0 μM and reductions of 1.8 to 0.9 log for 0.5 μM, respectively, for Tri-Py^+-Me-PF and Tetra-Py^+-Me, after 270 min of exposure to white light (Figure 4). Also, for both porphyrins, with this light source the pattern of phage inactivation was similar for the highest concentration (5.0 μM) (ANOVA, $p > 0.05$) and different for the other concentrations (0.5 and 1.0 μM) (ANOVA, $p < 0.05$) (Figure 4).

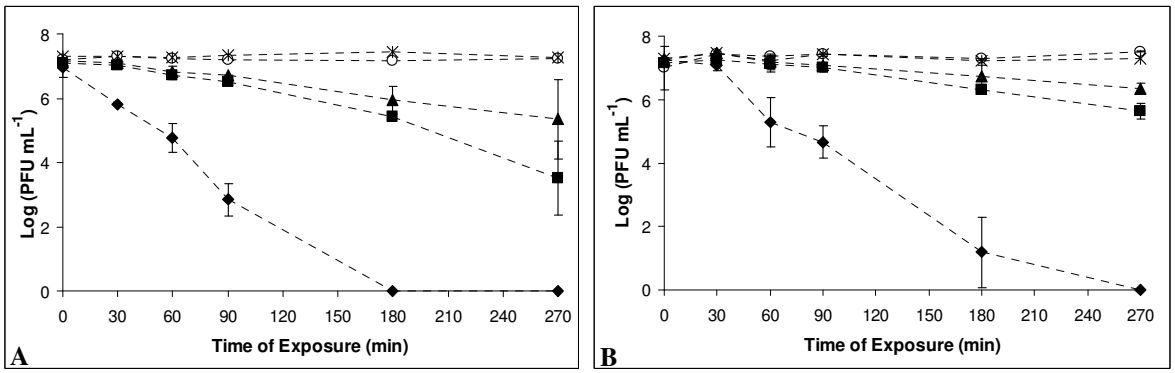

Figure 4. Variation of the numbers of the sewage bacteriophage after irradiation with the set of 13 fluorescent PAR lamps (40 W m^{-2}) in the presence of Tri-Py^+-Me-PF (A) and Tetra-Py^+-Me (B), respectively (-○- light control, -✳- dark control, -▲- 0.5 μM, -■- 1.0 μM, -♦- 5.0 μM). Error bars represent standard deviations.

The results of the comparison of the efficacy of sewage T4-like bacteriophage inactivation by Tri-Py^+-Me-PF using the same fluence rate delivered by different light sources are presented in Figures 5 and 6. The results show that the efficacy of phage inactivation at a low fluence rate (40 W m^{-2}) depends on the light source (Figure 5). However, at a high fluence rate (600 W m^{-2}) this dependence was not observed (Figure 6). At a fluence rate of 40 W m^{-2}, the phage was inactivated to the limit of detection (> 99.9999% of inactivation, corresponding to a reduction of 7.2 log) only when irradiated with fluorescent lamps (380–700 nm). With a halogen lamp coupled to an interchangeable fibre optic probe (400–800 nm), the rate of phage inactivation was considerably lower (reduction of 2.5 log) even after 270 min of irradiation

(Figure 5). However, the difference between the two sources was only significant for T180 and T270 (ANOVA, $p < 0.05$). At 600 W m^{-2}, the phage was inactivated to the limit of detection (> 99.9999% of inactivation, reduction of 7.2 log) after 90 min of irradiation with both light sources (solar irradiation and halogen lamp) (Figure 6). However, the pattern of inactivation for the two light sources was significantly different for T0 and T30 (ANOVA, $p < 0.05$ for T0 and T30 and $p > 0.05$ for T60, T90 and 120).

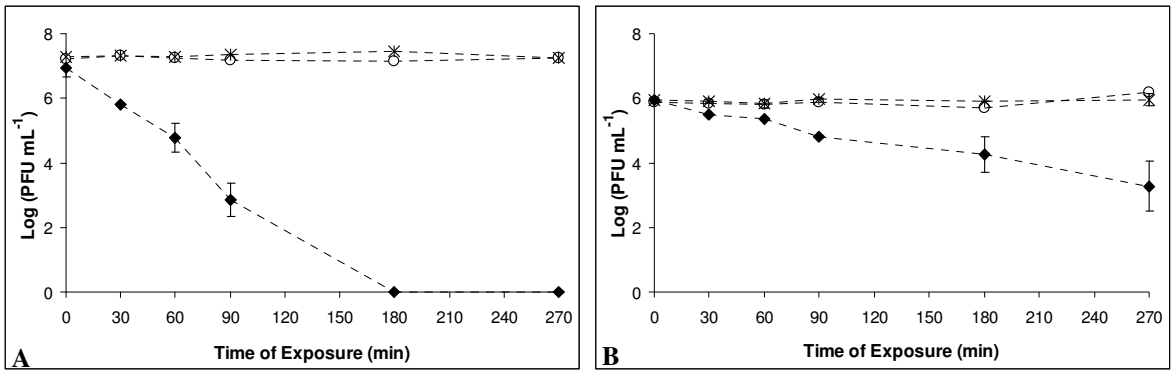

Figure 5. Variation of the numbers of the sewage bacteriophage after irradiation with the set of 13 fluorescent PAR lamps (A) and illumination system (halogen lamp) (B) at 40 W m^{-2} in the presence of porphyrin Tri-Py^{+}-Me-PF (-○- light control, -✳- dark control, -♦- 5.0 μM). Error bars represent standard deviations.

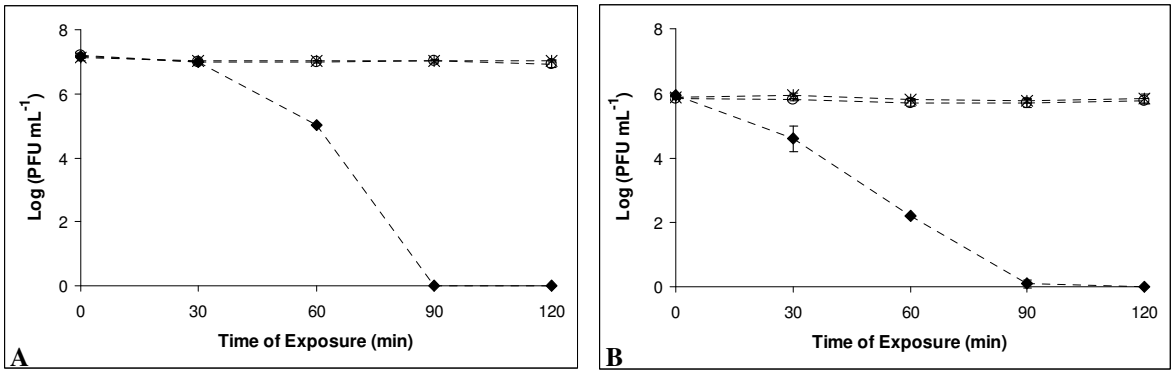

Figure 6. Variation of the numbers of the sewage bacteriophage after irradiation with solar light (A) and with the illumination system (halogen lamp) (B) at 600 W m^{-2} in the presence of porphyrin Tri-Py^{+}-Me-PF (-○- light control, -✳- dark control, -♦- 5.0 μM). Error bars represent standard deviations.

Influence of fluence rate using a specific light source on bacteriophage inactivation

The previous results lead us to evaluate how the bacteriophage inactivation would be affected by different fluence rates delivered by the same light source. This is to ensure that the obtained results were due only to the fluence rate, and not to the different emission spectra of the light sources. In this study we used the halogen lamp and the most efficient PS (Tri-Py^{+}-Me-PF).

For a total light dose of 216 J cm^{-2}, the rate of viral photoinactivation was more effective when the fluence rates were 150, 300 and 600 W m^{-2}. Under these conditions, the phage was inactivated to the limit of detection (> 99.9999% of inactivation) (Figure 7). However, for a fluence rate of 1200 W m^{-2} the bacteriophage was not totally inactivated (reductions of 5.4 log). The differences between the phage

inactivation with a fluence rate of 1200 W m^{-2} and other fluence rates were significant except for the highest light dose (ANOVA, $p > 0.05$).

For total light doses of 144 and 108 J cm^{-2}, the rate of phage inactivation was higher when the light was delivered at a lower fluence rate (reductions of 5.9 and 4.9 log, with 150 and 300 W m^{-2} for 144 J cm^{-2} and of 4.0 and 3.6 log for 108 J cm^{-2}, respectively) than when the light was delivered at 600 and 1200 W m^{-2} (reductions ranging from 1.8 to 3.1 log). The same pattern was obtained when a light dose of 54 J cm^{-2} was used (reductions of 2.3 and 1.8 log for 150 W m^{-2} and 300 W m^{-2} and of 1.4 and 1.1 log, respectively for 600 and 1200 W m^{-2}).

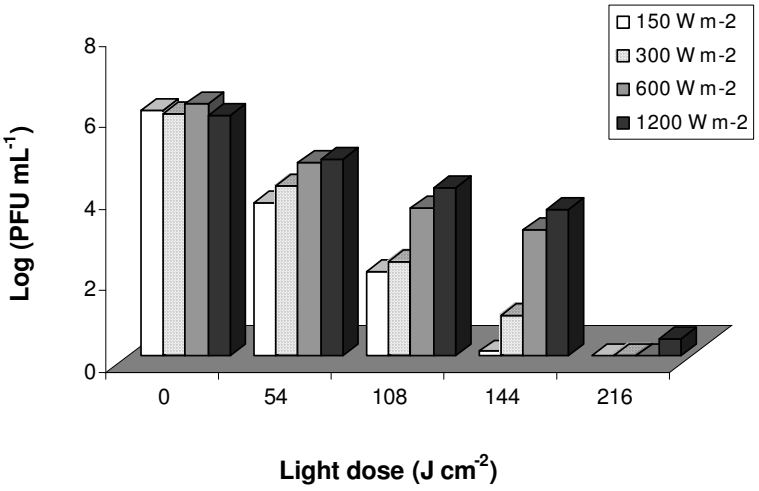

Figure 7. Variation of the numbers of the sewage bacteriophage after a total light dose of 54, 108, 144 and 216 J cm^{-2} delivered at the fluence rates of 150, 300, 600 and 1200 W m^{-2}.

Discussion

The present study demonstrates that the two cationic porphyrins Tetra-Py^{+}-Me and Tri-Py^{+}-Me-PF, when irradiated with different sources of light (fluorescent PAR lamps, sun light and halogen lamp) with fluence rates ranging from 40 W m^{-2} to 1690 W m^{-2}, can efficiently photoinactivate sewage non-enveloped viruses. All light sources tested lead to reductions of > 99.9999% for the somatic T4-like phage. However, the rate and the extent of inactivation are dependent on: (1) the structure and concentration of the photosensitizer; (2) the light source, namely when low fluence rates are used (40 W m^{-2}); and (3) the energy dose, being considerably more effective when light was delivered at a lower fluence rate.

At the highest concentration tested (5.0 μM) reductions of approximately 7 log are obtained with both porphyrins irrespective of the light source used. However, depending on the light source used, different irradiation periods are required to obtain these reductions. Irradiation with a halogen lamp (1690 W m^{-2}) for 45 min leads to an efficient phage inactivation (~ 7.2 log of reduction) with both sensitizers. Both PS when irradiated with solar light (600 W m^{-2}) or with a set of fluorescent lamps (40 W m^{-2}) for short periods gave a small phage inactivation (until 2.2 log after 60 min of irradiation) but are able to inactivate the phage to the limit of detection (reductions of about 7 log) after 180–270 min of irradiation.

It is important to note, however, that at the end of the experiments, for the lowest photosensitizer concentrations (0.5 and 1.0 μM), the rate of inactivation was higher with solar light and with fluorescent lamps rather than with the halogen lamp. This can be explained by the fact that less PS molecules are available in the microcosms for the photoinactivation process and a portion of the light energy is not used to generate cytotoxic oxygen species.

As observed before, the structure and concentration of the photosensitizer influences phage photoinactivation (Milanesio et al, 2003; Lazzeri et al, 2004; Tomé et al, 2005; Caminos et al, 2006; Costa et al, 2008; Almeida et al, 2009). The two cationic porphyrins, irradiated under the same conditions, inactivated the sewage T4-like phage differently. For concentrations of 5.0 μM, the reductions observed were almost 7 log (limit of detection) for both Tri-Py^+-Me-PF and Tetra-Py^+-Me although these reductions were reached at different irradiation times. However, at 1.0 and 0.5 μM the rate of inactivation was significantly lower (3.6 and 1.8 log, respectively). In fact, it is known that increasing the concentration of a sensitizer at a fixed light dose leads to increased viral inactivation as does an increase in the total light exposure at a fixed sensitizer concentration (Kasturi and Platz, 1992). Milanesio and co-workers (2003) also support the basic concept that, after irradiation with visible light, cell survival of human cells is dependent upon both intracellular sensitizer concentration and light exposure level. Similar results were obtained by other authors with bacteria (Lazzeri et al, 2004; Caminos et al, 2006).

The results also show that the efficacy of sewage T4-like bacteriophage inactivation using the same fluence rate is dependent on the light source used (for Tri-Py^+-Me-PF), in particular when the light is delivered at low fluence rate. At 40 W m^{-2} the phage inactivation with fluorescent lamps is much more efficient than with halogen lamps under the same experimental conditions (fluence rate, irradiation time and PS concentration). However, similar phage inactivation is observed when a halogen lamp at 600 W m^{-2} or solar irradiation is used. The difference in phage inactivation when the same fluence rate of 40 W m^{-2} is delivered by the two different light sources can be justified because the fraction of PS that is excited is not the same (Ghetti and Checcucci, 1996; Paula et al, 2009). Although the concentration of the PS used is the same, the emission spectra of the two lamps are different and, consequently, the energy available to excite the photosensitizer is different. The spectral ranges are: 380–700 nm for the fluorescent lamps and 400–800 nm for the interchangeable fibre optic probe coupled to the illumination system (halogen lamp). Since the absorption wavelengths for the porphyrin derivatives range from 400 to 650 nm, part of the energy provided by the halogen lamp (650–800 nm) is not used to excite the photosensitizer. On the contrary, for the fluorescent lamps most of the energy is emitted at 545 and 611 nm that coincides with the Q bands of the PS. When the illumination system (halogen lamp) at 600 W m^{-2} and solar irradiation are used this aspect is not so critical for the inactivation process due to the similarity of both emission spectra (Figure 8).

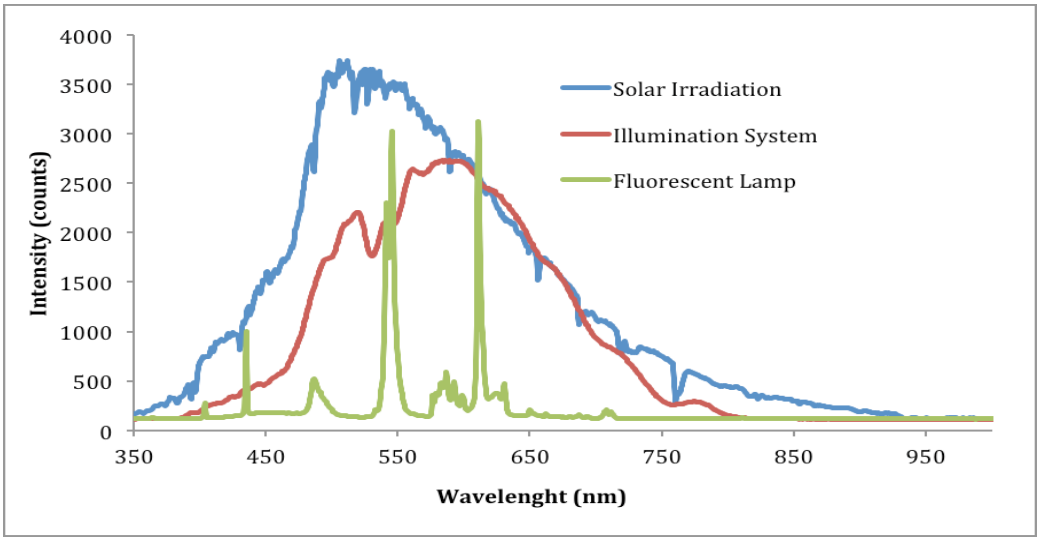

Figure 8. Emission spectra of the light sources used in this study.

When the same light dose is delivered by the same light source but at different fluence rates (150, 300, 600 and 1200 W m^{-2}) the efficacy of sewage T4-like bacteriophage inactivation is inversely proportional to the fluence rate used. Our results show (Figure 7) that the photoinactivation is more efficient if the same total amount of photons passing through a bacteriophage suspension is delivered during a longer illumination period (lower fluence rate). This is due to the fact that, when a high fluence rate is used, the PS in the suspension is not able to absorb the photons in excess (Qin et al, 2008; Prates et al, 2009). Similar results were reported by the study of Gábor and collaborators (2001) were they described a higher rate of *Escherichia coli* and *Enterococcus hirae* inactivation when a similar total light dose is received over a longer time period. Recently, Prates et al (2009) described dissimilar levels of inactivation on yeast cells when the same light dose is given in different fluence rates. At the same light dose, the low fluence rate was more effective than the higher fluence rate (Prates et al, 2009).

In conclusion, our results show that cationic porphyrins irradiated with three different light sources are able to efficiently photoinactivate environmental non-enveloped viruses. These compounds can be envisaged as a new, cheap and accessible technology for wastewater treatment. All light sources used here were effective in the excitation of the selected PS. Different levels of photoinactivation were achieved when the same light dose was received at different periods of irradiation. At higher doses (216 J cm^{-2}) the efficiency of the photoinactivation process was not significantly affected by the fluence rate, however, at lower light doses, the efficiency was strongly affected by the fluence rate. As the light parameters such as light source, fluence rate and total light dose play an important role in the effectiveness of antimicrobial photodynamic therapy, they should always be considered when establishing an optimal antimicrobial protocol.

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CHAPTER 3

Susceptibility of non-enveloped DNA- and RNA-type viruses to photodynamic inactivation

Abstract

The comparative susceptibility of "DNA- and RNA-type" viruses to photodynamic inactivation has not yet been clearly addressed. In this study the effect of the tricationic porphyrin Tri-Py^{+}-Me-PF on the inactivation of four DNA and three RNA non-enveloped phages was compared. The results obtained show that the photodynamic efficiency varied with the phage type, being the "RNA-type" phages much more easily photoinactivated than the "DNA-type" ones.

Introduction

The use of antivirals has largely increased in the last years and resistance to antiviral drugs is now well documented for several pathogenic viruses (Kimberlin and Whitley, 1996; Pillay and Zambon, 1998; Smee et al, 2002). Moreover, as viruses are genetically flexible, they may mutate quickly and, consequently, the emergence of antiviral drug resistance can become a great problem, possibly even higher than that observed for bacteria in relation to antibiotics. Consequently, the emergence of antiviral drug resistance requires alternative methods, unlikely to cause resistance. Microbial photodynamic inactivation (PDI), which uses a combination of a photosensitizer (PS), light and molecular oxygen to induce damages of important biological targets (Jori et al, 2011), has been described as a promising alternative for viral inactivation, namely for bacteriophages that are frequently used as surrogates of mammalian viruses.

After bacteriophage PDI the virus is rendered unable to penetrate a prospective host cell or it is unable to replicate after entry because its genome has been rendered defective, or the virus replication cycle is inhibited at some other step (Jockush et al, 1996; Lee et al, 1997).

Although several studies clearly indicate that enveloped viruses, including the non-usual enveloped bacteriophages, are more susceptible to PDI than their non-enveloped counterparts (Lenard et al, 1993; Wainwright, 2003), the comparative susceptibility of "DNA- and RNA-type" viruses to PDI has not been clearly addressed yet. Studies employing non-enveloped phages like T7 (DNA phage), PM2 (DNA phage with internal lipids) and MS2 (RNA phage), indicated that both DNA and RNA phages can be photoinactivated (Specht, 1994). An increase in T7 phage photoinactivation was observed with an increase in methylene blue concentration from 1.0 to 10 μM, while the photoinactivation rate of MS2 did not vary under the same experimental conditions. It was suggested that the critical targets for MS2 phage

were saturated at 1.0 μM of phenothiazines (Specht, 1994). Hotze et al (2009) also showed that PRD1 and T7 ("DNA-type" phages) are significantly more resistant to singlet oxygen inactivation than MS2 phage. The high propensity for MS2 phage photoinactivation was attributed to the damage of its A protein, necessary for infecting its host *Escherichia coli* since it contains highly reactive amino acids such as Met, Cys, His, and Tyr (Hotze et al, 2009).

The objective of this study was to compare the susceptibility to photodynamic inactivation of a series of "DNA- and RNA-type" bacteriophages (the designation "DNA- and RNA-type" is used in respect to the nature of the nucleic acid and capsid proteins). For that, a tricationic porphyrin, 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri- iodide (Tri-Py^{+}-Me-PF), a very promising PS already tested with success against several types of microorganisms (Almeida et al, 2011), was used.

Methods

Porphyrin synthesis

The 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri- iodide (Tri-Py^{+}-Me-PF) used as PS (Figure 1) was prepared in accordance with the methodology referred by Carvalho et al (2010). PS purity was confirmed by ^{1}H NMR spectroscopy. The PS was dissolved in dimethyl sulfoxide (500 μM stock solution) and sonicated for 30 min before use.

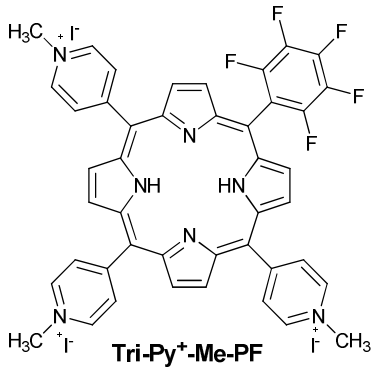

Figure 1. Structure of the PS used for bacteriophage photoinactivation.

Bacteriophages

Wastewater from a sewage secondary-treatment plant (Aveiro, Portugal) was used to select the somatic bacteriophages of *Escherichia coli* C (ATCC 13706) (Costa et al, 2008). Phages of *Aeromonas salmonicida* (AS) and *Vibrio anguillarum* (VA) were isolated from the aquaculture system Corte das Freiras (Ria de Aveiro, Portugal) (Pereira et al, 2011). *Pseudomonas aeruginosa* phage (PA) was obtained from a sewage water sample collected at the wastewater treatment plant of the University of Coimbra Hospitals (Vieira, 2011). MS2 and Qβ phages were purchased from DSMZ collection (Braunschweig, Germany). LAIST_PG002 phage was isolated from a sewage secondary-treatment plant (Lisboa, Portugal) (unpublished data).

T4-like, AS, PA and VA phages are non-enveloped double stranded (ds) DNA viruses with a simple capsid without lipids (Mesyanzhinov et al, 2004; Pereira et al, 2011; Vieira, 2011). MS2, Qβ and LAIST_PG002 phages are non-enveloped single stranded (ss) RNA viruses also with a simple capsid without lipids (Lee et al, 1997; Schneider et al, 1998; Cho et al, 2005).

Bacteriophage quantification

Bacteriophage quantification was done by the double agar layer technique (Adams, 1959), using *E. coli* C (ATCC 13706), *A. salmonicida*, *P. aeruginosa* and *V. anguillarum* as host strains, respectively for T4-like, AS, PA and VA phages and *Salmonella typhimurium* WG49 (Havelaar and Hogeboom, 1984) for MS2, Qβ and LAIST_PG002 phages. From each sample, duplicates of 1.0 mL of non-diluted or of serially diluted samples and 0.3 mL of bacterial host were added to a tube with 5.0 mL of soft TSA growth medium. The contents of the tube were mixed by manual rotation and then immediately poured onto a TSA monolayer on a Petri plate. After 5 (MS2 phage) and 18 hours (Qβ, LAIST_PG002, T4-like, AS, PA and VA phages) of dark incubation at 37 ºC, the number of phage plaques was counted on the most convenient series of dilutions and the number of plaque forming units per millilitre (PFU mL^{-1}) was determined.

Experimental setup

Phage photoinactivation by Tri-Py^{+}-Me-PF at 5.0 and 0.5 μM, respectively for "DNA- and RNA-type" phages, was achieved by exposing the bacteriophages in PBS (≈ 10^{7} PFU mL^{-1}) to white light (13 fluorescent lamps OSRAM 21 of 18 W each, 380-700 nm) of 40 W m^{-2} (measured with a light meter LI-COR Model LI-250) during 270 min, with agitation (100 rpm) at 25 ºC. Dark and light controls were also included in the experiment and were carried out simultaneously. In the light control (LC), the phage suspension without PS was exposed to the same irradiation protocol. In the dark control (DC), the beaker containing the phage suspension and the PS at the studied concentration (0.5 or 5.0 μM) was covered with aluminium foil to protect it from light exposure. Sub-samples of 1.0 mL of test and control samples were aseptically taken at time 0, 60, 90, 180 and 270 min (for "DNA-type" phages) and at time 0, 15, 30, 60, 90, 180 and 270 min (for "RNA-type" phages). The kinetics of phage inactivation was evaluated through the quantification of the number of phages according to Costa et al (2008). Three independent experiments were carried out with two replicates each.

Statistical analysis

SPSSWIN 14.0 was used for data analysis. The significance of difference in phage inactivation among different DNA and RNA phages was assessed using one-way ANOVA. Only the data with normal distribution (assessed by Kolmogorov-Smirnov test) and with homogeneity of variances (assessed by Levene test) were used.

Results

The results of LC and DC showed that white light or PS under dark did not significantly affect the phages viability (Figures 2 and 3), indicating that the reduction on phage survival was due to the PDI treatment.

The efficiency of bacteriophage photoinactivation by Tri-Py^{+}-Me-PF irradiated with 40 W m^{-2} during 270 min, was markedly different between DNA (T4-like, AS, PA and VA) and RNA (MS2, Qβ and LAIST_PG002) phages (Figures 2 and 3). All phages were efficiently photoinactivated (6-7 log), but the inactivation occurred earlier for "RNA-type" phages and at a PS concentration ten times lower than that required to efficiently photoinactivate the "DNA-type" ones (0.5 μM *versus* 5.0 μM and 60-90 min *versus* 180-270 min).

Although "DNA-type" phages exhibited different rates of photoinactivation (Figure 2), the differences were not statistically significant (ANOVA, $p > 0.05$). T4-like phage was the only DNA phage photoinactivated to the limits of detection (~ 7 log) after 180 min of irradiation at 5.0 μM PS. Reductions ranging from 5.7 to 6.1 log, after 270 min of irradiation, were attained for AS, PA and VA phages. "RNA-type" phages were all inactivated to the detection limit (6.2 to 7.2 log), after 60-90 min of irradiation, in the presence of 0.5 μM PS (Figure 3), and their rates of inactivation were not statistically different (ANOVA, $p > 0.05$).

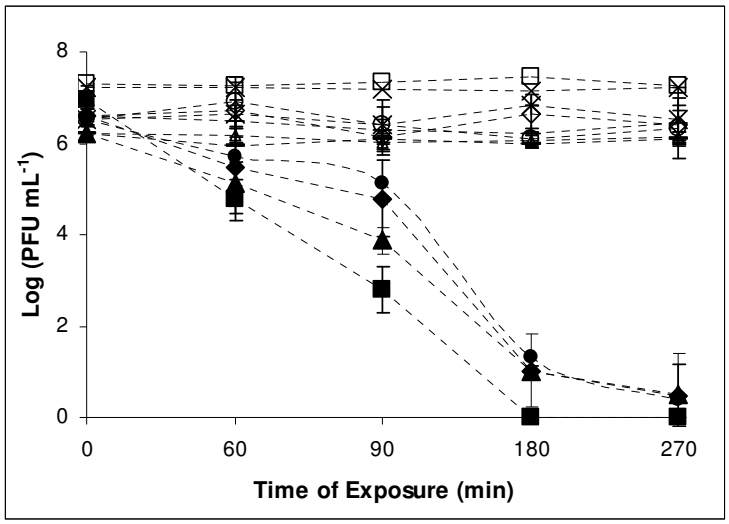

Figure 2. Variation of the number of the "DNA-type" phages after irradiation with white light (40 W m^{-2}) in the presence of 5.0 μM Tri-Py^{+}-Me-PF (–■– T4-like, –▲–PA, –•– AS, –♦– VA, –×– T4-like LC, –□– T4-like DC, –ˉ–PA LC, –Δ–PA DC, –✳– AS LC, –○– AS DC, –+– VA LC, –◊– VA DC). Each value represents mean ± standard deviation of three independent experiments, with two replicates each.

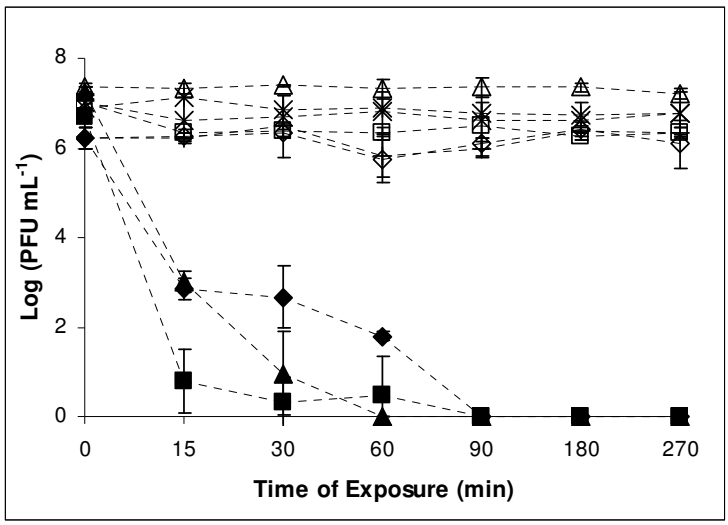

Figure 3. Variation of the number of the "RNA-type" phages after irradiation with white light (40 W m^{-2}) in the presence of 0.5 μM Tri-Py^{+}-Me-PF (–▲– MS2, –■– LAIST_PG002, –♦– Qβ, –✳– MS2 LC, –Δ–MS2 DC, –×– LAIST_PG002 LC, –□– LAIST_PG002 DC, –+– Qβ LC, –◊– Qβ DC). Each value represents mean ± standard deviation of three independent experiments, with two replicates each.

Discussion

The present study demonstrates that both "DNA- and RNA-type" bacteriophages can be efficiently photoinactivated by Tri-Py^{+}-Me-PF when irradiated with white light (40 W m^{-2}), and that "RNA-type" phages are more susceptible to PDI than the "DNA-type" ones. The rate and efficiency of the inactivation process seem to be dependent on the type of phages. "RNA-type" phages were inactivated to the detection limit, after 60-90 min of irradiation with white light at 0.5 μM of Tri-Py^{+}-Me-PF, while the "DNA-type" phages, although also efficiently inactivated by Tri-Py^{+}-Me-PF, required a concentration of PS ten times higher (5.0 μM) and a longer irradiation period (180 min for T4-like phage and 270 min for AS, PA and VA phages).

All "DNA- and RNA-type" phages tested in this study are non-enveloped, with a simple capsid without lipids. As the capsid of the studied "DNA-type" phages is composed by a higher diversity of proteins (Fokine et al, 2004; Mesyanzhinov et al, 2004), when compared with the capsid of "RNA-type" phages (Cho et al, 2005), the major difference between the two phage groups may not only reside on the type of nucleic acids, but also on the composition of their capsids, which can also explain the different pattern of PDI.

It has been shown that the main targets of PDI are the external microbial structures that, in the case of viruses, are the protein capsids and lipid envelopes (if present) (Hotze et al, 2009). The damages of the external viral structures can involve leakage of particle contents and/or inactivation of enzymes (Sieber et al, 1992; Zupán et al, 2008). Nucleic acid has been also identified as a PDI target (Abe and Wagner, 1995; Hotze et al, 2009). However, although nucleic acid damage occurs, it is not suggested as the main cause of viral PDI (Hotze et al, 2009). A demonstration of this is the bacterium *Deinococcus radiodurans*, which is known to have a very efficient DNA repair mechanism, but it is easily killed by PDI (Schafer et al, 1998). However, in contrast to DNA, short-lived RNA molecules are rapidly degraded

in living microorganisms by enzymes (RNase), which are very stable even in harsh environments (Sela et al, 1957; Sheridan et al, 1998). Consequently, in the case of viruses, for which the nucleic acid can be ss or dsDNA or RNA, and contrarily to bacteria and fungi, the type of nucleic acid can be an important and a determinant factor of the efficiency of viral PDI. Moreover, as for bacteria, the damages in the viral DNA can yet be repaired by the action of DNA repairing systems of the host cells (Imray and MacPhee, 1973), but it is impossible for the host cells to repair the damaged nucleic acid of RNA viruses. Therefore, "DNA-type" viruses tend to be more genetically stable than "RNA-type" viruses.

The studies so far conducted with "DNA- and RNA-type" phages (Specht, 1994; Hotze et al, 2009) showed that "RNA-type" phages were more easily inactivated than "DNA-type" ones under the same PDI protocol. However, in these studies, although several "DNA-type" phages were used, only one ssRNA phage (MS2) was tested, which precludes a clear picture of the effect of the nucleic acid type in viral PDI and generalizations are difficult to be made. In this study, three "RNA-type" phages were used, including the well-studied MS2 phage, and all of them were significantly more easily inactivated that any of the four "DNA-type" phages tested.

Associated with the higher stability of DNA strands, and the possibility of DNA repair by the host repair system, the more complex capsids of "DNA-type" phages could also contribute to the less effective PDI relatively to "RNA-type" phages, which have not RNA host repair mechanisms and have less complex capsids. In fact, it has been shown that the main targets of viral PDI are the capsid proteins (Hotze et al, 2009).

In conclusion, this work emphasizes that the efficiency of phages PDI is different for "DNA- and RNA-type" phages. However, more and detailed experimental work focusing on the main targets involved in the PDI of both "DNA-type and RNA-type" bacteriophages, including also dsRNA phages, is under progress.

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CHAPTER 4

Involvement of type I and type II mechanisms on the photoinactivation of non-enveloped DNA and RNA bacteriophages

Abstract

Microbial photodynamic inactivation (PDI), involving the use of a photosensitizer (PS), light and molecular oxygen, with the subsequent production of reactive oxygen species (ROS), has been considered a promising and effective technology for viral inactivation. Although singlet oxygen is generally accepted as the main damaging species in PDI, ROS like free radicals may also be involved in the process, inducing damages to proteins, lipids, nucleic acids and other molecular structures. In this study, the relative importance of each mechanism (type I and type II) on the photoinactivation of non-enveloped DNA (T4-like phage) and RNA (Qβ phage) viruses was evaluated. For this purpose, two cationic porphyrins (Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me) and four different ROS scavengers were used. The scavenging effect of sodium azide and L-histidine (singlet oxygen quenchers) and of D-mannitol and L-cysteine (free radical scavengers) was assessed by exposure of both phages (T4-like and Qβ) to each cationic porphyrin (5.0 μM for T4-like phage and 0.5 μM for Qβ phage) and white light (40 W m^{-2}) in the presence of different concentrations of the scavengers (5, 10, 50 and 100 mM). Sodium azide and L-histidine gave the best protection, reducing the phototoxic effect of Tri-Py^{+}-Me-PF on T4-like phage respectively by 80 and 72% and in the presence of Tetra-Py^{+}-Me by 90 and 78%. Free radical scavengers D-mannitol and L-cysteine did not significantly reduce the rate of T4-like phage photoinactivation (≤ 20% protection), for both PS). The sodium azide protection on Qβ phage photoinactivation, in the presence of Tri-Py^{+}-Me-PF, was lower (39%) when compared with T4-like phage. D-mannitol did not exert on Qβ phage any protective effect after 90 min of irradiation. The effect of the simultaneous presence of singlet oxygen and free radicals scavengers at 100 mM confirmed that singlet oxygen (type II mechanism) is clearly the main ROS involved in T4-like and Qβ phages photoinactivation by these two cationic PS. As RNA-type phages are more easily photoinactivated when compared with DNA-type ones, the protection conferred by the scavengers during the PDI process is lower and this should be taken into account when the main mechanism involved in PDI of different viruses is to be studied.

Introduction

Microbial photodynamic inactivation (PDI) is based on the ability of a photosensitizer (PS) to absorb energy from light and, from their excited state, lead to the production of reactive oxygen species (ROS), such as singlet oxygen and free radicals (Jori et al, 2011). In the first excited short-lived state,

achieved by absorption of visible light, the PS can loose its energy by fluorescence emission (radiative deactivation process), by internal conversion (radiationless deactivation process) or by transfer of an electron from an excited singlet to a long-lived triplet excited state (intersystem crossing process). Because of its longer lifetime, the triplet state of PS (^{3}PS*) has a good chance of interacting with other molecules. It can react with substrates by an electron and/or hydrogen atom transfer to give free radical forms (equations 1 and 2). The excited PS (^{3}PS*) can return to the ground state by reaction with oxygen, generating oxygen superoxide radical and other oxygenated products (type I mechanism) (equations 3-5). This type of mechanism is dependent on the substrate and PS concentrations, and is favoured when the PS is bound or strictly associated with readily oxidizable molecules (Bonnett, 2000; Ogilby, 2010; Jori et al, 2011). Type II mechanism involves an energy transfer process from the triplet state of PS to ground-state oxygen ($^3O_2$) giving a ground-state photosensitizer (^{1}PS) and a highly reactive singlet state of oxygen (singlet oxygen), which in turn attacks photosensitive targets in its surroundings (equation 6) (Via and Magno, 2001; Jori et al, 2011).

(eq. 1) $\text{SubstrateH}_2 + {}^3\text{PS}^* \rightarrow \text{PSH}^{\bullet} + \text{SubstrateH}^{\bullet}$

(eq. 2) ${}^3\text{PS}^* + \text{Substrate} \rightarrow \text{PS}^{\bullet-} + \text{Substrate}^{\bullet+}$ or ${}^3\text{PS}^* + \text{Substrate} \rightarrow \text{PS}^{\bullet+} + \text{Substrate}^{\bullet-}$

(eq. 3) $\text{PS}^{\bullet-} + {}^3\text{O}_2 \rightarrow \text{PS} + \text{O}_2^{\bullet-}$

(eq. 4) $\text{O}_2^{\bullet-} + \text{H}^+ \rightleftharpoons \text{HOO}^{\cdot}$

(eq. 5) $2\ \text{HOO}^{\cdot} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$

(eq. 6) ${}^3\text{PS}^* + {}^3\text{O}_2 \rightarrow {}^1\text{PS} + {}^1\text{O}_2$

The reactive oxygen species (ROS) formed *via* type I or II mechanisms are extremely reactive and strongly interact with a variety of biomolecules, like nucleic acids, lipids and proteins (Wainwright, 2004; Sagristá et al, 2009). Due to their high reactivity and short half-life, only molecules and structures near to the area of singlet oxygen and free radical species production by the PS are directly affected and subsequently destroyed (Ogilby, 2010).

Both type I and type II mechanisms may occur in parallel or exclusively, and the ratio between these processes depends on the PS used and on the concentrations of substrate and oxygen (Via and Magno, 2001). The competition between organic substrates and molecular oxygen for the triplet excited state of the PS determines whether the reaction pathway is type I or type II and the predominant mechanism can be changed during the course of the PDI process, as the oxygen in the system becomes depleted (Min and Boff, 2002).

An important goal in the investigation of photosensitization processes in PDI is the elucidation of the mechanism of action of a selected PS to determine whether it proceeds *via* a type I or a type II mechanism. The simple detection of ROS, such as singlet oxygen, does not necessarily explain the mechanism by which a specific PS induces the phototoxic effect (Ochsner, 1997; Maisch et al, 2005). It is

generally easier to draw a negative conclusion, *i.e.*, if singlet oxygen is absent, it cannot be the reactive species of interest. The simplest approach for determining whether singlet oxygen or free radicals are involved in the photodynamic process is to study the inhibitory effect of various scavengers, which can intercept the ROS at high rates and in a putatively selective manner (Ochsner, 1997; Girotti, 2001).

From the experimental work done until now, considerable evidence exists that singlet oxygen is the main mediator of biological damage in viral PDI, but pathways yielding other ROS are also possible and their participation cannot be excluded (Müller-Breitkreutz et al, 1995; Abe et al, 1997; Wainwright, 2004; Badireddy et al, 2007; Hotze et al, 2009; Sagristá et al, 2009). Although these works report which mechanisms are involved in the viral PDI process, studies using different types of phages (DNA and RNA phages), which are normally used as surrogates of mammalian viruses, and different types of scavengers and PS are still scarce and the results are sometimes contradictory, when the same PS and scavengers are used. Considering the PDI in the presence of singlet oxygen quenchers, the photoinactivation of M13 bacteriophage (DNA) by methylene blue (MB) was inhibited from 1.72 log to 0.54 log, by sodium azide, however, the photoinactivation occurred even in the presence of the quencher, suggesting that both type I and type II mechanisms may be involved in M13 photoinactivation by MB (Abe et al, 1997). Also, singlet oxygen was considered as the main responsible for the loss of biological activity of bacteriophage M13 by rose bengal (DiMascio et al, 1989). The phototoxic effect of 5,10,15-tris(4-β-D-galactosylphenyl)-20-(pentafluorophenyl)porphyrin on T7 phage was reduced by 38% in the presence of sodium azide (Egyeki et al, 2003). Photoinactivation of T7 phage with 5,10,15,20-tetrakis(4-β-D-glucosylphenyl)porphyrin was reduced by 42% in the presence of 1,3-diphenylisobenzofuran (DPBF), also used as singlet oxygen quencher. However, when 5,10,15-tris(4-β-D-glucosylphenyl)-20-phenylporphyrin was used as PS, the rate of protection substantially increased (74%) in the presence of DPBF, showing that the symmetric derivative seemed to exert its phototoxic effect mainly *via* free radicals generation, whereas the asymmetric gluco-derivative proceeded mainly by singlet oxygen production (Gábor et al, 2001). MS2 (RNA), PRD1 and T7 (DNA) phages photoinactivation by polyhydroxylated fullerene was shown to be significantly inhibited by the presence of β-carotene, a singlet oxygen quencher (Badireddy et al, 2007; Hotze et al, 2009). However, the results from Hotze et al (2009) do not exclude, during the PDI process in the presence of the singlet oxygen quencher, the occurrence of partial singlet oxygen quenching or that the observed photoinactivation was due to other mechanism than singlet oxygen production.

In what concerns to the effect of free radicals scavengers on phage PDI, it was shown that T7 phage photoinactivation by 5,10,15,20-tetrakis(4-β-D-glucosylphenyl)-porphyrin (Gábor et al, 2001) and 5,10,15-tris(4-β-D-galactosylphenyl)-20-(pentafluorophenyl)porphyrin (Egyeki et al, 2003) seemed to be mainly mediated by free radical species (62% and 89% protection in the presence of dimethylthiourea, respectively). Contrarily, superoxide dismutase (SOD) did not protect T7 phage from polyhydroxylated fullerene photosensitization, suggesting a negligible superoxide generation by this PS (Badireddy et al, 2007).

The aim of this study was to identify the main mechanism involved in T4-like (DNA) and Qβ (RNA) phages photoinactivation by two cationic porphyrin derivatives, 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^+-Me) and 5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py^+-Me-PF), which already proved to be effective for phage photoinactivation (Costa et al, 2008; Costa et al, *submitted*). In this context, two singlet oxygen quenchers (sodium azide and L-histidine) and two free radical scavengers (D-mannitol and L-cysteine) were used.

Material and Methods

The toxic effect of Tri-Py^+-Me-PF and Tetra-Py^+-Me on T4-like (5.0 μM) and Qβ (0.5 μM) bacteriophages was evaluated by exposing the phage suspensions to white light (40 W m^{-2}) during 270 and 90 min, respectively, in the presence of different concentrations (5, 10, 50 and 100 mM) of the singlet oxygen quenchers (sodium azide and L-histidine) and of free radical scavengers (D-mannitol and L-cysteine). Aliquots of the phage suspensions were aseptically collected at 0, 60, 180 and 270 min (for T4-like phage) and at 0, 10, 20, 30, 40, 60 and 90 min (for Qβ phage) after light exposition. The simultaneous presence of singlet oxygen and free radicals scavengers at 100 mM was also evaluated for T4-like and Qβ phages PDI by Tri-Py^+-Me-PF at 5.0 and 0.5 μM, respectively.

Photosensitizers

The porphyrin derivatives (Tri-Py^+-Me-PF and Tetra-Py^+-Me) used as PS (Fig. 1) were prepared in accordance with the methodology referred by Carvalho et al (2010). PS purity was confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy. Stock solutions of the PS in dimethyl sulfoxide at 500 μM were prepared and sonicated at room temperature for 30 min before use.

Scavengers

Stock solutions of sodium azide, L-histidine, D-mannitol and L-cysteine were prepared in ultra pure water for a final concentration of 500 mM and kept at 4ºC until use.

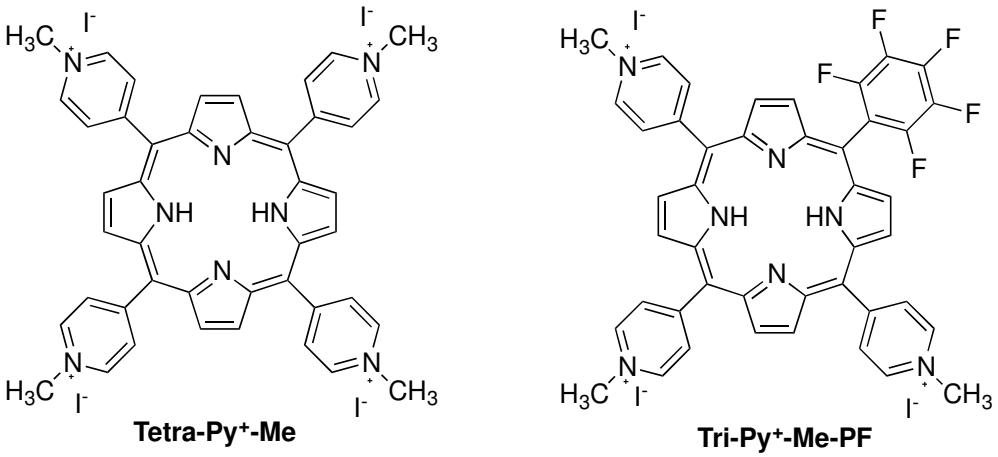

Figure 1. Structure of the PS used for T4-like and Qβ bacteriophages photodynamic inactivation.

Bacteriophages

T4-like phage was isolated from a sewage secondary-treatment plant of the city of Aveiro (Portugal) using *Escherichia coli* C ATCC 13706 (American Type Culture Collection, Rockville, MD, USA) as host strain (Costa et al, 2008). Qβ phage was purchased from DSMZ collection (Braunschweig, Germany). Phage suspensions with 10^9 particles per mL were used.

Bacteriophage quantification

The quantification of phages was determined, in duplicate, by the agar double layer technique (Adams, 1959), using *E. coli* C (ATCC 13706) and *Salmonella typhimurium* (WG49) (Havelaar and Hogeboom, 1984) as host strains, respectively, for T4-like and Qβ phages. For each phage, 1.0 mL of non-diluted or of serially diluted samples and 0.3 mL of bacterial host were added to a tube with 5.0 mL of soft TSA growth medium. After manually mixed, tube contents were immediately poured onto a confluent TSA monolayer on a Petri plate. After 18 h of dark incubation at 37 ºC, the number of lysis plaques was counted at the most convenient series of dilutions and the number of plaque forming units per millilitre (PFU mL^{-1}) was determined.

Experimental setup

Phage inactivation was achieved by exposing the phage suspensions to white light of 40 W m^{-2} (PAR radiation, 13 fluorescent lamps OSRAM 21 of 18 W each, 380-700 nm), in the presence of each PS and the selected scavengers, under stirring (100 rpm) at 25 ºC, during 270 (T4-like phage) and 90 min (Qβ phage). Dark and light controls were also included and were carried out simultaneously. Two light controls were prepared and exposed to the same irradiation protocol: one including the phage suspension without the PS and scavenger (LC), and the other one including the phage suspension and the scavenger under study (LCS). Three beakers containing the phage suspensions were prepared as dark controls. In one beaker was added the PS and the selected scavenger (DC), in another beaker was added the PS (DCPS), and at the third one was added the scavenger (DCS); all beakers were covered with an aluminium foil. A beaker with the phage suspension and the PS without any scavenger was also included

in the experiment (0 mM). Sub-samples of 1.0 mL from each test and control beakers were aseptically taken at established time intervals (0, 60, 180, and 270 min for T4-like phage, and 0, 10, 20, 30, 40, 60 and 90 min for Qβ phage). The protective effect afforded by singlet oxygen and free radicals scavengers was evaluated through quantification of the number of phages by the double layer technique, as described above. Three independent experiments were carried out with two replicates each. The protection of the scavengers was followed until the detection limit of the method was reached.

Effect of singlet oxygen and free radicals scavengers

The relative contribution of singlet oxygen (type II mechanism) and free radical species (type I mechanism) for bacteriophage PDI was evaluated by the presence of specific singlet oxygen (sodium azide and L-histidine) and free radical (D-mannitol and L-cysteine) scavengers. For T4-like phage, in the presence of Tri-Py^+-Me-PF, all four scavengers were tested at different concentrations (5, 10, 50 and 100 mM). In the presence of Tetra-Py^+-Me, sodium azide, L-histidine and D-mannitol were equally tested at the concentrations of 5, 10, 50 and 100 mM. T4-like phage PDI with Tri-Py^+-Me-PF at 5.0 μM was also evaluated in the simultaneous presence of singlet oxygen and free radicals scavengers at 100 mM: sodium azide and D-mannitol, sodium azide and L-cysteine, L-histidine and D-mannitol, and L-histidine and L-cysteine. For Qβ phage, singlet oxygen quencher sodium azide and free radicals scavenger D-mannitol were used, at the concentration of 100 mM (the concentration that exerted a significant effect for T4-like phage), in the presence of 0.5 μM of Tri-Py^+-Me-PF. The photodynamic inactivation of Qβ phage by 0.5 μM Tri-Py^+-Me-PF was also evaluated in the simultaneous presence of sodium azide and D-mannitol at the concentration of 100 mM. In all experiments, the scavengers and PS were added to the phage suspensions 30 min before irradiation and the suspensions were maintained in the dark during that time. The rate of protection afforded by singlet oxygen quenchers and free radicals scavengers in the presence of Tri-Py^+-Me-PF and/or Tetra-Py^+-Me was determined taking into account the irradiation time where T4-like and Qβ phages reached their maximum rate of photoinactivation.

Statistical analysis

SPSSWIN 15.0 was used for data analysis. The significance of difference in phages inactivation in the presence of different singlet oxygen and free radicals scavengers was assessed using one-way ANOVA. Only the data with normal distribution (assessed by Kolmogorov-Smirnov test) and with homogeneity of variances (assessed by Levene test) were used.

Results

Without light, the tested PS (DC, DCPS) did not exhibit any toxicity against any of the phages during the total time of the experiment. A similar trend was obtained with the phages in the absence of PS (with and without the scavengers) during the time course of the experiments (LC, LCS) (Figures 2 to 7).

The white light used in the experiments (380 – 700 nm) did not affect viral viability and the phototoxicity was due to the photodynamic effect of the PS.

In the absence of scavengers (0 mM), both cationic porphyrins were able to photoinactivate to the limit of detection (about 7 log of reduction) the T4-like and Qβ phages. T4-like phage was inactivated to the limit of detection (7.2 log of reduction) by Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me at the concentration of 5.0 μM after 180 and 270 min of irradiation, respectively (Figures 2 to 5). The efficiency of T4-like phage inactivation by Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me varied according to the type and concentration of the scavenger used. When sodium azide was used, in the presence of Tetra-Py^{+}-Me (Figure 2B), the rate of phage photoinactivation was considerably lower (0.7 to 1.9 log) for all the tested concentrations of sodium azide (5, 10, 50 and 100 mM), when compared with the control without quencher (0 mM) (ANOVA, $p < 0.05$). However, in the presence of Tri-Py^{+}-Me-PF there was only a significant protection effect (ANOVA, $p < 0.05$) when the highest concentration of sodium azide (100 mM) was used (1.4 log of reduction after 180 min of irradiation), compared to the samples in the absence of scavenger (0 mM) after the same time of light exposure (Figure 2A).

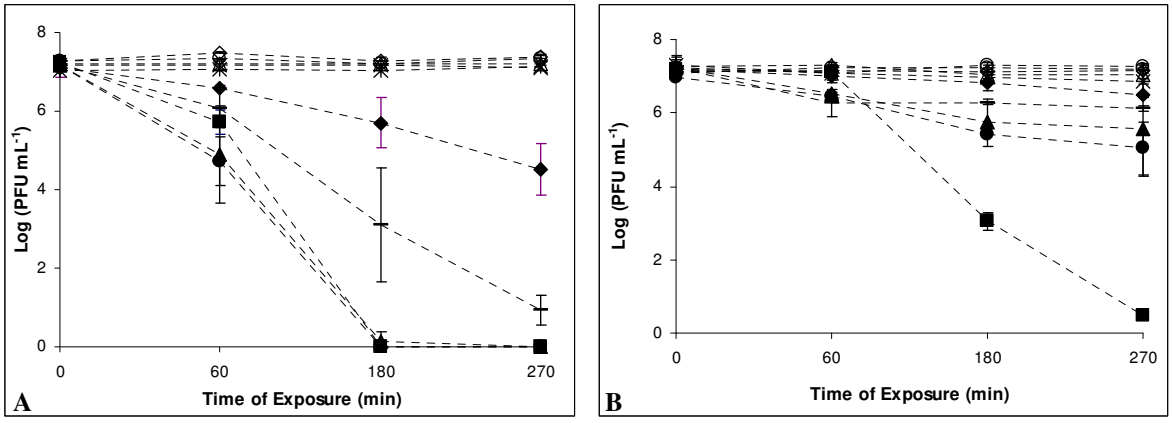

Figure 2. Variation of the numbers of T4-like phage after 60, 180 and 270 min of irradiation, in the presence of 5.0 μM Tri-Py^{+}-Me-PF (A) and Tetra-Py^{+}-Me (B), and different concentrations of sodium azide (–◊– LC, –△– LCS, –×– DCS, –○– DCPS, –✳– DC, –■– 0 mM sodium azide, –●– 5 mM sodium azide, –▲– 10 mM sodium azide, –▬– 50 mM sodium azide, –♦– 100 mM sodium azide). Each value represents mean ± standard deviation of three independent experiments, with two replicates each. Error bars represent standard deviations.

In the presence of L-histidine, the rate of phage inactivation was also considerably reduced for all the tested concentrations, when compared with the control without scavenger (0 mM) (ANOVA, $p < 0.05$). The highest photoinactivation inhibition was observed at 50 mM for Tri-Py^{+}-Me-PF (but the inhibition was not statistically different from that obtained at 100 mM of L-histidine, ANOVA, $p > 0.05$) and at 100 mM of quencher concentration for Tetra-Py^{+}-Me, reaching ~ 2 log of phages reduction for both PS after 180 and 270 min of white light irradiation, respectively for Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me (Figure 3). T4-like phage photoinactivation in the presence of singlet oxygen quenchers (sodium azide or L-histidine) was not statistically different for both PS (ANOVA, $p > 0.05$).

The two PS were less effective to photoinactivate T4-like phages in the presence of singlet oxygen quenchers than in the presence of free radicals scavengers. In the presence of D-mannitol at 100 mM and

Tri-Py$^+$-Me-PF, a smaller reduction in the photoinactivation rate was observed (5.7 log of phage inactivation), relatively to the sample without scavenger (7.2 log), after 180 min of irradiation (ANOVA, $p < 0.05$) (Figure 4). In the presence of Tetra-Py$^+$-Me, D-mannitol (100 mM) did not reveal any protective effect, exhibiting a phage reduction of 5.8 log, after 270 min of irradiation. Although the best protective effect of D-mannitol might be achieved at 5.0 mM (4.1 log), the differences between different D-mannitol concentrations were found to be not statistically significant (ANOVA, $p > 0.05$) (Figure 4). Similar results were obtained when L-cysteine was used as free radical scavenger in the presence of Tri-Py$^+$-Me-PF. In fact, slight protection of T4-like phage inactivation was observed with the highest concentrations tested (50 and 100 mM) relatively to the control sample without scavenger (5.6 and 6.3 log of phage inactivation, respectively, against 7.1 log observed for 0 mM) (ANOVA, $p < 0.05$) (Figure 5). The differences between free radical scavengers (D-mannitol and L-cysteine) for Tri-Py$^+$-Me-PF were not statistically significant (ANOVA, $p > 0.05$).

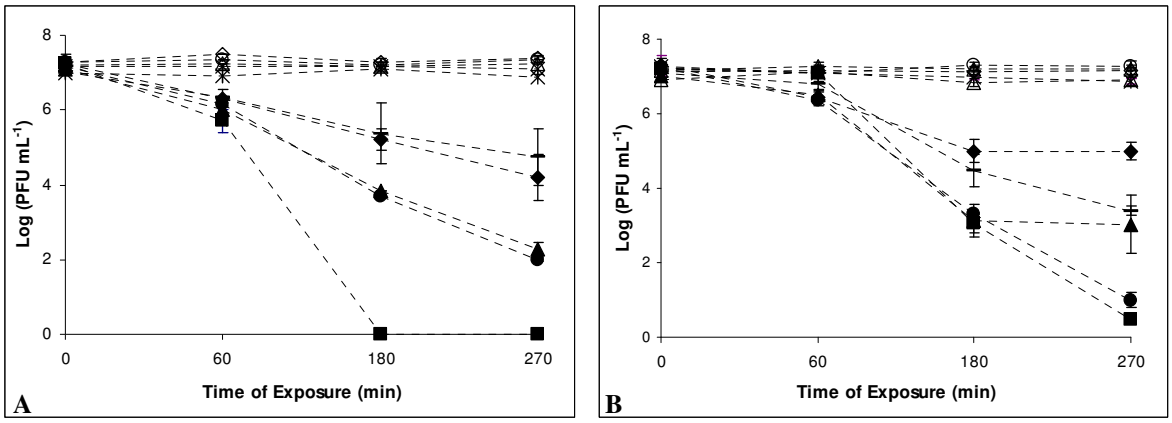

Figure 3. Variation of the numbers of T4-like phage after 30, 60, 180 and 270 min of irradiation, in the presence of 5.0 μM Tri-Py$^+$-Me-PF (A) and Tetra-Py$^+$-Me (B), and different concentrations of L-histidine (–◊– LC, –△– LCS, –×– DCS, –○– DCPS, –✳– DC, –■– 0 mM L-histidine, –●– 5 mM L-histidine, –▲– 10 mM L-histidine, –▬– 50 mM L-histidine, –♦– 100 mM L-histidine). Each value represents mean ± standard deviation of three independent experiments, with two replicates each. Error bars represent standard deviations.

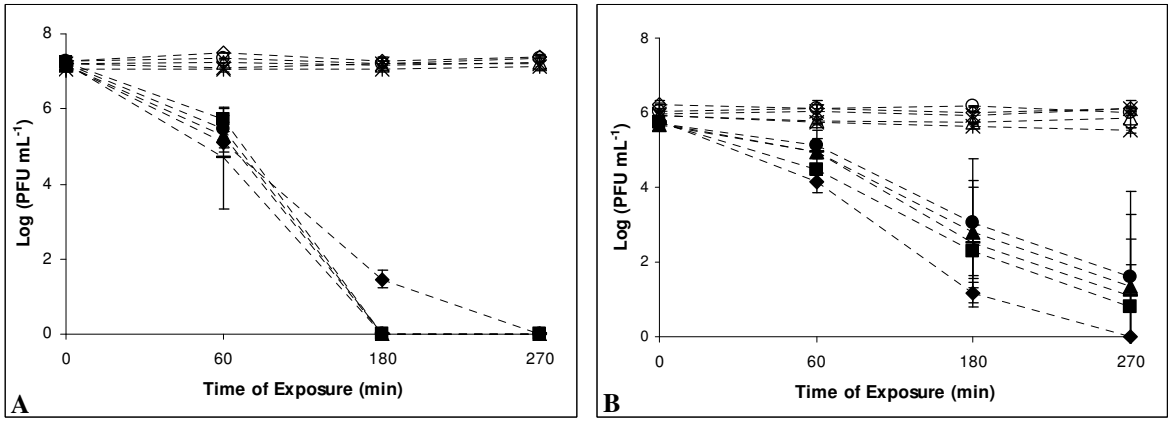

Figure 4. Variation of the numbers of T4-like phage after 30, 60, 180 and 270 min of irradiation, in the presence of 5.0 μM Tri-Py$^+$-Me-PF (A) and Tetra-Py$^+$-Me (B), and different concentrations of D-mannitol (–◊– LC, –△– LCS, –×– DCS, –○– DCPS, –✳– DC, –■– 0 mM D-mannitol, –●– 5 mM D-mannitol, –▲– 10 mM D-mannitol, –▬– 50 mM D-mannitol, –♦– 100 mM D-mannitol). Each value represents mean ± standard deviation of three independent experiments, with two replicates each. Error bars represent standard deviations.

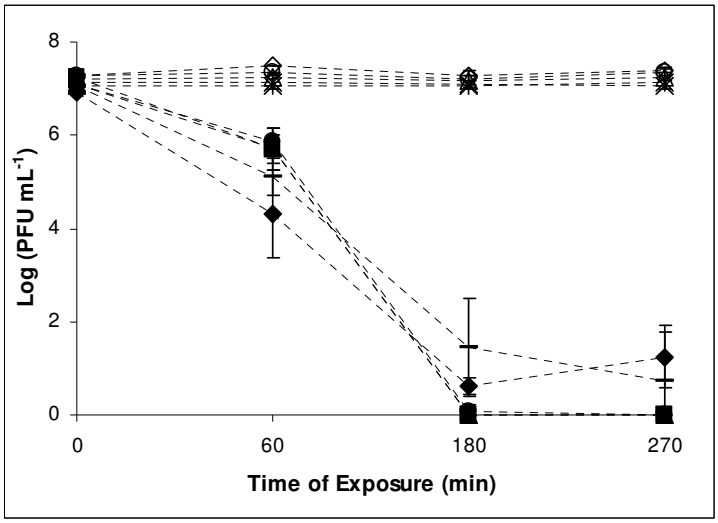

Figure 5. Variation of the numbers of T4-like phage after 30, 60, 180 and 270 min of irradiation, in the presence of 5.0 μM Tri-Py^{+}-Me-PF and different concentrations of L-cysteine (–◊– LC, –△– LCS, –×– DCS, –○– DCPS, –✳– DC, –■– 0 mM L-cysteine, –●– 5 mM L-cysteine, –▲– 10 mM L-cysteine, –‑‑– 50 mM L-cysteine, –♦– 100 mM L-cysteine). Each value represents mean ± standard deviation of three independent experiments, with two replicates each. Error bars represent standard deviations.

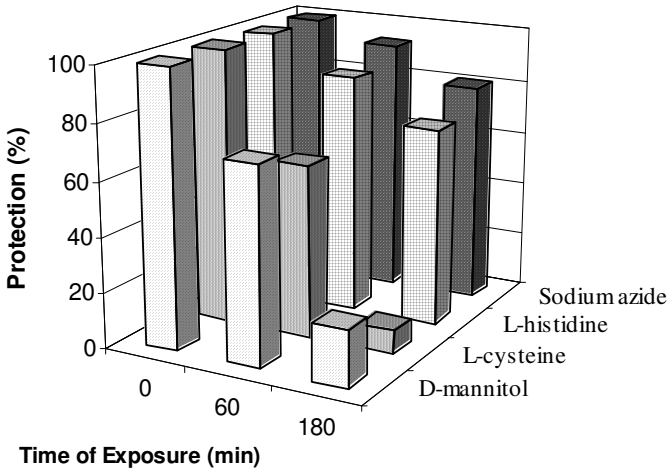

Figure 6. Percentage of protection of T4-like phage photoinactivation after 0, 60 and 180 min of irradiation, in the presence of 5.0 μM Tri-Py^{+}-Me-PF and 100 mM of singlet oxygen quenchers or 100 mM of free radicals scavengers. Each value represents mean ± standard deviation of three independent experiments, with two replicates each.

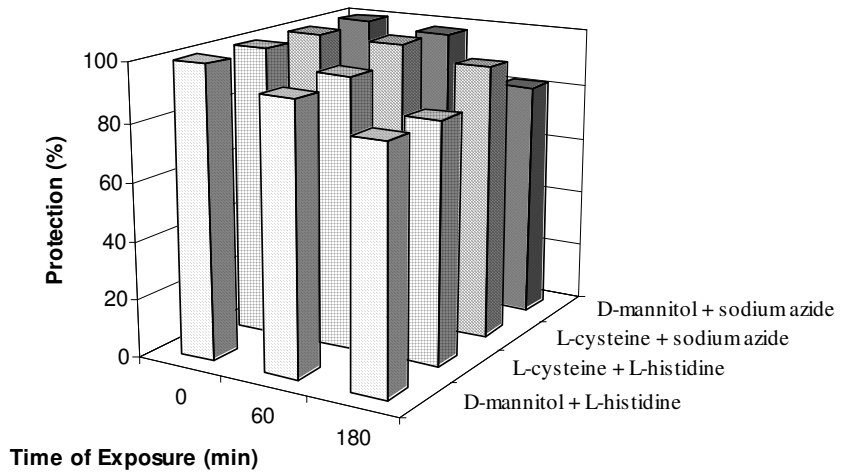

Figure 7. Percentage of protection of T4-like phage photoinactivation after 0, 60, and 180 min of irradiation, in the presence of 5.0 μM Tri-Py^{+}-Me-PF and 100 mM of singlet oxygen and free radicals scavengers. Each value represents mean ± standard deviation of three independent experiments, with two replicates each.

T4-like phage photoinactivation was also evaluated in the presence of two different scavengers at 100 mM (a singlet oxygen quencher plus a free radicals scavenger) (Figure 7). The highest protection of phage photoinactivation by the PS was observed for L-cysteine plus sodium azide (0.4 log of reduction, 93% of protection), and the lowest protective effect was observed for D-mannitol and sodium azide (1.3 log of reduction, 82% of protection). However, the differences between all tested combinations were not statistically significant (ANOVA, $p > 0.05$). The results also show that the rate of phage photoinactivation is almost the same, whether singlet oxygen and free radicals scavengers are tested in separate assays, or combined within the same sample (Figures 6 and 7).

In the absence of scavengers, Qβ phage was inactivated to the limit of detection by Tri-Py^{+}-Me-PF at the concentration of 0.5 μM (reductions of 7.2 log), after 90 min under white light irradiation (Figure 8). In the presence of sodium azide (100 mM), the rate of phage inactivation was considerably reduced (4.4 log of reduction, 39% of protection) when compared with the control without scavenger (0 mM) (ANOVA, $p < 0.05$) (Figure 8). D-mannitol (100 mM) did not exert any protective effect on Qβ phage inactivation after 90 min of irradiation (reductions of 7.2 log) (Figure 8). When sodium azide and D-mannitol (100 mM) were combined, in the same sample, the rate of phage inactivation after 90 min of irradiation was identical to that observed when the scavengers were tested in individual assays (Figure 8) (ANOVA, $p > 0.05$).

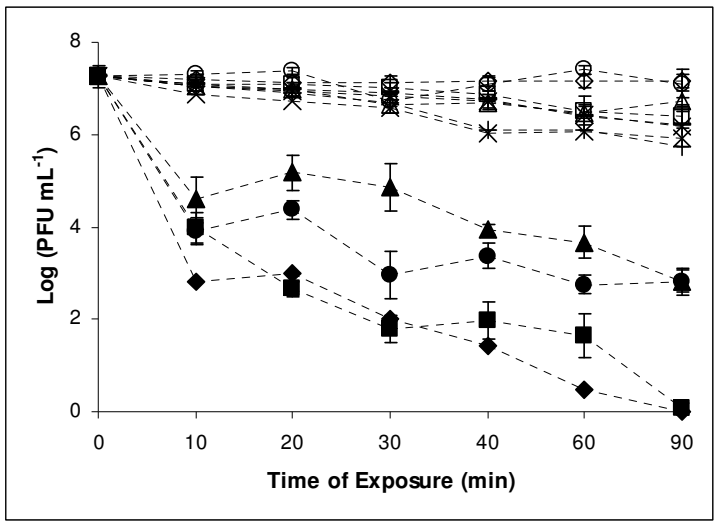

Figure 8. Variation of the numbers of Qβ phage after 10, 20, 30, 40, 60 and 90 min of irradiation, in the presence of 0.5 μM Tri-Py$^+$-Me-PF, singlet oxygen scavenger sodium azide and free radicals scavenger D-mannitol (–◊– LC, –✳– LC sodium azide, –×– LC D-mannitol, –○– DC PS, –△– DC sodium azide, –– DC D-mannitol, –□– DC sodium azide + PS, –+– DC D-mannitol + PS, –■– 0 mM, , –▲– 100 mM sodium azide, –♦– 100 mM D-mannitol, –●– 100 mM sodium azide + D-mannitol). Each value represents mean ± standard deviation of three independent experiments, with two replicates each. Error bars represent standard deviations.

Discussion

It is well established that the efficiency of microbial PDI is related to the ability of a PS to generate singlet oxygen (type II mechanism) and/or free radical species (type I mechanism) in the course of the photodynamic process. For this reason it is important to evaluate the type of ROS produced during PDI in order to determine the suitable conditions in which microbial photosensitization should operate and to design improved PS molecules. The simplest approach for determining whether singlet oxygen or free radicals are involved in a photodynamic reaction is to study the inhibitory effect of various scavengers or traps (Ochsner, 1997; Girotti, 2001).

The present study clearly demonstrates that (1) type II (production of singlet oxygen) is the main mechanism of T4-like and Qβ phages photoinactivation by Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me; (2) the protective effect of the scavengers varied with the concentration of the scavengers, as already shown in other studies (Abe et al, 1997; Gábor et al, 2001; Egyeki et al, 2003); and (3) the scavengers protective effect was higher in T4-like (DNA) than in Qβ (RNA) phages PDI, which was not yet emphasized in other studies.

According to the results obtained, type II (production of singlet oxygen) was clearly the main mechanism of T4-like phage photoinactivation by 5.0 μM Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me and of Qβ phage PDI in the presence of 0.5 μM Tri-Py$^+$-Me-PF. The PDI of T4-like phage was largely reduced by 100 mM of singlet oxygen quenchers sodium azide (80 and 90% of protection in the presence of Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me, respectively) and L-histidine (72 and 78% of protection of T4-like phage photosensitization by Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me, respectively). Qβ phage PDI was reduced by sodium azide (39% of protection). These results are in accordance with what was stated in previous papers about the main role of type II mechanism in viral PDI (O'Brien et al, 1992; Rywkin et al, 1992;

Lenard and Vanderoef, 1993; Müller-Breitkreutz et al, 1995; Dewilde et al, 1996; Wainwright, 2003; Badireddy et al, 2007; Mroz et al, 2007).

Although sodium azide seemed to be the most protective singlet oxygen quencher, the differences between both quenchers were not statistically significant (ANOVA, $p > 0.05$). In the presence of Tri-Py^+-Me-PF, the lowest quencher concentrations (5, 10 and 50 mM) conferred a protection less than 43% for sodium azide and a protection of 52-76% for L-histidine. In the presence of Tetra-Py^+-Me, the protection conferred by the same concentrations of sodium azide was considerably higher; however, the protection conferred by L-histidine was lower, with values ranging from 72 to 85% and 50 to 68%, respectively. These results are also in accordance with other studies (Abe et al, 1997; Gábor et al, 2001; Egyeki et al, 2003) that showed an increased degree of protection with the increase of the quencher concentration.

Free radicals scavengers were far less protective for T4-like phage, when compared with singlet oxygen quenchers. T4-like phage was reduced by 5.7 and 5.8 log in the presence of both PS and 100 mM D-mannitol (≤ 20% protection), after 180 min for Tri-Py^+-Me-PF and 270 min for Tetra-Py^+-Me. D-mannitol (100 mM) was slightly more protective than L-cysteine after 180 min, exhibiting a rate of T4-phage inactivation of 6.2 log (9% protection) with Tri-Py^+-Me-PF. The differences between both scavengers were, however, not statistically significant (ANOVA, $p > 0.05$).

Qβ phage PDI proceeded in a similar way, when sodium azide and D-mannitol were tested in the presence of Tri-Py^+-Me-PF. Singlet oxygen quencher sodium azide was also far more protective when compared with free radical scavenger D-mannitol. Qβ phage PDI by 0.5 μM Tri-Py^+-Me-PF in the presence of sodium azide at 100 mM after 90 min of irradiation with white light was of 4.4 log (39% protection) and of 7.2 log in the presence of 100 mM D-mannitol (0% protection). However, the protection conferred by the two scavengers for Qβ phage (RNA phage) was lower than that observed for T4-like phage (DNA phage). This can be attributed to the fact that non-enveloped RNA phages are more easily photoinactivated when compared with the DNA ones (Costa et al, *submitted*) and, consequently, the non-scavenged ROS produced during the PDI process can still be enough to achieve a substantial phage inactivation.

For both phages, the rate of phage inactivation was of the same order of magnitude whether the effect of singlet oxygen quenchers and free radicals scavengers was tested separately in independent assays or combined in the same sample, which means that there is not a synergistic effect of both types of scavengers, as observed in other studies (Gábor et al, 2001; Egyeki et al, 2003).

Photosensitization by type II mechanism was also observed when Tetra-Py^+-Me and Tri-Py^+-Me-PF were used for the photodynamic inactivation of bacteria. *E. coli* photosensitization was significantly reduced by the presence of sodium azide (84% and 87% of bacterial protection, respectively), but less reduced in the presence of free radical scavenger D-mannitol (8% and 3% protection, respectively for Tetra-Py^+-Me and Tri-Py^+-Me-PF) (Tavares et al, 2011).

However, in spite of the major contribution from singlet oxygen, the participation of free radical species cannot be ruled out. Some studies also indicate a contribution of free radicals in phage PDI, showing that type I mechanism may be equally or even more effective than type II mechanism (Gábor et

al, 2001; Egyeki et al, 2003). For this reason, it is important to take into account not only the PS excited states, but also the whole family of oxy-radicals, such as superoxide anion and hydroxyl, alkoxy and peroxy radicals, as intermediate reactive species (Sabbahi et al, 2008).

In conclusion, the results obtained in this study show that the inactivation of T4-like and Qβ phages by PAR white light (40 W m^{-2}) in the presence of Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me, at 5.0 (T4-like phage) and 0.5 μM (Qβ phage), occurs predominantly by a type II mechanism (singlet oxygen), independently of the phage type. However, as RNA-type phages are more easily photoinactivated when compared with DNA-type ones, the protection conferred by the scavengers during the PDI process is lower and this should be taken into account when the main mechanism involved in PDI of different viruses is to be studied by the use of scavengers.

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CHAPTER 5

A fast screening method (IR spectroscopy) to evaluate viral protein damage induced by photosensitization

Abstract

Microbial photodynamic inactivation (PDI) results in the production of reactive oxygen species (ROS), like free radicals and singlet oxygen. Toxic effects of ROS include severe damage to molecular structures such as proteins, lipids and nucleic acids. Viral structures like the envelope lipids and proteins, the capsid proteins and the nucleic acids, are targets for the PDI of viruses. In this study, the effect of viral PDI on phage proteins was studied by two different methodologies, the traditional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the rapid-screening infrared (IR) spectroscopy. A non-enveloped DNA bacteriophage (T4-like phage) was exposed to white light (40 W m^{-2}) during 270 min, in the presence of 5.0 μM Tri-Py^{+}-Me-PF. The damages induced after PDI on phage proteins were evaluated by SDS-PAGE and by IR spectroscopy. Changes on T4-like phage protein profile were clearly revealed by both methods, when compared with the dark (IR spectroscopy) and light (SDS-PAGE) controls. After SDS-PAGE analysis, T4-like phage protein profile was found to be considerably altered by PDI after 90 and 270 min of irradiation. After 270 min of irradiation, seven protein bands presumably corresponding to capsid and tail tube proteins exhibited a weakened intensity, and two other protein bands showed an increased intensity, effects that are related to phage PDI. IR spectroscopy also revealed alterations induced by PDI on phage proteins. After irradiation, the phototreated T4-like phage samples, when compared with the dark controls, revealed a time-dependent alteration on the protein profile, exhibiting an increase at 1595 cm^{-1} and a decrease in the 1629 cm^{-1} peak which is indicative of considerable protein alterations induced by PDI. Both methods reveal significant alterations on T4-like phage proteins after PDI in the presence of Tri-Py^{+}-Me-PF. Data from IR spectra analysis is in good accordance with the ones obtained by SDS-PAGE; since IR spectroscopy is a simple and cost effective method, it can be considered a promising and innovative approach for analysis of viral protein damage induced by photosensitization. Besides, one single IR spectrum can put in evidence damages induced to all viral molecular structures, thus overcoming the delays and complex protocols associated with conventional methods.

Introduction

Photodynamic inactivation (PDI) procedures involving the generation of short-lived reactive oxygen species (ROS) after the irradiation of photosensitizers (PS), in the presence of molecular oxygen,

have been shown as a very promising method for viral inactivation (Wainwright, 2003). The ROS produced by light-activated PS are shown to react with several biomolecules, which happens by two mechanisms: (i) type I mechanism involves electron/hydrogen transfer directly from the excited PS, producing ions, or electron/hydrogen removal from a substrate molecule to form free radical species. These radicals rapidly react with oxygen, resulting in the production of highly reactive species like superoxide and hydroxyl radicals; (ii) type II mechanism involves the production of the electronically excited and highly reactive state of oxygen known as singlet oxygen, which is considered to be the major toxic species in PDI (Jori and Coppellotti, 2007; Konopka and Goslinski, 2007). Due to the high reactivity and short half-life of singlet oxygen and hydroxyl radicals, only molecules and structures that are proximal to the area of their production (areas of PS localization) are directly affected by PDI and subsequently destroyed (Castano et al, 2004). ROS are shown to attack lipids, proteins, carbohydrates and nucleic acids inducing oxidation, cleavage, cross-linking and other modifications, which eventually lead to damages in important biological molecules (Niki, 1991). Different viral structures, such as the envelope, the capsid or the nucleic acid, can be targets for PDI leading to loss of infectivity (Müller-Breitkreutz et al, 1995) by inhibiting the adhesion of the virus to the host cell and its penetration (O'Brien et al, 1992; Lenard and Vanderoef, 1993), or by inducing damages to viral nucleic acids (Specht, 1994). Due to the short half-life and high reactivity of ROS, especially of singlet oxygen, the main targets of PDI are the microbial external structures (Ben-Hur et al, 1992; Lenard et al, 1993; Malik et al, 1993; Schneider et al, 1998; Smetana et al, 1998; Gábor et al, 2001; Egyeki et al, 2003; Floyd et al, 2004; Hotze et al, 2009). It has been shown that enveloped viruses are significantly more sensitive to photosensitization than their non-enveloped counterparts, as the former are inactivated due to oxidation of their external envelope lipids and proteins (Käsermann and Kempf, 1997), that are not present in the non-enveloped viruses. For non-enveloped viruses, capsid proteins are the main targets of PDI (Schneider et al, 1998; Gábor et al, 2001; Egyeki et al, 2003; Floyd et al, 2004; Zupán et al, 2008; Hotze et al, 2009). Core proteins (Müller-Breitkreutz et al, 1995; Egyeki et al, 2003) are also important targets of PDI.

PDI induced protein damage occurs by backbone fragmentation, photooxidation of essential amino acid residues, such as cysteine, histidine, tyrosine, methionine and tryptophan, and by the formation of cross-links among different proteins or among proteins and DNA or RNA, thereby disrupting the proteins folding conformation and leading to the formation of molecular aggregates (Verweij and Steveninck, 1982; Afonso et al, 1999; MacDonald and Dougherty, 2001; Davies, 2003). Of the naturally occurring amino acids only those that possess aromatic or sulphur-containing side chains are readily oxidized by PDI, leading to protein denaturation and loss of functionality (Min and Boff, 2002; Jori and Coppellotti, 2007). The protein analysis by SDS-PAGE and Western blot of herpes simplex virus type 1 (HSV-1) treated with phthalocyanine derivatives revealed irreversible changes in the proteins of the viral envelope, which were thought to be responsible for HSV-1 inactivation. Some phthalocyanine derivatives have also been shown to induce protein cross-links in HSV that might be responsible for the observed loss of infectivity (Malik et al, 1996). Treatment of viruses with the PS merocyanine 540, hypericin or rose bengal lead to extensive cross-linking of envelope proteins, which may impair the

capacity of viruses, such as human immunodeficiency virus (HIV), vesicular stomatitis virus (VSV), HSV, human cytomegalovirus, sindbis virus and friend erythroleukemia virus, to adhere to and to penetrate the host cells (Sieber et al, 1992; Lenard et al, 1993).

Damages in the capsid protein and/or loosening of the protein-DNA interaction were shown to be responsible for T7 phage PDI when treated with a glycoconjugated *meso*-tetraarylporphyrin derivative (Gábor et al, 2001). Nucleic acids of PRD1 and T7 phages were relatively less affected than their proteins after photosensitization in the presence of polyhydroxylated fullerene (Hotze et al, 2009). Cross-linking of phage capsid proteins was shown to be a main cause of phage PDI (Hotze et al, 2009). Photoinactivation of MS2 in the presence of this PS is possibly due to the damages in its A protein which is necessary for infecting its host *Escherichia coli*, since it contains highly reactive amino acids (Hotze et al, 2009). Irradiation of Qβ phage in the presence of increasing concentrations of methylene blue (MB) resulted in exponentially increasing amounts of viral RNA-protein cross-links, suggesting this lesion as the most important event in viral PDI. Oxidative damage of the RNA alone did not directly account for Qβ phage inactivation (Floyd et al, 2004). RNA-protein cross-links were also shown to be the most likely inactivating lesion after treating Qβ phage with MB (Schneider et al, 1998).

The studies focusing on the evaluation of proteins as targets of viral PDI are based on conventional laborious methods, such as SDS-PAGE and Western blot (Melki et al, 1994; Abe and Wagner, 1995; Bachmann et al, 1995; Smetana et al, 1998; Hotze et al, 2009). New fast and cost-effective methodologies are needed to determine the main targets of PDI. Since the late 1940s, IR spectroscopy has become an accepted tool for the characterization of biomolecules (Margarita and Quinteiro, 2000). IR spectroscopy is a form of vibrational spectroscopy and the IR spectra reflect both molecular structure and molecular environment (Duygu et al, 2009). An advantage of IR spectroscopy is that this method can be applied to powdered, dehydrated or aqueous samples and a single spectrum reflects all microbial constituents, such as proteins, lipids, carbohydrates and DNA (Sacksteder and Barry, 2001). IR spectroscopy not only provides a competitive and rapid identification but, since it allows the study of the microorganism in its intact state, it appears to be a very promising tool also for the study of microbial metabolism, antimicrobials susceptibility and other interactions with drugs (Orsini et al, 2000; Duygu et al, 2009).

As far as it is known, there is only one study focusing on the use of IR spectroscopy to evaluate the damages induced by PDI on phage proteins (Hotze et al, 2009). The aim of the present study is to assess if IR spectroscopy is a suitable rapid-screening methodology to evaluate the major damages induced to T4-like phage proteins after PDI. SDS-PAGE was used as a standard methodology. In this context, phage PDI was performed in the presence of 5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py^{+}-Me-PF), which already proved to be very effective for viral inactivation (Costa et al, 2008; Costa et al, 2010).

Material and Methods

Porphyrin synthesis

The porphyrin (Tri-Py^{+}-Me-PF) used as PS (Figure 1) was prepared in accordance with the methodology referred by Carvalho et al (2010). PS purity was confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy. The PS was dissolved in dimethyl sulfoxide (500 μM stock solution) and sonicated for 30 min before use.

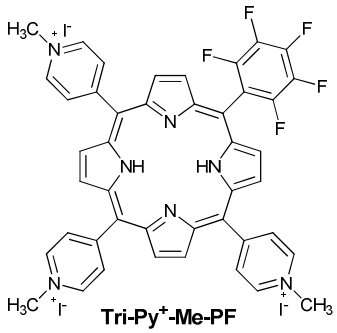

Figure 1. Structure of the cationic porphyrin derivative used in this study to photoinactivate the T4-like bacteriophage.

Phage selection and identification

T4-like phage was isolated from a wastewater-treatment plant of the city of Aveiro (Portugal) using *Escherichia coli C* ATCC 13706 (American Type Culture Collection, Rockville, MD, USA) as host strain (Costa el al, 2008). Phage suspensions with 10^{9} particles per mL were used.

Bacteriophage quantification

T4-like phage quantification was determined by the agar double layer technique (Adams, 1959), using *E. coli* C (ATCC 13706) as host strain. Duplicates of 1.0 mL of non-diluted or of serially diluted samples and 0.3 mL of bacterial host were added to a tube with 5.0 mL of soft TSA growth medium. After manually mixed, tube contents were immediately poured onto a confluent TSA monolayer on a Petri plate. The number of phage plaques was counted after 18 h of dark incubation at 37 ºC in the most convenient series of dilutions and the number of plaque forming units per millilitre (PFU mL^{-1}) was determined.

Experimental setup

Phage inactivation was achieved by exposing the phage suspensions, in the presence of 5.0 μM Tri-Py^{+}-Me-PF, to white light of 40 W m^{-2} (13 fluorescent lamps OSRAM 21 of 18 W each, 380-700 nm), under stirring (100 rpm) at 25ºC, during 270 min. Dark and light controls were also included and were carried out simultaneously. Light control (LC) included the phage suspension without the PS and was exposed to the same irradiation conditions. Dark control (DC) included the phage suspension in the presence of the PS and was covered with an aluminium foil.

Aliquots of test and control samples were aseptically taken at selected times (0, 30, 60, 90, 180 and 270 min) in order to evaluate the efficiency of Tri-Py^{+}-Me-PF for phage inactivation, and for protein analysis. The toxic effect of the ROS produced by Tri-Py^{+}-Me-PF on T4-like phage proteins was

evaluated by SDS-PAGE analysis and by IR spectroscopy, by comparison of the porphyrin-treated samples with the controls. The inhibitory effect of the singlet oxygen quencher sodium azide at 100 mM on phage PDI was also evaluated by SDS-PAGE analysis. Three independent experiments were carried out.

Protein quantification

Protein concentration was determined by the Micro BCA protein assay kit (Pierce, Rockford, USA) according to the manufacturer instructions.

Protein analysis by SDS-PAGE

Phage suspensions (4.0 mL) were centrifuged at 100.000 g for 1 h at 4 ºC. The pellet was recovered and washed with sodium chloride 0.9% (w/v). After a new centrifugation at 100.000 g for 1 h at 4 ºC the pellet was washed with 50 mM Tris-HCl (pH 7) and centrifuged for 1 h at 100.000 g at 4 ºC. The pellet was re-suspended in a denaturing solution containing 8 M urea, 100 mM Tris, 100 mM bicine, 2% 2-mercaptoetanol (v/v) and 2% SDS (w/v), sonicated for 5 cycles (5 s each) and kept at -20 ºC until analysis.

Viral proteins were separated (5.0 µg protein/lane) by SDS–PAGE according to Laemmli (1970). The separation was performed in the Mini-PROTEAN 3 (Bio-Rad) with lab casted SDS polyacrylamide gels (15%). Gels ran for 2 h, at 120 V at 4 ºC. Proteins were visualized by silver staining (O'Connell and Stults, 1997). Each gel image was acquired using the GS-800 calibrated imaging densitometer (Bio-Rad). Apparent molecular weights and band intensities were determined using the Quantity One v4.1 software (Bio-Rad). The apparent molecular weight of the proteins was determined using a molecular weight calibration kit as marker, consisting of a mixture of proteins with 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa (Precision Plus Protein Standards All Blue, from Bio-Rad). Band optical density (OD) was determined as OD per mm^2, subtracted for background and corrected for OD differences between gels, as described earlier (Lemos et al, 2010). All samples were analysed in triplicate.

Infrared spectroscopy and principal component analysis

Infrared spectra were obtained with a mid-infrared spectrometer (Perkin-Elmer Spectrum BX) equipped with a Golden Gate single reflection diamond ATR system. Spectra acquisition of the samples was carried out in the mid-infrared region (between 4000 and 600 cm^{-1}) with a spectral resolution of 8 cm^{-1} and co-adding 32 scans. Phage suspensions (4.0 mL) were centrifuged at 100.000 g during 1 h at 4 ºC. The pellet was recovered, and with the help of a spatula directly placed onto the diamond element, air-dried under aseptic conditions at the sampling accessory and immediately used for spectra acquisition. A total of 9 spectra (3 from each experiment that was conducted in triplicate) were obtained and the spectral region between 1800 and 1300 cm^{-1} was chosen because it was the one that showed differences between treatments.

Results

Without light (DC), Tri-Py^{+}-Me-PF did not exhibit activity against T4-like phage, during 270 min. A similar trend was obtained with the phage in the absence of the porphyrin during the 270 min of irradiation with white light (LC) (Figure 2). T4-like phage was inactivated to the detection limits (7 log of reduction) by 5.0 µM Tri-Py^{+}-Me-PF after 270 min of irradiation. In the presence of singlet oxygen quencher sodium azide (100 mM), the rate of T4-like phage PDI was considerably reduced (1.4 log of reduction) when compared with the phage sample reduction without the presence of the quencher (Figure 2).

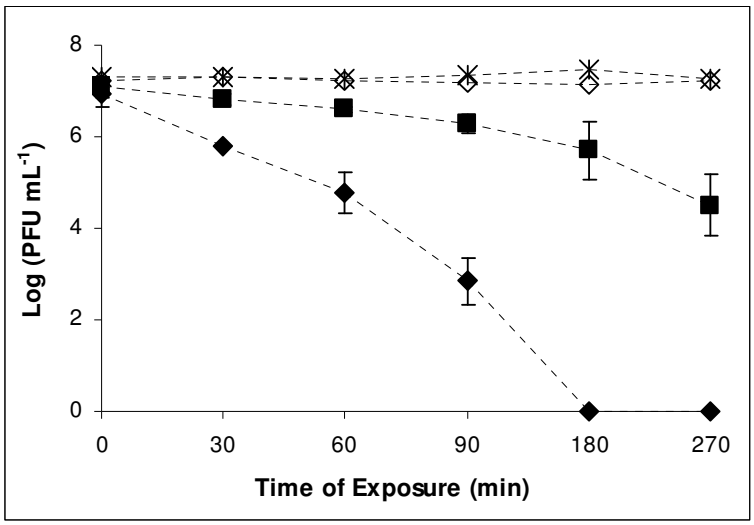

Figure 2. Variation of the numbers of infectious T4-like phage after 30, 60, 90, 180 and 270 min of irradiation, in the presence of 5.0 µM Tri-Py^{+}-Me-PF and 100 mM sodium azide (–◊– LC, –*– DC, –♦– 5.0 µM Tri-Py^{+}-Me-PF, –■– 100 mM sodium azide). Each value represents mean ± standard deviation of three independent experiments, with two replicates each. Error bars represent standard deviations.

SDS-PAGE analysis

Similar results were obtained with the phage protein profile, when the test and control samples were analysed by SDS-PAGE. After 90 and 270 min of irradiation, light and dark controls revealed a protein profile identical to that observed at the beginning of the experiment (Figures 3 A and B).

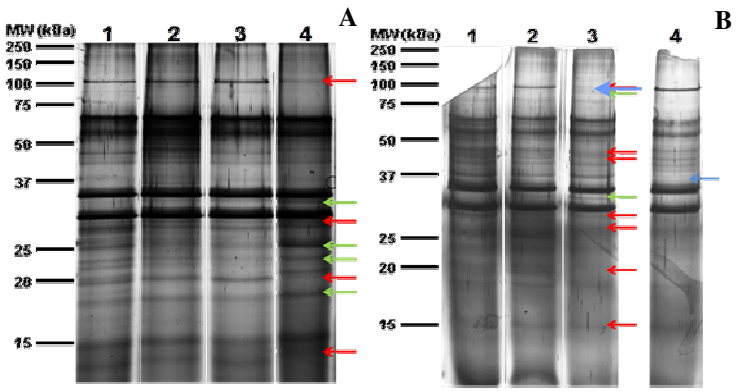

Figure 3. SDS-PAGE analysis of T4-like phage proteins after 90 min of PDI by 5.0 µM Tri-Py^{+}-Me-PF (A). Lane 1: T4-like phage before irradiation (0 min of irradiation); lane 2: light control; lane 3: dark control; lane 4: T4-like phage PDI treated

sample; and after 270 min of PDI by 5.0 μM Tri-Py^{+}-Me-PF (B). Lane 1: light control; lane 2: dark control; lane 3: T4-like phage PDI treated sample; lane 4: T4-like phage PDI treated sample in the presence of 100 mM sodium azide. Green arrows indicate bands with increased protein intensity; red arrows indicate bands with weakened protein band intensity; blue arrows show the protective effect of sodium azide.

Differences in the protein profiles of T4-like phage were distinguishable by SDS-PAGE after phage PDI, when compared with the control samples. The observed differences reflected a change on protein bands intensity after PDI. After 90 min of irradiation, in the presence of 5.0 μM Tri-Py^{+}-Me-PF, the results from SDS-PAGE analysis revealed substantial alterations on phage proteins. Protein bands presumably corresponding to the long tail fiber protein (109 kDa), intron-homing endonuclease (28.2 kDa), head-proximal tip of tail tube (19.7 kDa), and to valyl-tRNA ligase modifier (13.1 kDa) (Miller, 2003; Kurzepa et al, 2009) revealed a weakened intensity, whereas bands presumably corresponding to the proximal tail sheath stabilizer (31.6 kDa), site-specific DNA endonuclease (24.6 kDa), assembly catalyst of distal tail fiber (22.3 kDa), and the baseplate distal hub subunit (20.1 kDa) (Miller, 2003) increased their intensity after PDI, when compared with the light control (Figure 4A). At the end of the experiment (270 min of irradiation), T4-like phage protein profiles exhibited a similar pattern of alterations, with a decrease of the concentration of seven proteins and the increase of two proteins: phage proteins like the long tail fiber protein (109 kDa), vertex head subunit (46 kDa), RNA ligase and catalyst of tail fiber attachment (43.5 kDa), intron-homing endonuclease (28.2 kDa), site-specific DNA endonuclease (24.6 kDa), head-proximal tip of tail tube (19.7 kDa), and valyl-tRNA ligase modifier (13.1 kDa) (Miller, 2003; Kurzepa et al, 2009) revealed a weakened intensity after phage photosensitization. Two protein bands presumably corresponding to the proximal tail sheath stabilizer (31.6 kDa) (Miller, 2003) and one uncharacterized protein of 99.1 kDa exhibited an increase in their intensity, when compared with the control without PS (LC) (Figure 4B).

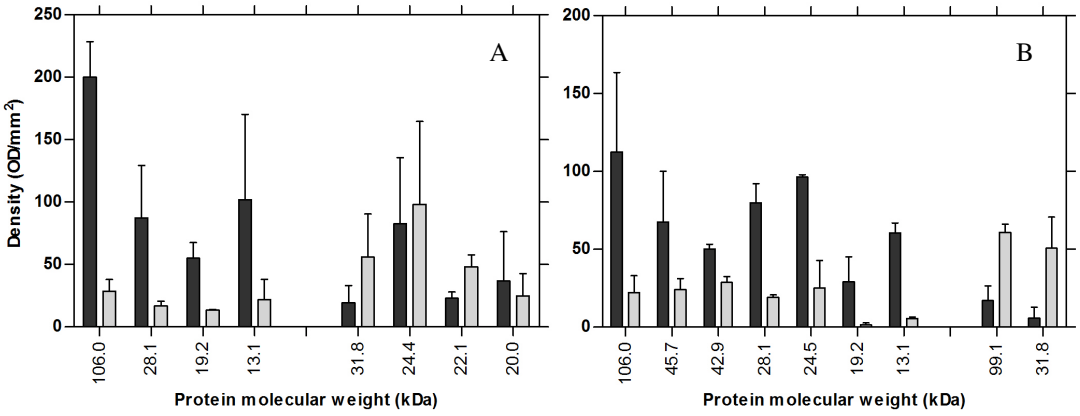

Figure 4. Induced alterations on T4-like phage proteins, as assessed by SDS-PAGE analysis, after 90 (A) and 270 min (B) of irradiation in the presence of 5.0 μM Tri-Py^{+}-Me-PF. Black bars represent the light control (LC); grey bars represent the photo-treated phage samples. Each value represents mean ± standard deviation of three independent experiments.

When the phage samples were treated with singlet oxygen quencher sodium azide, significant alterations were observed on T4-like phage proteins profile, when compared with the porphyrin-treated samples after 270 min of irradiation (Figure 3B).

IR spectroscopy

The data from IR spectroscopy, after 30, 60, 90 and 270 min of irradiation in the presence of 5.0 μM Tri-Py^{+}-Me-PF, revealed several and important alterations on T4-like phage protein profile, when compared with the DC and the PS-treated sample at time 0. Immediately after addition of the PS and without irradiation, a clear change was detected and the maximum of the amide I band was shifted to 1629 cm^{-1}. After 30, 60, 90 and 270 min of white light irradiation, T4-like phage profile revealed changes with the peak maximum shifting to 1595 cm^{-1} and time-dependent alterations, with the phototreated samples at time 270 min being considerably more affected by PDI than the samples after 30 min of irradiation. After 270 min, a clear increase of the peak corresponding to 1595 cm^{-1} and a decrease of the peak corresponding to 1629 cm^{-1} were obtained when compared with the dark control (DC) and the phototreated sample at time 0, revealing significant alterations on peak on the zone of T4-like phage proteins. DC, which was also added of DMSO, at times 0, 60 and 90 min revealed no changes in T4-like phage proteins during the course of the experiment (Figure 5).

Chemical modifications and increasing and decreasing peak areas upon Tri-Py^{+}-Me-PF photosensitization of T4-like phage after 270 min are summarized in Figure 6.

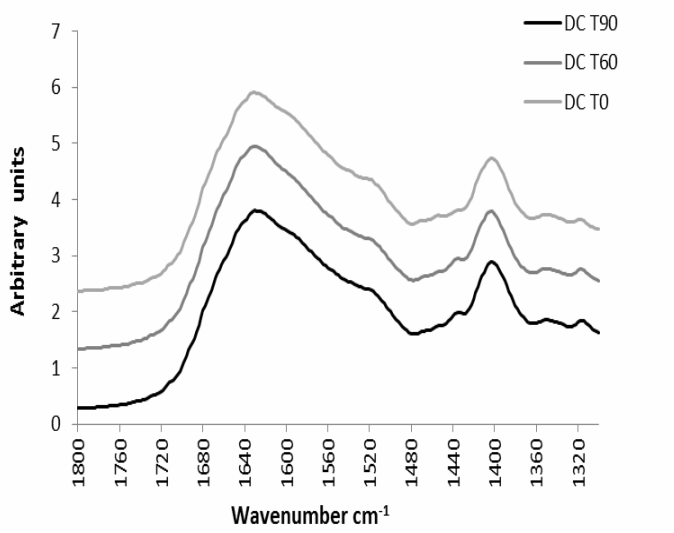

Figure 5. IR spectra of T4-like phage in the dark (DC) after 0, 60 and 90 min in the presence of 5.0 μM Tri-Py^{+}-Me-PF.

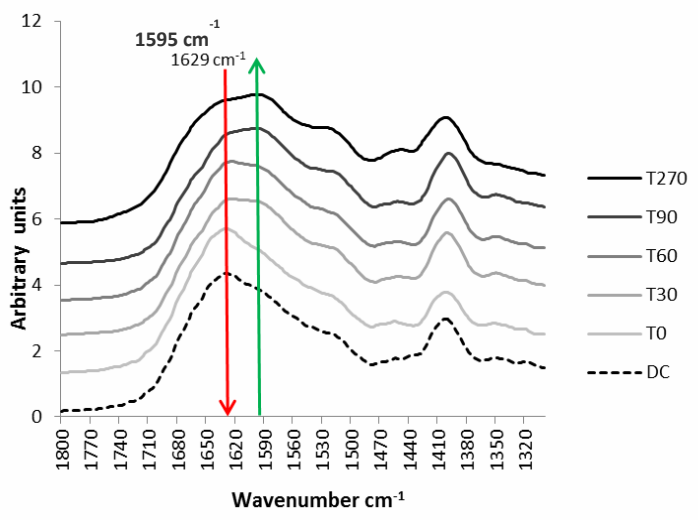

Figure 6. IR spectra of T4-like phage after white light irradiation (40 W m^{-2}) after 0, 30, 60, 90 and 270 min in the presence of 5.0 µM Tri-Py^{+}-Me-PF.

Discussion

It is well established that the ROS generated by PDI are extremely toxic and induce alterations on viral molecular structures, such as protein capsids, lipid envelopes and nucleic acids (Käsermann and Kempf, 1997; Almeida et al, 2011). In fact, it has been shown that both enveloped and non-enveloped viruses can be efficiently inactivated due to the damages induced by PDI on viral proteins (Lenard et al, 1993; Malik et al, 1993; Moor et al, 1997; Smetana et al, 1998; Hotze et al, 2009; Wong et al, 2010). For this reason, it is important to persist in the development of novel, convenient and fast-preparation methods to evaluate the damages induced by photosensitization on viral molecular structures.

The results from this work are in good accordance with what has been stated in the literature, about the effect of PDI on viral proteins (Moor et al, 1997; Lim et al, 2002; Hotze et al, 2009), and demonstrate that T4-like phage proteins were significantly damaged by photosensitization in the presence of 5.0 µM Tri-Py^{+}-Me-PF for 270 min. These results also show that IR spectroscopy can be a promising and fast screening method for the detection of protein damages after PDI, which can be used as a first approach before the traditional methodology of SDS-PAGE analysis.

Since a similar number of bands in SDS-PAGE analysis was observed in all samples, protein degradation does not appear to be a major consequence of ROS attack. SDS-PAGE analysis revealed significant alterations on the density of several protein bands after 90 and 270 min of white light irradiation in the presence of 5.0 µM Tri-Py^{+}-Me-PF. Protein bands putatively corresponding to the long tail fiber protein (109 kDa), vertex head subunit (46 kDa), head-proximal tip of tail tube (19.7 kDa), and four T4-like phage enzymes (Miller, 2003; Kurzepa et al, 2009) revealed a weakened intensity after phage photosensitization. On the other hand, two protein bands, one with the same molecular weight of the proximal tail sheath stabilizer (31.6 kDa) (Miller, 2003) and other uncharacterized protein of 99.1 kDa exhibited an increase in their intensity, when compared with the control without porphyrin, after 270 min of irradiation with white light.

When T4-like phage was photosensitized in the presence of 100 mM of sodium azide (a well known singlet oxygen quencher) the toxic effect of Tri-Py^+-Me-PF was considerably reduced (Figure 2). The protein band presumably corresponding to the long tail fiber protein (109 kDa) (Kurzepa et al, 2009), which exhibited an accentuated weakening of its intensity in the phototreated sample without quencher, showed an intensity identical to the control samples after 270 min or irradiation when sodium azide was added to the treated sample (Figure 3B), confirming that sodium azide protected the T4-like phage from the light induced Tri-Py^+-Me-PF toxic effects.

Studies from other authors also support the same conclusions of the results obtained with this work. Illumination of VSV in the presence of phthalocyanine derivatives caused a decrease in the intensity of the glycoprotein (G protein) band and a slight decrease in the intensity of the matrix protein (M protein) band. Formation of protein cross-links were also detected, after SDS-PAGE analysis (Moor et al, 1997). Contrarily, the formation of protein cross-links was not observed after T4-like photosensitization. In another study (Abe and Wagner, 1995), only minor changes on the relative abundance of VSV G protein and M13 phage coat protein were observed after photosensitization with MB and phthalocyanine derivatives after SDS-PAGE. Also, no additional bands were detected (Abe and Wagner, 1995). Protein damage in T7 phage treated with polyhydroxylated fullerene was revealed by changes in proteins structure, weakening of some protein bands intensity and by the formation of protein cross-links (Hotze et al, 2009).

The results from IR spectroscopy are in agreement with SDS-PAGE data, confirming the existence of significant protein alterations on the photo-treated T4-like phage. Immediately after the addition of the PS, in the dark or before irradiation, a change in the peak of amide I with a maximum at 1629 cm^{-1} was detected. It is consistent with the attributed effect of dimethyl sulfoxide on proteins, which was used to dissolve the porphyrin derivative (Tri-Py^+-Me-PF) in this study. It is well-known that the addition of organic solvents, like dimethyl sulfoxide, to aqueous solutions of proteins greatly affects the conformation of these biomacromolecules (Voets et al, 2010; Fraga et al, 2012). Although at a very small percentage (0.05%), when the dimethyl sulfoxide-dissolved porphyrin was added to the phage sample it could have affected the proteins, inducing aggregation and increase of β-sheets (Voets et al, 2010), which may explain the peak at 1629 cm^{-1} obtained at time 0 and in the dark controls. However, after 30, 60, 90 and 270 min of irradiation in the presence of Tri-Py^+-Me-PF, IR spectra revealed some important peak alterations in the zone corresponding to the region of amides (the same area affected by the DMSO) leading to the conclusion that this particular area of the spectrum is extremely sensitive to the toxic action of the ROS generated by PDI. Modification in amide I can be attributed to oxidative modifications after PDI in protein secondary structures (like β-structures and α-helices), especially since they are told to be important structural constituents of T4-like phage capsid, baseplate and tail tube (Leiman et al, 2003; Mesyanzhinov et al, 2004), which is in good accordance with the data obtained by SDS-PAGE that suggests that some structural phage proteins were changed during the photoinactivation process.

The increase of the protein peak of 1595 cm^{-1} and the decrease on the amide I peak of 1629 cm^{-1} after PDI may be correlated with the observed alterations of phage protein profile as observed by SDS-

PAGE analysis, revealing significant protein alterations that might be related with T4-like phage photoinactivation.

Although not as specific as SDS-PAGE, which gives us a detailed vision of all the proteins that were affected by photosensitization, IR spectroscopy can be efficiently used as an advantageous rapid-screening method, when the effects of photosensitization on the structure of phage proteins are to be studied. In a less time-consuming and in a cost-effective manner, IR analysis can reveal the alterations on all viral molecular structures.

Furthermore, IR spectroscopy has the additional advantage of revealing alterations induced on all viral molecular targets, including the proteins, the nucleic acids and the envelope lipids (whether present) in one single IR spectrum. Thus, when evaluating the relative importance of different molecular targets, IR spectroscopy is suited to reveal the damages induced to all viral constituents, conferring the advantage of combining in the same spectrum the results otherwise revealed by three different laborious conventional methodologies, turning this methodology less time-consuming and cost-effective.

In conclusion, since IR spectroscopy was successfully used to assess the protein damages induced by 5.0 μM Tri-Py^{+}-Me-PF when irradiated with white light (40 W m^{-2}) during 90 and 270 min, and correlated well with the results from SDS-PAGE analysis, it can be used in the future as a promising fast-screening and cost-effective methodology to evaluate the damages induced by viral PDI. After the initial screening, however, to get a further insight into the target damages induced by PDI, conventional methodologies like the SDS-PAGE for proteins may also need to be performed.

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CHAPTER 6

Evaluation of resistance development and viability recovery by a non-enveloped virus after repeated cycles of PDI

Abstract

Nowadays, the emergence of drug resistant microorganisms is a public health concern. The microbial photodynamic inactivation (PDI) has an efficient action against a wide range of microorganisms and can be viewed as an alternative approach for treating microbial infections. The aim of this study was to determine if a model target virus (T4-like bacteriophage), in the presence of the tricationic porphyrin 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py^+-Me-PF), can develop resistance to PDI and recover its viability after photodynamic treatments. To assess the development of resistance to PDI after repeated treatments, a suspension of T4-like bacteriophage was irradiated with white light (40 W m^{-2}) for 120 min in the presence of 5.0 μM of Tri-Py^+-Me-PF (99.99% of inactivation) and new phage suspensions were produced from the surviving phages, after each cycle of light exposure. The procedure was repeated ten times. To evaluate the recovery of viral viability after photoinactivation, a suspension of T4-like bacteriophage was irradiated with white light for 120 min in the presence of 5.0 μM of Tri-Py^+-Me-PF on five consecutive days. In each day, an aliquot of the irradiated suspension was plated and the number of lysis plaques was counted after 24, 48, 72, 96 and 120 h of dark incubation at 37 ºC. The profile of bacteriophage photoinactivation did not change after ten consecutive cycles and no recovery of viability was detected after five accumulated cycles of photodynamic treatment. The results suggest that PDI represents a valuable and promising alternative therapy to treat viral infections, overcoming the problem of microbial resistance.

Introduction

The introduction of antimicrobial drugs in clinic, in the 1930s, coupled with the use of safe and effective vaccines, suggested that infectious diseases could be controlled and, eventually, eradicated. Although antimicrobials have saved countless lives and transformed the practice of medicine, the initial widespread optimism has proven to be premature and infections still remain the leading cause of mortality worldwide. Pathogenic microorganisms are widely spread in nature, there are numerous infection sources, and the unfit and prolonged antibiotic and antiviral treatments have led to greater microbial resistance to these substances.

Antimicrobial-resistant pathogens pose an enormous threat to the treatment of a wide range of serious infections. Nosocomial and community-acquired agents have developed resistance to a wide range

of antimicrobials and have proven to be highly successful in their ability to develop resistance mechanisms, often transferable, against virtually all commonly used antimicrobial drugs (Kimberlin and Whitley, 1996; Pillay, 1998; Taylor et al, 2002; Hamblin and Hasan, 2004; Maisch, 2007; Wainwright, 2009).

Although there are now many different antimicrobial drugs on the market, the vast majority of antimicrobials are antibacterials and, consequently, resistance to antimicrobial drugs is now well documented in bacteria (Nishijima et al, 1993; Cookson, 1998; Kömerik et al, 2000; Fridkin, 2001; Sievert et al, 2002; Konopka and Goslinski, 2007; Maisch, 2007). Human and animal viruses can also develop resistance to antiviral drugs. This may not seem an issue of major concern due to the lack of antivirals available, but it should be kept in mind that the use of antiviral drugs is increasing and that viruses, being genetically flexible, may mutate quickly and, consequently, the emergence of antiviral drug resistance will come as no surprise and will be an everlasting issue. Moreover, the growing availability of antiviral drugs and the larger access to combination therapy can lead to the transmission of resistant viruses. Transmission of viruses with major resistance mutations may indeed result in partial virological response or even therapy failure (Tamalet et al, 2000; Duwe et al, 2001).

The emergence of antiviral drug resistance requires the research of new alternative methods unlikely to cause resistance. Photodynamic inactivation of microorganisms represents a potential alternative to meet that need. The main advantages of PDI are the non-target specificity and the potential absence of resistance mechanisms due to the mode of action and type of biochemical targets (multi-target process) (Jori et al, 2006; Winckler, 2007). Both oxidative mechanisms responsible for the photoinactivation of microorganisms, *i.e.*, type I and type II pathways (Wainwright, 1998; Calin and Parasca, 2009), lead to highly toxic reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$) and free radicals (superoxide and hydroxyl radicals), that are able to irreversibly alter microorganisms' vital constituents, resulting in oxidative lethal damage (DeRosa and Crutchley, 2002; Ergaieg et al, 2008).

The main targets of the viral photodynamic activity are the external structures, such as capsid proteins and envelope lipids, and nucleic acids (Vzorov et al, 2002; Egyeki et al, 2003; Wainwright and Crossley, 2004; Zupán et al, 2004). The damages to the external microbial structures are sufficient to photoinactivate the target microorganism through leakage of microbial contents or inactivation of enzymes (Li et al, 1997; Mettath et al, 1999). As the main targets of PDI are the external microbial structures, the photosensitizer (PS) does not need to penetrate in the microorganism, having little possibility to create or operate any kind of anti-drug mechanisms (Winckler, 2007).

Until now, the majority of research in this field was done essentially with bacterial strains. Photosensitization-resistant mutants have not been found for bacteria (Lauro et al, 2002; Oliveira et al, 2009; Pedigo et al, 2009). *Actinobacillus actinomycetemcomitans*, *Peptostreptococcus micros*, *Vibrio fischeri* and *Escherichia coli* did not develop resistance to PDI after at least ten cycles of subtotal photodynamic inactivation (Lauro et al, 2002; Pedigo et al, 2009; Tavares et al, 2010). Besides that, both *V. fischeri* and *E. coli* were unable to recover their metabolic activity (Tavares et al, 2010). Moreover, all studies that have examined the inactivation of antibiotic resistant bacteria by PDI have found them to be

as susceptible as their native counterparts (Wainwright, 1998; Embleton et al, 2004; Maisch et al, 2005; Trannoy et al, 2006; Maisch, 2007; Grinholc et al, 2008). In spite of all the work with antibiotic resistant bacteria, however, there are few studies of PDI using antiviral resistant strains of viruses. The PS benzoporphyrin derivative was investigated regarding its ability to destroy azidothymidine (AZT)-resistant strains of HIV-1. The results showed that AZT-resistant strains appear to be as susceptible to photodynamic inactivation as AZT-sensitive strains of HIV (North et al, 1994). Although the recovery of viral viability of hepatitis C virus after PDI has already been tested (Cheng et al, 2010), studies concerning the development of resistance after repeated exposure to photodynamic treatment have not been performed yet.

The aim of this study is to determine if a model target virus (T4-like bacteriophage), in the presence of the tricationic porphyrin 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl) porphyrin tri-iodide (Tri-Py^{+}-Me-PF), a very promising PS, can develop resistance to PDI after repeated photodynamic cycles and recover its viability after photodynamic treatments. The T4-like phage was chosen because this phage is used as a model system for understanding general features of viral multiplication (Madigan and Martinko, 2006) besides having some important characteristics that make it useful as an indicator of enteric viruses, namely the same lifetime, similar structure, size and morphology.

Material and Methods

Photosensitizer

The tricationic porphyrin Tri-Py^{+}-Me-PF (Figure. 1) used in this work was prepared in accordance to the literature (Carvalho et al, 2010). The tricationic porphyrin Tri-Py^{+}-Me-PF was selected as PS because this porphyrin, already described by our group, has shown promising results on the photoinactivation of several types of microorganisms (Costa et al, 2008; Oliveira et al, 2009; Carvalho et al, 2010; Tavares et al, 2010).

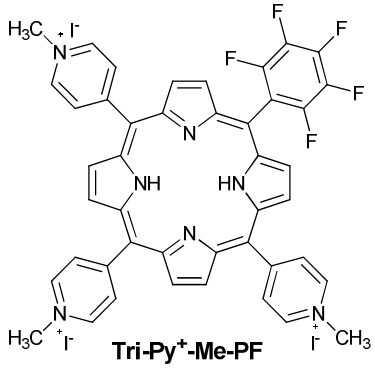

Figure 1. Structure of the PS used for bacteriophage photoinactivation.

Phage selection and quantification

A sample of wastewater from a secondary-treated sewage plant near the city of Aveiro (Portugal) was used to select the somatic bacteriophages of *E. coli* C (ATCC 13706) (Costa et al, 2008). The

quantification of bacteriophages was conducted, in duplicate, by the agar double layer technique (Adams, 1959) using the aforementioned strain of *E. coli* (Costa et al, 2008).

Photoinactivation assay

Phage photoinactivation by Tri-Py^{+}-Me-PF, at the concentration of 5.0 μM, was achieved by exposing the T4-like phage in laboratory conditions to white light (13 fluorescent lamps OSRAM 21 of 18W each, 380–700 nm) with a fluence rate of 40 W m^{-2} (measured with a light meter LI-COR Model LI-250) during defined time intervals, with agitation (100 rpm). Dark and light controls were also included in the experiment and were carried out simultaneously. In the light control (LC), the phage suspension without PS was exposed to the same irradiation protocol. In the dark control (DC), the beaker containing the phage suspension and the PS at the studied concentration (5.0 μM) was covered with aluminium foil to protect it from light exposure. Sub-samples of 1.0 mL of test and control samples were aseptically taken at times 0, 60, 90, 180, and 270 min. The rate of phage inactivation was evaluated through the quantification of the number of phages as previously referred (Costa et al, 2008). Two independent experiments were carried out with two replicates each.

PDI resistance assay

To check for the development of resistance to PDI, new phage suspensions were produced after each cycle of exposure to photodynamic treatment. In order to obtain a modest phage inactivation, the phage suspension in the presence of Tri-Py^{+}-Me-PF (5.0 μM) was exposed to white light in cycles of 120 min (28.8 J cm^{-2}) in the same conditions of the aforementioned photoinactivation assay. This will allow to test if the phages affected by the PS, though not in such a drastic way as occurs when they are irradiated for a long period (270 min), are able to develop resistance to PDI. After each cycle of 120 min, sub-samples of 1.0 mL were aseptically taken and plated by the double agar layer technique. Three phage plates were picked up with an inoculation loop and added to 1.0 mL of an *E. coli* exponential phase culture in TSB medium. After 4–5 h of incubation at 37 ºC, the suspension was centrifuged at 10.000g during 10 min. The pellet was discharged and the supernatant was collected into a new tube, and kept as a new phage stock. An aliquot of 50 μL of the new phage stock was diluted 1:100 in PBS and submitted to a new cycle of photodynamic treatment, using the previously described irradiation protocol. Phage enumeration was also performed as previously described. Dark and light controls were included in all inactivation cycles. This procedure was repeated for ten consecutive cycles. The experiment was performed three times and the results were presented as the average means.

PDI viability recovery assay

Phage photoinactivation in the presence of 5.0 μM Tri-Py^{+}-Me-PF was achieved by exposing the phage to white light (40 W m^{-2}) irradiation in the conditions described above, for 120 min, in order to obtain only a modest phage inactivation. This procedure allows us to check if the phages affected by the PS, though not in such a drastic way as occurs when they are irradiated during 270 min, are able to

recover their viability. Dark and light controls were also prepared as described above and included in all inactivation cycles. Five sub-samples of 1.0 mL of the irradiated phage suspension in the presence of PS, LC and DC samples were aseptically taken at time 120 min and plated by the double agar layer technique. All Petri plates were incubated at 37 ºC, in the dark, and the number of lysis plaques was counted in each of the five Petri plates, after 24, 48, 72, 96 and 120 h, in order to detect the delayed development of lysis plaques. The remaining phage suspensions, after the first cycle of irradiation, were kept at room temperature in dark conditions for 24 h. After this period, phage suspensions were submitted to a new cycle of irradiation (120 min, 40 W m^{-2}) and sub-samples taken and plated following the same procedure. This methodology was repeated five times intercalated by 24 h of dark incubation at room temperature.

Statistical analysis

SPSSWIN 15.0 was used for data analysis. The significance of differences in phage inactivation was assessed using one-way ANOVA. Only the data with normal distribution (assessed by Kolmogorov-Smirnov test) and with homogeneity of variances (assessed by Levene test) was used. A value of $p < 0.05$ was considered significant. All experiments were done at least in duplicate.

Results

Bacteriophage response to photoinactivation

After 270 min of white light irradiation, in the presence of 5.0 μM Tri-Py^{+}-Me-PF, the phage was almost completely inactivated (~ 7 log) (Figure 2). Consequently, in the PDI resistance studies and recovery assays, a shorter irradiation time (120 min) was used, in order to obtain a modest phage inactivation (~ 3 log). It was possible, with such procedure, to obtain a sizeable number of lysis plaques, which were used to study the possible development of resistance and phage recovery.

The results of light and dark controls show that phage viability was neither affected by irradiation itself nor by the direct effect of the PS in the dark (Figure 2). This indicates that the reductions obtained on phage survival after irradiation of the treated samples are only due to the photosensitizing effect of the porphyrin.

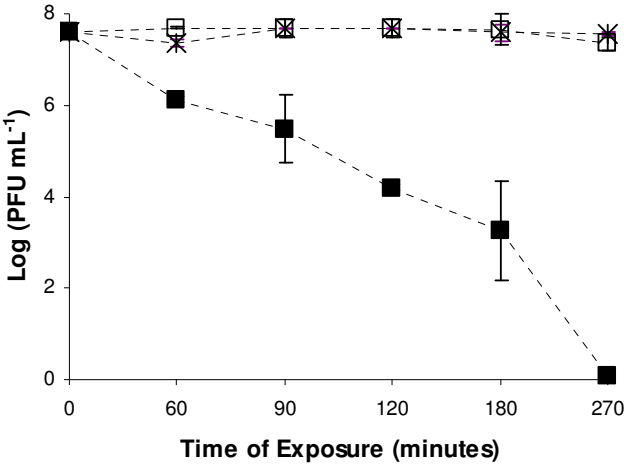

Figure 2. Variation of the number of bacteriophages after irradiation with white light (40 W m^{-2}) in the presence of Tri-Py^{+}-Me-PF at 5.0 μM. (open square: LC, asterisk: DC, filled square: 5.0 μM). The values are expressed as the mean of two independent experiments; error bars represent the standard deviation (small bars are overlapped by the symbols).

PDI resistance study

The rate of phage inactivation in ten consecutive treatments with Tri-Py^{+}-Me-PF at 5.0 μM was almost the same (Figure 3). The number of surviving phages was approximately the same in all cycles of irradiation (~ 5 log of surviving phages). That means no appreciable development of resistance in partially inactivated phages was observed. In fact, the efficiency of photoinactivation underwent no significant change in ten subsequent irradiation cycles (ANOVA, $p > 0.05$). In each phototreatment, the differences between replicates were not significant either (ANOVA, $p > 0.05$).

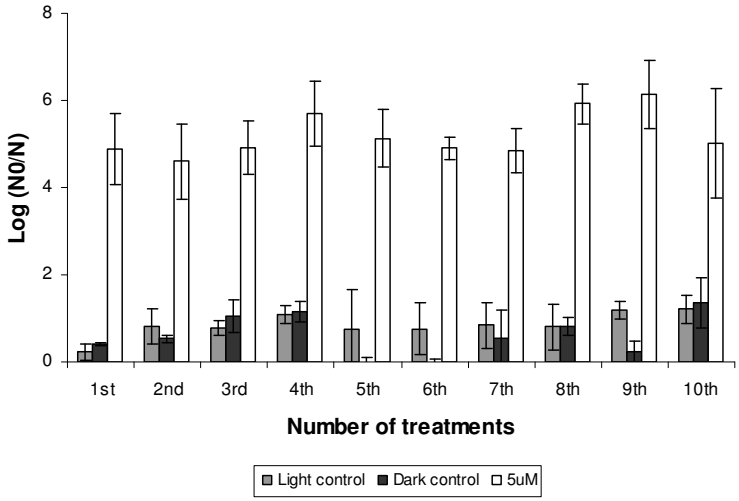

Figure 3. Photodynamic inactivation efficiency during ten consecutive T4-like phage generations, in the presence of 5.0 μM Tri-Py^{+}-Me-PF after 120 min of irradiation with white light (40 W m^{-2}). The values are expressed as the mean of three independent experiments (three different phage plates); N_0 represents the number of initial phages determined by phage plaque counts before the irradiation and N represents the number of surviving phages determined by phage plaque counts after irradiation of each experiment; error bars represent the standard deviation.

PDI viability recovery study

After the first cycle of partial photodynamic inactivation, the phage numbers were reduced to ~ 4 log and the number of lysis plaques did not vary significantly (ANOVA, $p > 0.05$) for each sample during the following 120 h (5 days) of incubation at 37 ºC in the dark (Figure 4). After the second cycle of irradiation, the phage numbers were reduced to ~ 2 log and, as for the first cycle, the number of phage plaques did not increase after 120 h of incubation (ANOVA, $p > 0.05$) (Figure 4). After the third cycle of irradiation, the phages were inactivated to the detection limit and the formation of new lysis plaques was not observed during the following 120 h. The number of lysis plaques was similar in both controls during the 120 h of incubation at 37 ºC, in the five cycles of irradiation (ANOVA, $p > 0.05$).

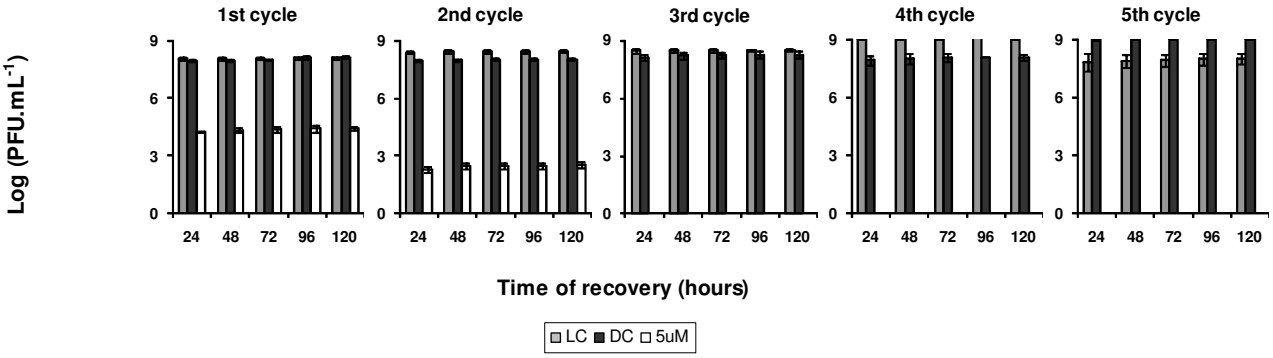

Figure 4. Variation of the number of T4-like bacteriophages after irradiation with white light (40 W m^{-2}) during 120 min in presence of Tri-Py^{+}-Me-PF at 5.0 μM, after 24, 48, 72, 96 and 120 h, for five cycles of treatment. For each cycle five Petri plates were used for both controls and for the sample. Error bars represent the standard deviation.

Discussion

In this study, it was demonstrated that: (1) the tricationic porphyrin Tri-Py^{+}-Me-PF has antiviral activity and effectively inactivates T4-like phage (reduction of ~ 7 logs) after irradiation with white light. The inactivated phages do not recover their viability after 120 h of dark incubation; (2) viruses that survived to short treatments, in the presence of 5.0 μM Tri-Py^{+}-Me-PF, do not develop resistance after ten repeating cycles of PDI.

In general, the development of resistance to photosensitization by microorganisms should be considered as an unlikely event, because this is typically a multi-target process, which marks the difference between PDI and the mechanism of action of most antimicrobial drugs (Maisch et al, 2004). Moreover, it is questionable if microorganisms can develop resistance to ROS, which mediate photodynamic inactivation. Up to now, there are only a few reports concerning a potential specific resistance mechanism against ROS. Although antioxidant enzymes, such as superoxide dismutase, catalase and peroxidase, give protection against some ROS, they do not protect against singlet oxygen (Wainwright and Crossley, 2004) that, according to the literature, is the main ROS through which the PS

exert their photodynamic action (Nitzan et al, 1989; Müller-Breitkreutz et al, 1995; Hadjur et al, 1998; Maisch et al, 2005; Maclean et al, 2008). Moreover, these enzymes are also inactivated by singlet oxygen (Kim et al, 2001). The results of this study corroborate the literature (Lauro et al, 2002; Tavares et al, 2010) in what concerns to the absence of resistance mechanisms in bacteria exposed to photodynamic inactivation events. This finding is of particular interest mostly because the effect of consecutive phototreatments on the possible gain of viral resistance had not yet been addressed.

The results of the resistance assay show that, after ten consecutive phototreatments, the T4-like phage did not exhibit any evident signs of resistance to PDI. The porphyrin Tri-Py^{+}-Me-PF can potentially lead to simultaneous binding to several components/ subunits of the viral capsid and to the nucleic acid (DNA), avoiding the development of resistance to photosensitization. The virus would require multi-site mutations to become resistant, an event with significantly lower probability than single-site mutation which is often sufficient for conferring resistance to small molecule inhibitors.

When the phage was subjected to ten consecutive PDI cycles, the fraction of non-inactivated viruses did not show strong variation between cycles. After the first exposure to white light, the phage plaques produced after irradiation of the viral suspension were used to produce new phage stocks in the presence of host bacterial cells. After ten consecutive sessions of 120 min with 5.0 μM Tri-Py^{+}-Me-PF, any reduction in the efficacy of the photosensitization of T4-like phage was observed. If phage resistance to PDI would occur, significant reductions on phage photoinactivation efficiency would be detected between experiments. This would be interpreted as an indication of enhanced resistance of the phages of later generations in relation to the ones in the initial stocks. However, this was not observed, thus invalidating the hypothesis of the acquisition of resistance to PDI by bacteriophages. Although there were some variations between treatments and even between the three replicas of each treatment, these differences were not significant (ANOVA, $p > 0.05$) and were possibly due to different concentrations of the distinct suspensions of phages produced before each cycle of photoinactivation.

The multi-target property of PDI can also explain the irreversible effects on the bacteriophage viability by the tricationic porphyrin Tri-Py^{+}-Me-PF after 120 min of irradiation. T4-like bacteriophage was irradiated for 120 min in order to obtain only a modest phage inactivation. This allowed us to test if the phages were affected by the PS, though not in such a drastic way, as happened when irradiated for long period of time (270 min), during which all phages are inactivated and are able to recover their viability. During 120 h of incubation after phototreatment, the phages, by being in the presence of their host, have all the necessary conditions to recover from the photodynamic treatment. If new phage plaques would appear in the Petri plates during the 5 days of dark incubation it would mean that the bacteriophages, previously not able to produce plaques, after the ‘‘lethargy period’’ become competent to recover from the PDI treatment. However, the phages were not able to recover their viability in the 120 h post-phototreatment period. This indicates that when the phages are efficiently inactivated they cannot recover from the photodynamic effect. Using a period of irradiation of 120 min, the phages were inactivated to the limits of detection only after three consecutive cycles and did not recover within 120 h. This result indicates that, although one cycle of 120 min of photodynamic treatment with Tri-Py^{+}-Me-PF

is not enough to inactivate all the phages in the suspension, the phages inactivated after this time do not recover their viability. This is in accordance with what was found for hepatitis C virus, which did not recover its viability after exposure to 5,10,15,20-tetraki(3,5-dicarboxy-4,4'-biphenyl)porphyrin (Cheng et al, 2010).

In conclusion, the results of this study show that Tri-Py^{+}-Me-PF has potential use as an efficient alternative to conventional antimicrobial drugs to treat viral infections. With this PS, viral resistance and the potential recovery of viral viability after photodynamic treatment are not a problem.

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CHAPTER 7

DISCUSSION

Several factors including the PS concentration, the number of charges and the nature and location of the *meso*-substituent groups on the PS macrocycle (Costa et al, 2008), can affect the microbial photoinactivation. Among other factors, phage PDI is also influenced by the light source and the light dose and fluence rate used to excite the PS, as demonstrated by the results of the present study. The rate and extent of T4-like phage PDI increased with the increase of the light fluence rate and, for the same PS concentration, a higher light fluence rate considerably reduces the time needed to achieve the same amount of inactivation (~ 7 log). When the same light dose was delivered by the same light source at different fluence rates, the rate of T4-like phage PDI was higher for lower fluence rates, that is, longer irradiation times.

The two cationic porphyrin derivatives, at relatively low concentrations (0.5 and 5.0 μM), when irradiated with white light (40 Wm^{-2}) can efficiently photoinactivate DNA- and RNA-type non-enveloped viruses (reductions of 6-7 log). Bacteriophage photosensitization proceeds mainly *via* the mechanism involving the production of singlet oxygen (type II mechanism), as was revealed by the presence of singlet oxygen quenchers. Free radical scavengers also indicate the participation of radical species (type I mechanism), although at a minor scale. For that reason, their participation on phage PDI cannot be ruled out. Singlet oxygen and free radicals are known to induce damages to several viral molecular structures which include the viral proteins, lipids and nuclei acids (Käsermann and Kempf, 1997). In this study, the ROS produced after irradiation of a cationic porphyrin induced significant alterations on T4-like phage proteins, as revealed by SDS-PAGE analysis and IR spectroscopy. Since viral PDI is a multi-target process, the possibility of resistance development is very improbable and, in fact, T4-like phage did not develop any kind of resistance to viral PDI, even after consecutive cycles of photosensitization. Besides this, once inactivated by photodynamic procedures, T4-like phage did not recover its viability.

In order to elaborate an efficient protocol for viral PDI, it must inevitable focus on several factors influencing the photodynamic effect. The rate and extent of viral photosensitization, among other factors, is affected by the type and concentration of PS and also by the light source used to excite the PS, as well as by the fluence rate throughout the process. The energy dose is also an important parameter that must be taken into account in a viral PDI episode.

Tetra-Py^{+}-Me and Tri-Py^{+}-Me-PF, when irradiated with different sources of light, including fluorescent PAR lamps (40 W m^{-2}), sun light (600 W m^{-2}) and a halogen lamp (1690 W m^{-2}), can efficiently photoinactivate the T4-like phage. However, the rate and the extent of T4-like phage inactivation varied with the nature and concentration of the PS, the light source used to irradiate the phage suspensions, and with the applied energy dose.

Depending on the light source used, different irradiation periods are required to obtain the same rate of phage inactivation. Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me, irradiated under the same conditions,

differently inactivated the T4-like phage. At a PS concentration of 5.0 μM, sources providing higher fluence rates required shorter periods of irradiation to achieve a 7 log reduction. However, when the lowest PS concentrations were used (0.5 and 1.0 μM), the rate of T4-like phage PDI was lower for the halogen lamp, when compared with the other two light sources. Increasing the PS concentration at a fixed light dose leads to an increased rate of inactivation, as does an increase in the irradiation period at a fixed PS concentration. Besides this, the rate of phage inactivation with Tri-Py^+-Me-PF was also dependent on the light source used, even when the given fluence rate was the same. At 40 W m^{-2}, phage inactivation with fluorescent lamps is much more efficient than with the halogen lamp, under the same experimental conditions (fluence rate, irradiation time and PS concentration). Although the PS concentration is the same, the emission spectra of these two lamps are different and, consequently, the energy available to excite the PS is not the same. Part of the energy provided by the halogen lamp (650-800 nm) is probably not used to excite the PS. On the other hand, most of the energy emitted by the set of fluorescent lamps (380-700 nm) coincides with peaks of PS light absorption. Contrarily, the fluence rate of 600 W m^{-2} produced a similar amount of inactivation whether supplied by the halogen lamp or the sun itself. The light source used for PDI must exhibit suitable spectral characteristics that coincide with the maximum absorption wavelength range of the PS in order to generate enough ROS to produce an efficient toxic effect (Robertson et al, 2009).

When the same energy dose is delivered by the same light source, but at different fluence rates (150, 300, 600 and 1200 W m^{-2}), the efficacy of T4-like phage PDI is inversely proportional to the light intensity. Phage PDI is more effective if the energy dose is received during longer irradiation periods (low fluence rates). An increase in the fluence rate enhances the killing effect; however, it seems to be an upper limit of photons to observe this effect. In fact, if the number of photons is higher than this limit, the antimicrobial effect will decrease because the PS in the suspension will not be able to absorb all the photons in excess. This may explain what happened when the high light intensity was used (Qin et al, 2008; Prates et al, 2009). Other authors reached the same conclusion, that low fluence rates caused more damage than high fluence rates for the same total light dose (Foster et al, 1993; Veenhuizen and Stewart, 1995; Robinson et al, 1998). However, another study suggested that the rate of inactivation is a function of the energy dose, irrespective of the fluence rate used to provide that dose. The inactivating light may be applied at high fluence rate over a short time or at a lower fluence rate over a longer time (Maclean et al, 2008).

According to the results obtained with this study, both DNA- and RNA-type bacteriophages can be efficiently photoinactivated by Tri-Py^+-Me-PF when irradiated with white light (40 W m^{-2}) during 270 min; however, RNA-type phages are considerably more susceptible to PDI than the DNA-type ones, requiring ten times lower porphyrin concentration (0.5 μM) and only 60-90 min of irradiation, when compared with the 180-270 min required to produce the same rate of inactivation (6-7 log) on DNA-type phages. According to the observed differences, it seems reasonable to assume that the inactivation process is dependent on the nucleic acid type of phages. It is well known that the capsids of the DNA-type phages used in this work are far more complex and composed of a higher number of different proteins (Fokine et

al, 2004; Mesyanzhinov et al, 2004) than those of RNA-type phages (Cho et al, 2005). The major difference between DNA- and RNA-type phages PDI may not only reside on their nucleic acids, but also on the nature of their capsid proteins, which are considered to be the main targets of non-enveloped viruses PDI (Hotze et al, 2009). The differences in the inactivation kinetics, which were observed between DNA- and RNA-type phages, may be attributed to dissimilarities in their structure and composition. Contrarily to DNA, short-lived RNA molecules are rapidly degraded in living microorganisms by enzymes (RNase), which are very stable even in harsh environments (Sela et al, 1957; Sheridan et al, 1998). Consequently, in the case of viruses, the type of nucleic acid can be an important and determinant factor of the efficiency of viral PDI. Moreover, as for bacteria, the damages in the viral DNA can yet be repaired by the action of DNA repairing systems of the host cells (Imray and MacPhee, 1973), but it is impossible for the host cells to repair the damaged RNA of viruses. Therefore, DNA-type viruses tend to be more genetically stable than RNA-type viruses.

As far as it is known, the results of viral PDI with DNA- and RNA-type phages showed that, under the same protocol, RNA-type phages are more easily inactivated than DNA-type ones (Specht, 1994; Hotze et al, 2009). However, is not easy to draw a clear picture of the effect of the viral nucleic acid type on phage photosensitization due to the lack of diversity of RNA-type phages used in phage PDI (only MS2 phage was tested) in assays were several DNA-type phages are used. PDI effect on phage nucleic acids was already tested for non-enveloped DNA-type phages like M13 (Abe and Wagner, 1995). After methylene blue (MB) treatment, M13 phage DNA was damaged, which correlated with phage photoinactivation, leading to the conclusion that DNA might be an important target of PDI (Abe and Wagner et al, 1995). DNA-type phages like PRD1 and T7 were also studied, but their DNA was shown to be relatively less affected than the proteins after photosensitization in the presence of fullerene derivatives (Hotze et al, 2009). T7 phage PDI in the presence of porphyrin derivatives was shown to be mediated by protein oxidation (Gábor et al, 2001; Zupán et al, 2008) and also by damages on the DNA (Zupán et al, 2008). Oxidative damage of Qβ phage RNA alone by MB did not directly account for Qβ phage inactivation (Floyd et al, 2004). Instead, photoinactivation was mainly mediated by protein damages as revealed by the formation of viral RNA-proteins cross-links (Schneider et al, 1998; Floyd et al, 2004). Although enveloped phages are told to be more sensitive to viral PDI than their non-enveloped counterparts (Lytle et al, 1991; Smetana et al, 1994; Käsermann and Kempf, 1998), due to damages on their external structures, nothing is known about the effect of viral PDI on their lipids. However, the results from Lytle et al (1991) with the enveloped RNA-type ϕ6 phage, although indirectly, are in good accordance with what is said in the literature about the major contribution from lipids for the viral photoinactivation process.

In this study, four DNA- and three RNA-type phages were used, and all RNA-type phages were significantly more easily photoinactivated than any of the DNA-type ones.

It is well established that the efficiency of viral PDI is strongly correlated with the ability of a PS to generate singlet oxygen (type II mechanism) and/or free radical species (type I mechanism), after being excited by light in the presence of molecular oxygen. In order to define the optimal conditions of a PDI

protocol and to design improved and more efficient PS, it is important to determine which ROS are produced during a photodynamic procedure and the main mechanism by which phage PDI occurs.

After several experiments with Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me at 0.5 and 5.0 μM, respectively for T4-like and Qβ phages, irradiated by white light of 40 W m^{-2} during 270 and 90 min, respectively, in the presence of singlet oxygen and free radicals scavengers, it was concluded that type II mechanism (production of singlet oxygen) is the main process responsible for the inactivation of both phages by these PS. Phage PDI was dependent on the PS used and on the concentration of the tested scavengers. T4-like and Qβ phages were mainly photoinactivated by type II mechanism, as revealed by the presence of singlet oxygen quenchers sodium azide and L-histidine. However, the protective effect of the scavengers was lower for Qβ, when compared with T4-like phage. Due to the easier photoinactivation of Qβ phage, it may be assumed that the ROS present in the phage suspension during the irradiation period had enough time to exert their toxic effect on the phage molecular structures before starting to be scavenged.

The results obtained with this study corroborate well with what is stated in the literature (O'Brien et al, 1992; Rywkin et al, 1992; Lenard and Vanderoef, 1993; Müller-Breitkreutz et al, 1995; Dewilde et al, 1996; Wainwright, 2003; Badireddy et al, 2007; Mroz et al, 2007). However, although singlet oxygen is shown as the main ROS on viral PDI, the participation of free radical species cannot be ruled out. In some cases, type I mechanism may be equally or even more effective than type II mechanism (Abe et al, 1997; Gábor et al, 2001; Egyeki et al, 2003; Hotze et al, 2009).

Phage PDI was largely reduced by sodium azide (100 mM). Sodium azide protected T4-like and Qβ phages from the toxic effect of the singlet oxygen generated by Tri-Py$^+$-Me-PF, reducing the rate of phage PDI by 80% and 39%, respectively. In the presence of Tetra-Py$^+$-Me, T4-like phage was considerably more protected by 100 mM of sodium azide (90% of protection). L-histidine (100 mM) was slightly less efficient in protecting T4-like phage from the toxic effect of the singlet oxygen generated by Tri-Py$^+$-Me-PF (72% of protection) and Tetra-Py$^+$-Me (78% of protection). These results show that both PS (Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me) have similar behaviour on viral PDI.

Contrarily, free radical species did not seem to be very active participants in this particular study due to the reduced degree of protection against Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me, conferred by D-mannitol and L-cysteine, when compared with singlet oxygen quenchers. D-mannitol (100 mM) protected T4-like phage by 20%, in the presence of Tri-Py$^+$-Me-PF, after 180 min of irradiation. D-mannitol also did not protect Qβ phage from photosensitization. L-cysteine at a concentration of 100 mM protected T4-like phage by 9% from the free radicals generated by Tri-Py$^+$-Me-PF. Besides this, the rate of T4-like and Qβ phages PDI was identical whether the effect of singlet oxygen and free radicals scavengers was tested separately in independent assays or combined in the same sample, excluding the possibility of a synergistic effect between both types of scavengers, as observed in other studies (Gábor et al, 2001; Egyeki et al, 2003).

It can then be concluded that, although T4-like and Qβ phages PDI proceeds at different times and rates, the mechanism of photosensitization is not affected by the phage type. In fact, T4-like and Qβ

phages PDI occurred in a very similar way, when the same PS and scavengers were used, leading us to conclude that the main mechanism by which viral photosensitization takes place is the same (type II mechanism), whether using a DNA- or a RNA-type phage, depending however on the type of PS which is used.

It is well known that the ROS produced during viral PDI are extremely reactive and induce damages on several important biomolecules, such as proteins, lipids and nucleic acids (Käsermann and Kempf, 1997), to induce oxidation, cleavage, cross-linking and modification, which eventually cause damage (Niki, 1991). As the understanding of a particular PS toxicity implies the knowledge of its microbial targets, it is important to evaluate the damages on viral molecular structures, namely on viral proteins, which are considered to be one of the main targets of viral PDI.

According to SDS-PAGE and IR spectroscopy analysis, T4-like phage proteins were considerably affected by photosensitization after 270 min of white light irradiation (40 W m^{-2}), in the presence of 5.0 μM Tri-Py^{+}-Me-PF, showing that they can be important targets of viral PDI. Though not as specific as SDS-PAGE, which allows a detailed analysis of all the affected proteins, the similar results obtained by SDS-PAGE and IR spectroscopy make the latter a promising and innovative fast screening methodology for the detection of protein damages after viral photosensitization, overcoming the delays associated with SDS-PAGE analysis, in a less time-consuming and cost-effective way. These results are in accordance with what is stated on the literature, which imply the phage proteins as critical targets of viral PDI (Abe and Wagner, 1995; Moor et al, 1997; Lim et al, 2002; Egyeki et al, 2003; Hotze et al, 2009).

SDS-PAGE analysis of T4-like phage proteins after 90 and 270 min of PDI, in the presence of 5.0 μM Tri-Py^{+}-Me-PF, revealed significant alterations on the density of several protein bands. Protein bands presumably corresponding to the long tail fiber protein (109 kDa), vertex head subunit (46 kDa), head-proximal tip of tail tube (19.7 kDa), and four T4-like phage enzymes (Miller, 2003; Kurzepa et al, 2009) revealed a weakening of their intensity after phage photosensitization. Contrarily, other protein bands, like those putatively corresponding to the proximal tail sheath stabilizer (31.6 kDa) (Miller, 2003) and one uncharacterized protein of 99.1 kDa exhibited an increasing in their intensity, when compared with the control without porphyrin, after 270 min of irradiation with white light.

Since the same number of bands was observed in all lanes and the formation of protein agglomerates was not observed, neither protein fragmentation nor the formation of protein cross-links seemed to be a major consequence of ROS attack on T4-like phage proteins. The phototreatment of T4-like phage by Tri-Py^{+}-Me-PF pointed to significant alterations in the proteins structure, which may be the cause of phage photodynamic inactivation.

After 270 min of irradiation in the presence of sodium azide (100 mM), the protein band presumably corresponding to the long tail fiber protein (109 kDa), which is important for host recognition, was not affected by photosensitization. At the end of the experiment, the protein band intensity is identical to that of the control without porphyrin (LC), revealing a significant protective effect of singlet oxygen quencher sodium azide, as was already shown.

In what concerns to the results obtained with the IR spectroscopy, it can also be concluded that T4-like phage exhibits significant protein alterations after 30, 60, 90 and 270 min of irradiation in the presence of 5.0 μM Tri-Py^{+}-Me-PF. Immediately after the addition of the PS, in the dark or before irradiation (time 0), a change in the peak of amide I with a maximum at 1629 cm^{-1} was detected. It is consistent with the attributed effect of dimethyl sulfoxide on proteins (Voets et al, 2010), which is used to dissolve the porphyrin derivative (Tri-Py^{+}-Me-PF) in this study. However, after 30, 60, 90 and 270 min of irradiation, IR spectra revealed some important peak alterations in the zone corresponding to amides (the same area affected by the DMSO) leading to the conclusion that this particular area of the spectrum is extremely sensitive to the toxic action of the ROS generated by PDI. Reductions in amide I peak areas can be attributed to oxidative modifications in protein secondary structures (like β-structures and α-helices), specially since they are told to be important structural constituents of T4-like phage capsid, baseplate and tail tube (Leiman et al, 2003; Mesyanzhinov et al, 2004).

Damages in external components, as revealed by SDS PAGE analysis and IR spectroscopy, suggest that T4-like phage PDI resulted from the loss of ability to bind to the host receptors and subsequent impairment of infectivity, as observed by Hotze et al (2009) after T7 phage PDI in the presence of polyhydroxylated fullerene.

The increase in the peak area corresponding to 1595 cm^{-1} and the decrease in the region of 1629 cm^{-1}, as revealed by the results of IR spectroscopy analysis may be correlated with the observed protein damages by SDS-PAGE analysis.

From the similar results obtained with both methods, it can be concluded that, although not specific and detailed as SDS-PAGE analysis, which allows the determination of proteins molecular weights and reveals the changes on proteins density and charge and the formation of protein cross-links (Schneider et al, 1999; Floyd et al, 2004; Hotze et al, 2009), IR spectroscopy can be seen as a promising and fast screening method, when the damages on viral proteins after PDI are to be detected. Besides, one single IR spectrum reveals the alterations in all viral molecular structures, which include the nucleic acids, capsid and internal proteins and lipids (whether present). Consequently, IR spectroscopy represents a very promising tool for the evaluation of the molecular damages induced by PDI, since one sample is just enough to reveal the most damaged targets and which one is being the most damaged one under the same PDI conditions. For this reason, IR spectroscopy is less time-consuming and a cost-effective procedure, when compared with the laborious and more expensive SDS-PAGE analysis.

Since viral PDI is typically a multi-target process, the development of resistance to photosensitization is considered to be a very unlikely event (Maisch et al, 2004). However, to make use of viral PDI as an efficient alternative to conventional antiviral drugs, all aspects concerning the possibility of resistance development and viral viability recovery have to be cleared up.

The results from this study demonstrated that T4-like phage does not recover its viability after 120 h of dark incubation at 37 ºC, when treated with 5.0 μM Tri-Py^{+}-Me-PF for 120 min under white light irradiation (40 W m^{-2}). When applied to the same PDI protocol, the T4-like phages that survived to a sub-

lethal photosensitization episode, did not develop resistance, even after ten repeating cycles of porphyrin phototreatment.

These findings are of extreme importance since, as far as it is known, this is the first report focusing on the issue of viral resistance after several episodes of viral photosensitization. Although there are no other studies focusing on the possibility of viral resistance development and/or viability recovery after PDI, the results of this study are in accordance with what is known about bacterial PDI (Lauro et al, 2002; Tavares et al, 2010). These studies also showed that the bacteria exposed to consecutive PDI events did not develop any resistance mechanisms.

As stated above, the ROS produced by Tri-Py^{+}-Me-PF (especially singlet oxygen) can potentially attack several viral molecular structures, such as the capsid proteins and the DNA, which avoids the development of resistance to photosensitization. The phage would require multi-site mutations to become resistant, an event with significantly lower probability than single-site mutation, which is often sufficient for conferring resistance to small molecule inhibitors (like antiviral drugs). On the other hand, the PS used in PDI are not consumed (a situation that only happens when they are not stable under light, undergoing a process called photobleaching) during the process of photosensitization differing from what happens with the conventional antimicrobial drugs, which are consumed after the antiviral treatment. The PS are only used as catalysts for the production of toxic ROS, which also contributes to avoid the development of resistance. Moreover, it has been shown that the PS do not need to penetrate into the microorganisms to achieve an efficient inactivation, which is also another factor favouring the lack the viral resistance (Zupán et al, 2008). After ten consecutive cycles of PDI, of 120 min each, T4-like phage did not suffer any reduction on the rate of inactivation. If phage resistance to photosensitization would occur, significant reductions on phage PDI efficiency would be detected between subsequent experiments. Although there were some variations between treatments and even between the three replicas of each treatment, the differences were not statistically significant (ANOVA, $p > 0.05$) and were possibly due to different concentrations of the distinct suspensions of phages produced before each new cycle of photoinactivation.

The multi-target nature of viral PDI can also explain the irreversible effects on the bacteriophage viability by Tri-Py^{+}-Me-PF after 120 min of irradiation. During the 120 h of incubation after phototreatment, the phages are incubated in the presence of their bacterial host, which offers them all the necessary conditions to recover from the photodynamic treatment. If new phage plaques would appear in the Petri plates, during the 5 days of dark incubation, it would mean that the bacteriophages, previously not able to infect their host, after the ‘‘lethargy period’’ become competent to recover from PDI. As expected, the phages were not able to recover their viability in the 120 h post-phototreatment period. When the phages are efficiently inactivated they cannot recover from the photodynamic effect.

In conclusion, it can be shown that (i) for the same PS concentration, higher fluence rates required shorter irradiation times to produce the same amount of inactivation. Also, the same light dose is more effective if given for longer periods of irradiation. For these reasons, light parameters such as light source,

fluence rate and total light dose played an important role in the effectiveness of phage PDI, and they should always be considered when establishing an optimal antiviral protocol; (ii) bacteriophage PDI in the presence of cationic porphyrin derivatives under white light irradiation, although very efficient for both DNA- and RNA-type phages, is considerably different for these two phage types, with the latter being considerably more susceptible to PDI; (iii) bacteriophage PDI in the presence of Tri-Py^+-Me-PF and Tetra-Py^+-Me proceeds mainly by singlet oxygen generation (type II mechanism). However, the participation of free radicals species (type I mechanism) cannot be ruled out; (iv) PDI significantly affected the phage proteins, as revealed by the changes in proteins density after SDS-PAGE analysis, and by the changes on 1629 and 1595 cm^{-1} peak areas as revealed by IR spectra analysis; and (v) Tri-Py^+-Me-PF can be efficiently used for phage PDI without the risk of viral resistance development or the possibility of the phage to recover its viability, as revealed after consecutive episodes of photodynamic treatment.

FUTURE WORK

Although bacteriophage PDI is already successfully in use, the understanding of several aspects of the process of photosensitization is still in its infancy. Future work will supply a tool to clear up and get further knowledge of the following points:

- evaluation of the damages induced by photosensitization on phage nucleic acids (DNA and RNA);
- evaluation of the damages induced by photosensitization on the lipids of enveloped phages;
- effectiveness of immobilized photosensitizers for phage photosensitization and evaluation of their possible re-utilization;
- evaluation of the applicability of photosensitization in the inactivation of mixed viral populations;
- evaluation of the efficiency of photosensitization in animal models.

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