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Liliana Andreia dos Santos Costa

Fotoinactivação de bacteriófagos de águas residuais por porfirinas livres e imobilizadas

Sewage bacteriophage photoinactivation by free and immobilized porphyrins

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o júri

presidente

Prof. Dr. António Carlos Matias Correia
professor associado com agregação da Universidade de Aveiro

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Prof. Dr.ª Sónia Alexandra Leite Velho Mendo Barroso
professora auxiliar da Universidade de Aveiro

Prof. Dr.ª Maria Adelaide Pinho de Almeida
professora auxiliar da Universidade de Aveiro

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palavras-chave

Terapia fotodinâmica, porfirinas, bacteriófagos, águas residuais, suportes magnéticos

resumo

A terapia fotodinâmica tem sido usada para inactivar microrganismos através do uso de fotosensibilizadores. Recentemente, tem sido referida a inactivação de bactérias em águas residuais mas não se sabe nada acerca da fotoinactivação de bacteriófagos ambientais, que são frequentemente utilizados como indicadores de vírus entéricos humanos. O obejctivo deste estudo foi avaliar a utilização de compostos porfirínicos para a inactivação de bacteriófagos de águas residuais. Para isso determinou-se o efeito do número de cargas (compostos com duas a quatro cargas) bem como da sua distribuição nos derivados porfirínicos na inactivação dos fagos. Avaliou-se ainda a influência de diferentes intensidades luminosas e de vários tempos de exposição à luz no processo de fotoinactivação. O melhor fotossensibilizador foi ainda testado na forma imobilizada em nano-partículas magnéticas com carga positiva e neutra. Suspensões de fagos tipo T4 isolados na ETAR de Aveiro (5×10^7 UFP ml^{-1}) foram expostas a luz branca (40 W m^{-2}), luz solar (600 W m^{-2}) e a um feixe luminoso com 1690 W m^{-2}, durante 270, 180 e 60 minutos, respectivamente, a três concentrações do fotossensibilizador livre (0.5, 1.0 e 5.0 μM) e a três concentrações do fotossensibilizador suportado (5.0, 20 e100 μM). As porfirinas tetra- e tricatiónicas inactivaram o fago tipo T4 até aos limites de detecção, mas as porfirinas dicatiónicas não produziram um decréscimo significativo na viabilidade do fago. Qualquer uma das três intensidades luminosas testadas inactivou completamente o fago (> 99.9999%, reduções na ordem dos 7 log), mas em tempos diferentes. Com o feixe luminoso, a inactivação ocorreu aos 25 minutos, mas para os dois outros tipos de luz (solar e branca) a inactivação do fago só foi observada aos 90 e 180 minutos de irradiação, respectivamente. As três formas de porfirina suportadas inactivaram o fago até aos limites de detecção, assim como observado para a forma livre da porfirina. A taxa de inactivação bacteriofágica variou não só com a carga e concentração do fotosensibilizador, mas também com a natureza dos grupos *meso*, a dose de intensidade luminosa e ainda com o tipo de materiais de suporte utilizados para imobilizar as porfirinas. A inactivação total de vírus, mesmo a baixas intensidades luminosas, indica que esta metodologia pode vir a ser aplicada na desinfecção de águas residuais sob condições de irradiação natural (luz solar), mesmo nos dias mais escuros. Este facto, associado à recuperação e possível re-utilização dos fotossensibilizadores imobilizados em matrizes sólidas, facilita a aplicação desta técnica, tornando-a mais económica e sem efeitos nocivos para o ambiente.

keywords

Photodynamic therapy, porphyrins, bacteriopages, wastewater, magnetic supports

abstract

Photodynamic therapy has been used to inactivate microorganisms through the use of targeted photosensitizers. Recently, the inactivation of bacteria in residual waters has been reported, but nothing is known about photoinactivation of environmental bacteriophages, which are often used as indicators of human enteric viruses. The goal of this study was to evaluate the utilization of porphyrinic compounds for sewage bacteriophage photoinactivation. For that purpose we studied the effect of the number of charges (compounds with two to four charges), as well as their distribution on porphyrinic derivatives in phage inactivation. The influence of different light intensities and different irradiation periods in the photoinactivation process were also assessed. The best sensitizer was also tested in the supported form on magnetic nano-particles with positive and neutral charge. T4-like phage suspensions isolated from the ETAR of Aveiro (5 x 10^7 PFU ml^{-1}) were exposed to white light (40 W m^{-2}), solar light (600 W m^{-2}) and to a led light (1690 W m^{-2}), during 270, 180 and 60 minutes, respectively, at three concentrations of the free (0.5, 1.0 and 5.0 μM) and immobilized photosensitizers (5.0, 20 and 100 μM). Tetra- and tricationic porphryins inactivated the T4-like sewage phage to the limits of detection, but dicationic porphyrins did not lead to significant decrease in phage viability. All light sources tested completely inactivated the phage (> 99.9999%) after the respective total irradiation period, with reductions of about 7 log. With the led light, the inactivation occurred in 25 minutes, but for the other two sources of light (solar and white light) the phage inactivation was only observed after 90 and 180 minutes of irradiation. The three forms of immobilized porphyrin inactivated the phage to the limits of detection, as observed for the free form of the porphyrin. The rate of bacteriophage inactivation appeared to vary not only with the photosensitizers charge and concentration, but also with the nature of the meso-substituent groups, the light intensity dose and with the nature of the support materials used for the porphyrin immobilization. A complete viral inactivation, even at low light intensities, means that this methodology can be applied to the disinfection of residual waters under natural irradiation conditions (solar irradiation), even at dark days. This fact, associated to the recovery and possible re-utilization of the immobilized photosensitizers, turns this a less-expensive, easy applicable and environmental-friendly technology.

CONTENTS

CHAPTER 1

INTRODUCTION

1. Wastewater disinfection

It is well known that the high growth of the population in urban areas increases the amount of wastewater to treat. The lack of new technologies for the wastewater treatment induces the reduction of water resources, increasing the environmental pollution and the removal of harmful microorganisms, such as bacteria, viruses and protozoa, assumes greater significance. The wastewater treatment includes processes that permit the physical removal of the microorganisms and/or their destruction or inactivation. Most treatment plants have primary treatment (physical removal of floatable and settleable solids) and secondary treatment (the biological removal of dissolved solids). Secondary treatment typically utilizes biological treatment processes, in which microorganisms convert nonsettleable solids to settleable solids. Sedimentation typically follows, allowing the settleable solids to settle out. After primary and secondary treatment, wastewater is usually disinfected by using chlorine (or other disinfecting compounds, or occasionally ozone or UV light). An increasing number of wastewater facilities also employ tertiary treatment, often using advanced treatment methods. Tertiary treatment may include processes to remove nutrients such as nitrogen or phosphorous, carbon adsorption to remove chemicals and disinfection to inactivate microorganisms.

The microorganisms' elimination, during tertiary treatment, can be reached by a variety of methods, mostly using UV light, ozone, chlorine, hypochlorites and chlorine dioxide (Jemli et al, 2002). Chemical methods, that are the most common technology of ensuring microbiological safety in tertiary effluents, depend mostly on selected chemicals with oxidizing and biocidal properties. Their practical applications range from removing undesirable constituents to disinfecting wastewater effluents. These solutions have not been yet implemented due to environmental and economical imperatives. In the wake of the discovery that chlorination of residual organic material can generate chlorinated-organic compounds that may be carcinogenic or harmful to the environment (Rook, 1974; Williamson, 1981; Carpenter and Beresford, 1986) chlorine credibility is diminishing. Residual chlorine may also be capable of chlorinating organic

material in the natural aquatic environment. Further, because residual chlorine is toxic to aquatic species, the treated effluent must also be chemically dechlorinated, adding complexity and costs to the treatment. With UV light, as no chemicals are used, the treated water taste is more natural as compared to other methods. UV radiation causes damage to the genetic structure of bacteria, viruses, and other pathogens, making them incapable of reproduction. The key disadvantages of UV disinfection are the need for frequent lamp maintenance and replacement and the need for a highly treated effluent to ensure that the target microorganisms are not protected from the UV radiation (by the solids present in the treated effluent). Ozone is considered to be safer than chlorine because, unlike chlorine which has to be stored on site, ozone is generated onsite as needed. Ozonation also produces fewer disinfection by-products than chlorination. A disadvantage of ozone disinfection is the high cost of the ozone generation equipment and the requirements for highly skilled operators.

Photodynamic antimicrobial chemotherapy may provide an approach to meet that need, both in terms of therapy and in terms of sterilization. Phototreatment presents advantages for pathogen inactivation in that the photosensitizing agent does not need to be highly reactive in its native state, in contrast to direct disinfection by chemical oxidants such as chlorine and the resulting reactive oxygen species and free radicals are unlikely to foster microbial resistance (Casteel et al, 2004). Besides, when the photosensitizer is immobilized on solid matrixes, it can be easily recovered and re-utilized, which turns this technology a less-expensive, easy-applicable and an environmental friendly one.

2. Photodynamic therapy and photodynamic antimicrobial chemotherapy

The photodynamic effect was first described by Raab (1900) in the photokilling of unicellular organisms (Protista) in the presence of acridine dyes. Subsequently, there have been many reports of the photomicrobicidal effect of sensitizers (Gábor et al, 2001a; Jemli et al, 2002; Lukšiene, 2005; Banfi et al, 2006; Bonnett et al, 2006; Carvalho et al, 2007). Although photodynamic therapy (PDT) was first designed for cancer treatment, it as shown a huge potential for several other diseases like psoriase, atheromatous plaque, bacterial and viral infections, including HIV (Jemli et al, 2002). The photodynamic antimicrobial chemotherapy (PACT) is based on the PDT concept (Carvalho et al, 2007). PDT is a process in which the activation of

photoreactive compounds (photosensitizers) by light energy results in the production of singlet oxygen and free radicals that are cytotoxic and can damage proteins, lipids, nucleic acids and other cellular components. Due to the highly reactive nature of the radicals and singlet oxygen formed through this process, activity is confined to their immediate environment (Rovaldi et al, 2000). So, the photosensitizer needs to be in close proximity with the target.

The initiating step of photosensitizing mechanism is the absorption of a light photon by the sensitizer, causing a promotion of the drug molecule from its ground state to the extremely unstable excited singlet state with a half-life in the range of 10^6-10^9 s (Lukšiene, 2005). The singlet excited photosensitizer either decays back to the ground state, resulting in the fluorescence or undergoes intersystem crossover to the longer lived triplet excited state. The interaction of the triplet sensitizer with surrounding molecules results in two types of photoactive reaction (Lukšiene, 2005). Both reactions occur simultaneously and in competition. The type I pathway involves electron-transfer reactions from the photosensitizer triplet state with the participation of a substrate to produce radical ions that can then react with oxygen to produce cytotoxic species, such as superoxide, hydroxyl and lipid-derived radicals. The type II pathway involves energy transfer from the photosensitizer triplet state to the ground state molecular oxygen (triplet) to produce excited-state singlet oxygen, which can oxidize many biological molecules, such as proteins, nucleic acids and lipids and lead to cytotoxicity (Hamblin and Hasan, 2004). Whether the type I or the type II pathway will be followed depends on the relative rates of reaction between the sensitizer and microorganism and the sensitizer and oxygen, since these reactants compete for triplet state dye molecules, which are the common substrates of the two reaction pathways. Also important are the relative concentrations of oxygen and substrate. Singlet oxygen is generally accepted as the main damaging species in photodynamic action, although the other reactive oxygen species may also be involved in the process (Capella and Capella, 2003). At the subcellular level, the membranous systems and nuclei are the main targets of the photoprocess (Milanesio et al, 2003).

Some results support the view that the type II reaction plays a major role in virus inactivation by photoactive dyes (Gábor et al, 2001a; Wainwright, 2004). Besides the predominant type II reaction, the type I mechanism can also contribute to the virucidal effect of photosensitization (Gábor et al, 2001a).

3. Characteristics of porphyrinic photosensitizers

As a rule, photosensitizers are usually aromatic molecules that can form long-lived tiplet excited states (Lukšiene, 2005). Porphyrins are tetrapyrrole compounds that present opportunities for synthetic manipulation to modulate physicochemical properties and have potential for PACT (Casteel et al, 2004). These tetrapyrroles contain hydrophobic aromatic rings and can be synthesized with sulfonate groups (Caughey et al, 1998). Tetrapyrroles can bind strongly and selectively to proteins and affect changes in protein conformation, potentially critical properties of an effective inhibitor (Caughey et al, 1998). The large planar core aromatic ring system is likely to be an important feature because it is common to all the tetrapyrrole inhibitors, whereas the peripheral substituents and metal ions (or lack thereof) can vary widely (Caughey et al, 1998). The identification of the most effective inhibitor and therapeutic agent among tetrapyrrole structures will require the optimization of the combination of core structures and substituents (Caughey et al, 1998).

The biochemical and functional effects of porphyrin photosensitization include cross-linking and photo-oxidation of membrane proteins, peroxidation of lipids, inhibition of transport of some essential metabolites, leakage of lysossomal enzymes and increased cellular uptake of porphyrins. Such damage probably permits the penetration of photosensitizer molecules into the cytoplasm (Jemli et al, 2002). Another important feature of porphyrins is their ability to be metalated and demetalated (Scranton and Gou, 2004).

Important parameters in the make-up of the photosensitizer include lipophilicity (relative solubilities in water and lipids), degree of ionization, electric charge, molecular size, non-specific protein binding (Maisch et al, 2004) and other, more specialized factors such as light absorption characteristics (the maximum wavelength of absorption and the intensity of the absorption) and the efficiency of formation of the triplet excited state or of singlet oxygen production and free radicals (Wainwright, 2000).

The toxicity of photosensitizers is nearly universal. In general, all organisms are sensitive to inactivation by the photodynamic effect, namely viruses, bacteria, protista, yeasts, algae, insects and cultured mammalian cells (Wainwright, 1998; Makowski and Wardas, 2001; Lukšiene, 2005). The photocatalytic method can also be applied to the degradation of toxins secreted to water by bacteria (Makowski and Wardas, 2001).

4. Factors affecting photodynamic antimicrobial chemotherapy

The effects of PACT on microorganisms depend on at least four factors: the concentration of the dye, the concentration of O_2, and the appropriate wavelength and intensity of light (Capella and Capella, 2003). However, other factors like the photosensitizer charge, the charge distribution and the presence of *meso* substituent groups can also affect the photodynamic effect (Merchat et al, 1996). The differences in the photoinactivation are also the consequence of the degree of sensitizer hydrophobicity (Jemli et al, 2002). Lipophilicity, ionization and the efficiency of singlet oxygen and of free radicals production must be also included in a putative photoantimicrobial profile (Lukšiene, 2005).

Increasing the concentration of a sensitizer at a fixed light dose leads to increased viral inactivation (Kastury and Platz, 1992). Other authors observed that an increase in the sensitizer concentration led to an increase in the photosensitizing activity (Alouini and Jemli, 2001; Jemli et al, 2002). The results of Maisch and colleagues (2004), demonstrated that the porphyrin-based photosensitizers had concentration-dependent differences in their efficacies of killing the methicillin resistant staphylococcal strains. A tetraphenyl porphyrin inactivated the T7 phage in a concentration-dependent manner. However, at over 2 μM, the process was saturated (Egyeki et al, 2003). Further increase in porphyrin concentration did not lead to higher inactivation rate of T7. Aggregation of porphyrin derivatives in polar solvent and/or photobleaching of photosensitizer can be considered as possible reasons for such behaviour (Egyeki et al, 2003).

Photodynamic effect considerably diminishes in anoxic conditions, indicating that an oxygen atmosphere is required for the mechanism of microorganism photoinactivation (Caminos et al, 2005). In the case of more oxygenated wastewater, it was observed an increased photosensitivity of *Taenia* eggs (Alouini and Jemli, 2001). Dissolved oxygen concentration in water plays an important role in the production of the oxidative species required for the photodisinfection process (Alouini and Jemli, 2001).

A very wide selection of light sources is available, ranging from laser technology to basic tungsten-filament lamps. However, PACT uses low-power light rather than the lasers used in ablative therapy: microbial killing is attained with milliwatts rather than tens (or hundreds) of watts (Wainwright, 1998). Higher light intensity leads to higher rate of photoinactivation after the same irradiation time

(Gábor et al, 2001b). However, an identical incident dose can lead to a different rate of cell survival, depending on the intensity of the emitted light. A similar dose results in a higher rate of inactivation if it is received over a longer time period (Gábor et al, 2001b). Increasing the duration of the irradiation period will improve the wastewater yield treatment. It can compensate for a low concentration of sensitizer or a less efficient sensitizer type or a mediocre quality wastewater (Jemli et al, 2002).

In terms of molecular structure, molecular charge is important in determining antiviral activity. Thus, it is more likely that positively charged photosensitizers will be effective in causing nucleic acid damage than will neutral or anionic congeners (Wainwright, 2004). Positively charged photosensitizers are generally more efficient and can act at lower concentrations than neutral and anionic photosensitizer molecules (Demidova and Hamblin, 2005). The positive charges on the photosensitizer molecule appear to promote a tight electrostatic interaction with negatively charged sites at the outer surface of bacterial cells. In a study by Soukos and collaborators (1998), the pronounced photodynamic effect of the cationic conjugate on *Actinomyces viscosus* may be due to the electrostatic attraction between the conjugate and the negatively charged membrane of the bacterium. It has been found that the efficacy of a photosensitizer in sensitizing Gram-negative bacteria is related to the charge on the photosensitizer itself (Hamblin et al, 2002). This behaviour also appears to apply to nonenveloped viruses such as hepatitis A virus and bacteriophage MS2, whose viral capsids and proteins are negatively charged at physiological pH (Casteel et al, 2004). Hence, in the case of nonenveloped viruses the affinity of the cationic porphyrins may be the result of electrostatic attraction to the negatively charged viral capsids. This kind of associations increases the efficiency of the photoinactivation process (Casteel et al, 2004; Lazzeri et al, 2004). Cationic porphyrins are potential candidates for microbial photoinactivation, because photodamage can be induced to DNA or membranes by sensitizers binding to it or by sensitizers localized in its vicinity (Zupán et al, 2004). Besides this, it is likely that the presence of one or more positively charged groups play an essential role in orientating the photosensitizer toward sites which are critical for the stability of cell organization and/or the cell metabolism (Merchat et al, 1996). However, other studies have shown that *meso* substituted cationic porphyrins can efficiently inactivate bacteria independently of

the number of positive charges (Merchat et al, 1996; Maisch et al, 2004).

Porphyrins with opposite charged groups are more symmetric than porphyrins with adjacent charged groups. The adjacent positive charges in the porphyrin macrocycle should result in a molecular distortion, due to electrostatic repulsion. Actually, studies on the localization and photodynamic efficacy of two cationic porphyrins varying in charge distribution on Murine L 1210 cells showed that the efficacy of the sensitizer with the charged groups in adjacent positions was greater than that of the sensitizer with the charged groups in opposite positions (Kessel et al, 2003). The asymmetric charge distribution at the peripheral position of the porphyrin produces an increase in the amphiphilic character of the structure, which can help a better accumulation in cells (Lazzeri et al, 2004). However, in a study by Maisch and colleagues (2004), the presence of a symmetric orientation of positive charges could bring about an additional effect. It has also been shown that the intracellular localization and binding site of the photosensitizer, which is an important factor in photodynamic antimicrobial chemotherapy, is highly affected by the structure and intramolecular charge distribution of the photosensitizer (Merchat et al, 1996; Minnock et al, 1996).

The symmetry and the size of the *meso* substituent groups also affect the photodynamic effect. In a study by Gábor and collaborators (2001a), the derivative with symmetrical groups was found to be twice as effective as the asymmetrical on the photoinactivation process. Casteel and colleagues (2004) have also observed differences in the photoinactivation rate of hepatitis A virus and MS2 phage when they used tetracationic porphyrins with different alkyl substituent groups. Reddi and colleagues (2002), found a direct correlation between the length of linear hydrocarbon chain of the mono-*N*-alkyl-4-pyridyl porphyrin and the extent of porphyrin accumulation in both Gram-positive and Gram-negative bacteria.

A limited increase in the hydrophobicity of the photosensitizer molecule enhances its affinity for bacterial cells, hence promoting a more efficient photoinactivation (Reddi et al, 2002). Activity data suggest that a moderate degree of lipophilicity may improve photosensitizer efficiency (Banfi et al, 2006). The partial lipophilicity can be achieved either introducing aromatic hydrocarbon side chains or modulating the number of positive charges on photosensitizer (Banfi et al, 2006). Overall, an important role may be performed by the presence of the positive charge(s), since this structural feature could orientate the porphyrin towards specific

sites which are critical for cell functions (Rovaldi et al, 2000). The combination of hydrophobic and hydrophilic substituents in the sensitizer structure results in an intramolecular polarity axis, which facilitates membrane penetration, and results in better accumulation of a new cationic porphyrin in subcellular compartments. It enhances the effective photosensitization (Milanesio et al, 2003). Accordingly to Lazzeri and colleagues (2004), an increase in the amphiphilic character of the photosensitizer, given by the presence of a trifluorophenyl group, appears to enhance its affinity for *E. coli* cells.

The efficiency of PACT is highly dependent on the level and localization of singlet oxygen molecules produced by the photosensitizer (Salmon-Divon et al, 2004). The ability of a molecule to instigate redox reactions and/or to form singlet oxygen depends on the production of a sufficient population of triplet state molecules. This in turn depends on the decay rates of both the triplet and initially formed singlet states (Wainwright, 1998). Other important factors that define the microbial inactivation efficiency are the intracellular localization and binding site of the photosensitizer. Both of them are highly affected by the chemical structure of the photosensitizer (Lukšiene, 2005).

An inherent disadvantage of most dyes is their water solubility, which turns their removal from solution extremely difficult (Käsermann and Kempf, 1998). To overcome this disadvantageous situation, the use of immobilized photosensitizers on inert solid matrixes can be a promising solution for a real situation. Otherwise, the possibility of removing the supported porphyrins from the environment allows the re-utilization of the sensitizers, diminishing the cost and raising the advantage of using an environmental-friendly technology.

Bonnet and colleagues (2006) purposed the use of immobilized photosensitizers on polymeric supports (chitosan membrane) to inactivate bacteria in drinking water. *E. coli* at a level of 10^5 cells mL^{-1}, in a water flow, was inactivated by more than 2 log. They concluded that the use of immobilized sensitizers on inert solid matrixes can be a good solution for a real situation. It has been shown that the photosensitizer can be covalently coupled to solid matrixes without impairment of its photobiological activity (Bonnet et al, 2006). Noticeable disinfection was either observed with the RDP^{2+}/silicone system, for which a rate of cell inactivation by singlet oxygen up to 1.1×10^5 CFU h^{-1} L^{-1} has been measured with *E. coli* and 0.7×10^5 CFU h^{-1} L^{-1} with *Enterococcus faecalis* (Jiménez-Hernández et al, 2005).

Artarsky and collaborators (2006) observed that phthalocyanines can be successfully immobilized on a silicate matrix and used for the photodisinfection of microbially polluted waters. They obtained reductions of about 1 log for *E. coli*, after 120 minutes of exposure. Regenerated cellulose impregnated with 5,10,15,20-tetrakis(*N*-methylpyridinium)porphyrin tetra-p-tosylate has shown some photobactericidal activity against *Staphylococcus aureus*, *E. coli.*, *Proteus vulgaris* and *Bacillus subtilis* (Bonnett et al, 1997). A photosensitizer with piridinium groups coupled to methyl polymetaculate polymer was showed to have effective inhibitory effect in the photoinactivation of *Deinococcus radiodurans* (Faust et al, 1999). Krouit and collaborators (2006), showed the inactivation of Gram-positive and Gram-negative bacterial strains by films with an immobilized porphyrin. Fluorescent dyes have also been incorporated into silica shells by covalently coupling these organic compounds with the sol-gel precursor (Lu et al, 2002).

Suitable supports, as ideal carriers for microbial inactivation, must include the following characteristics: compatibility with the photosensitizer, allowing easy and reproducible immobilization procedures and avoiding leaching out to water; mechanical strength and stability towards sunlight; good oxygen permeability for efficient singlet oxygen quenching; high biocompatibility to maximize the interaction between the immobilized sensitizer and the microorganism; and commercial availability and low cost (Jiménez-Hernández et al, 2005).

Nanoparticles can be ideal carriers of photosensitizer molecules for the photodynamic effect (Wang et al, 2004). Nanomaterials are promising in that they could be made hydrophilic; possess enormous surface areas, and their surface can be modified with functional groups possessing a diverse array of chemical or biological properties; and, since numerous strategies for the preparation of nanomaterials are already in place, sensitizer-loaded nanoparticles can be desirously made by numerous different methods, like chemical covalent grafting or self-assembly (Wang et al, 2004). Magnetic supports usually consist of inorganic magnetic cores and organic or polymeric shells that are either biocompatible or possessing active groups, which can be conjugated to biomolecules such as proteins and antibodies (Liu et al, 2004). The most important parameters of magnetic supports are their size, size distribution, structure, hydrophobicity/hydrophilicity and density of reactive surface groups and the superparamagnetic property (Liu et al, 2004). After being coated with silica, the magnetic nanospheres can be well-dispersed in aqueous

solution with no naked magnetite exposure (Liu et al, 2004). Silicate matrixes have some advantages in comparison with other organic matrixes. They are insoluble in water, resistant towards microorganisms, easy to fabricate, and might be developed successfully for the photodisinfection of water (Artarsky et al, 2006). Excited state lifetimes are longer in the case of cationic supports. In order to have high singlet oxygen production quantum yield, sensitizers with long excited state lifetimes are required since the probability of excited state quenching increases (Jiménez-Hernández et al, 2005).

5. Inactivation of microorganisms by porphyrinic photosensitizers

It has been known since the first days of PACT, later in the last century, that certain microorganisms can be killed by the combination of dyes and light *in vitro*. Initial studies were focused on bacterial photoinactivation but nowadays there is a plethora of several other microorganisms which can be efficiently inactivated by this methodology.

Various types of neutral and anionic photosensitizers exhibit a pronounced phototoxic activity against Gram-positive bacteria, whereas they exert no appreciable cytotoxic activity against Gram-negative bacteria unless the permeability of the outer membrane is altered by treatment with ethylene diamine tetra-acetic acid (EDTA) or polycations (Reddi et al, 2002).

Positively charged photosensitizers, including porphyrins, promote efficient inactivation of Gram-negative bacteria without the need for modifying the natural structure of the cellular envelope and can act at lower concentrations than neutral and anionic photosensitizers (Reddi et al, 2002; Demidova and Hamblin, 2005). It appears that the positive charge favours the binding of the photosensitizer molecule at critical cellular sites that once damaged by exposure to light causing the loss of cell viability (Reddi et al, 2002).

Viral photoinactivation is also dependent on the target microorganisms because it appears to be different for enveloped and nonenveloped viruses. Several viral components, including nucleic acids and lipid-rich envelopes, are potential targets for photodynamic attack (Egyeki et al, 2003). No generalization can be made as to the primary target of sensitized virus inactivation. However, it has been shown that enveloped viruses are significantly more sensitive to photodynamic destruction than

nonenveloped viruses (Käsermann and Kempf, 1998; Wainwright, 2000; Egyeki et al, 2003; Demidova and Hamblin, 2005). It is supposed that the lipids and proteins in the envelope act as photosensitizer binding-sites and viruses can be inactivated due to damages caused in their protein molecules (Egyeki et al, 2003). Porphyrins have been demonstrated as effective virucidal agents *in vitro*, apparently causing photodamage to the viral envelope (Wainwright, 1998). It is more likely that positively charged photosensitizers cause nucleic acid damage (oxidation of guanosine residues), whereas anionic photosensitizers act against the viral envelope (Lukšiene, 2005). Aminolipids and peptides in the viral envelope are potential targets, leading to the inactivation of membrane enzymes and receptors, whereas lipid peroxidation is detrimental to membrane integrity, leading to loss of fluidity and increased membrane permeability (Lukšiene, 2005). For nonenveloped viruses, the photoinactivation effects depend mainly on damages in the protein capsid and/or loosening of protein-DNA interaction like in the photodynamic inactivation of nonenveloped T7 phage (Egyeki et al, 2003). Photomodification of core proteins can also lead to phage inactivation, even if the primary structure of the DNA is preserved, since these proteins play an important role in the early events of infection and DNA penetration (Egyeki et al, 2003). Accordingly to Gábor and colleagues (2001a), both type I and type II mechanisms play a role in the inactivation of T7 when it is sensitized by porphyrins with either symmetrical or asymmetrical *meso* substituent groups. In the case of nonenveloped viruses, the affinity of the cationic or amphiphilic porphyrins may be the result of electrostatic attraction to the negatively charged viral capsids (Casteel et al, 2004). So far, photodynamic inactivation has been proven to be a powerful method for inactivating enveloped viruses, such as murine retroviral vectors (Ben-Hur et al, 1992), human immunodeficiency viruses (HIV-1 and -2) (Schagen et al, 1999; Vzorov et al, 2002), herpes simplex viruses (Silva et al, 2005; Tomé et al, 2007), hepatitis-B (Wagner et al, 2001), and vesicular stomatitis virus (Horowitz et al, 1991) and also for the inactivation of nonenveloped viruses, like the adenovirus (Schagen et al, 1999), hepatitis A virus (Casteel et al, 2004), human papillomavirus (Wainwight, 2004) and T7 (Egyeki et al, 2003), lambda (Kastury and Plaz et al, 1992) and MS2 (Casteel et al, 2004) phages.

6. Applications of photodynamic antimicrobial chemotherapy

Nowadays, the major use of PACT is in the clinical area (Wainwright, 1998; Bonnett, 2000; Wainwright, 2000). PACT is mainly used for the disinfection of blood and blood products, particularly for viral inactivation, although more clinically-based protocols are being developed, namely in the treatment of oral infections (Wainwright, 1998; Egyeki et al, 2003; Hamblin and Hasan, 2004). Porphyrins represent also a promising class of compounds for further development as microbicides to prevent HIV transmission. Modified porphyrins exhibit substancial activity against this virus and their target is the HIV Env protein (Vzorov et al, 2002).

Besides this, the lethal photosensitization of microbes is one of several alternatives to antibiotics that are being developed for use in the treatment of infections due to antibiotic-resistant organisms. A number of studies have demonstrated its effectiveness in animal models (Embleton et al, 2005) and in the treatment of aquaculture waters (Magaraggia et al, 2006), where the use of antibiotics is restricted to a few chemical types.

Recent studies have shown that photoinactivation of bacteria in drinking (Bonnett et al, 2006) and residual waters (Jemli et al, 2002; Carvalho et al, 2007; Alves et al, submitted) is possible under natural irradiation (solar light irradiation). However, to apply this technology to the disinfection of residual and drinking waters, it is necessary to use photosensitizers immobilized on solid supports. Up to our knowledge, the study of Bonnett and colleagues (2006) is the only one concerning the use of immobilized porphyrins on the treatment of drinking water (> 2 logs of reduction even at significant levels of contamination with *E. coli*). In what concerns to the wastewater treatment, as far as we know, there is only one study. It uses a free cationic porphyrin in the photoinactivation of fecal coliforms (2.9 log of reduction), when irradiated with sunlight (Jemli et al, 2002). As far as we know, there are no studies using free or immobilized sensitizers to inactivate viruses in residual waters.

7. Objectives of the work

Although PACT is already in use, the efficiency of the present treatments is not sufficient and the mechanism of viral photoinactivation is still not clear. In what

concerns to PACT applications to wastewater disinfection, as far as we know, there is only one study focused on bacterial photoinactivation by free sensitizers (Jemli et al, 2002). But there is also a lack of information about the best light sources, most efficient porphyrin charges and the efficacy of immobilized photosensitizers for sewage viral inactivation and the efficacy of immobilized photosensitizers. Although there is already one study about the effect of supported sensitizers on bacteria from residual water, much more studies are necessary to understand PACT mechanisms when immobilized porphyrins are applied to wastewater disinfection, namely to viruses inactivation. Free porphyrins when immobilized on solid matrixes represent a promising alternative to this kind of treatment since it allows the recovery and future re-utilization of the photosensitizer. This obviously turns it an easy applicable, less expensive and an environmental safe technology.

The present work intends to clear up some of the last topics, namely:

- Investigation of the effect of six cationic porphyrins containing between two to four charges on the photoinactivation of a somatic sewage bacteriophage of *Escherichia coli*;
- Investigation of the effect of three sources of light with different intensities (40 W m^{-2}, 600 W m^{-2} and 1690 W m^{-2}) on the photoinactivation of a somatic sewage bacteriophage of *E. coli*, by different concentrations of three cationic sensitizers;
- Investigation of the effect of three immobilized porphyrins on solid matrixes on the photoinactivation of a somatic sewage bacteriophage of *E. coli*, by different concentrations of these photosensitizers.

CHATER 2

Sewage bacteriophage photoinactivation by cationic porphyrins: a study of charge effect

Liliana Costa,[1] Eliana Alves,[1] Carla M. B. Carvalho,[1,2] João P. C. Tomé,[2] Maria A. F. Faustino,[2] Maria G. P. S. Neves,[2] Augusto C. Tomé,[2] José A. S. Cavaleiro,[2] Ângela Cunha,[1] and Adelaide Almeida[1*]

[1]CESAM and Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal

[2]Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

* Corresponding author: aalmeida@ua.pt

Summary

Photodynamic therapy has been used to inactivate microorganisms through the use of targeted photosensitizers. Recently the inactivation of bacteria in residual waters has been reported, but nothing is known about photoinactivation of environmental bacteriophages, which are often used as indicators of human enteric viruses. In this study we tested the effect of six cationic porphyrin derivatives with two to four charges on the photoinactivation of a sewage bacteriophage. A phage suspension of 5 x 10^7 PFU mL^{-1} was exposed to white light (40 W m^{-2}), during 270 minutes, at three concentrations of the photosensitizer (0.5, 1.0 and 5.0 μM). Tetra- and tricationic porphyrins inactivated the T4-like sewage phage to the limits of detection, but dicationic porphyrins did not lead to significant decrease in phage viability. At the highest photosensitizer concentration (5.0 μM), the phage was completely inactivated (>99.9999% of inactivation, reduction of 7.2 log) after 270 minutes by the tetracationic porphyrin. Two of the tricationic derivatives also led to phage inactivation to the limit of detection. The rate and extent of bacteriophage photoinactivation appeared to vary with the photosensitizer charge and also with the substituents in the *meso*-positions of the porphyrin macrocycle. Tetra- and tricationic porphyrins can be used with increased efficiency as a new methodology for inactivating sewage bacteriophages that are

frequently used as human enteric viruses' indicators. The complete inactivation of viruses with low light intensity means that this methodology can be used even in cloudy days and during winter, opening the possibility to develop new technologies for wastewater treatment.

Introduction

Disposal of raw or inadequately treated sewage is the main source of pathogens in the aquatic environment. Thus, the processes of disinfection of wastewater and sewage have been a subject of growing scientific interest and public concern. New legislation implies the implementation of procedures to reduce the amount of microorganisms in treated waters. However, there is a lack of new environment-friendly technologies for wastewater treatment.

Chlorination is the most common method of ensuring microbiological safety in tertiary effluents since it effectively inactivates bacteria and viruses. However, its massive utilization may lead to the formation of disinfection byproducts with potential health hazard.[1-3] Moreover, chlorine in addition to killing cells reacts with organic compounds affecting also water taste and smell, which is an inconvenient in water supply. Porphyrin compounds associated to photodynamic therapy can be a promising chemical disinfectant for the inactivation of pathogens as they are effective in inactivating microbial cells without formation of potentially toxic products.[4-6] When photosensitizers are exposed to light in the presence of oxygen, they produce singlet oxygen and free radicals, that are cytotoxic to pathogen population with very limited damage to the host tissue. The oxygen cytotoxic species have been shown to be effective in vitro against bacteria (including antibiotic-resistant strains), viruses, fungi and protozoa.[7-11]

It is documented that, in general, Gram-positive bacteria are efficiently photoinactivated by a variety of porphyrins whereas Gram-negative bacteria are usually resistant to the action of neutral photosensitizers.[12-14] However, cationic porphyrins or analogues have been shown to efficiently photoinactivate Gram-negative bacteria.[9,10,14,15] It has also been shown that the intracellular localization and binding site of the photosensitizer, which is highly affected by the structure and intramolecular charge distribution of the photosensitizer, is an important factor in photodynamic antimicrobial chemotherapy.[16,17] The positive charges promote an electrostatic binding

of the porphyrin to the negatively charged sites at the outer membrane of Gram-negative bacteria, inducing damage that enhance the penetration of the photosensitizer.[16,18]

The mechanism of viral photoinactivation is still not clear but, in general, the phototreatment results suggest that both singlet oxygen (type II reactions) and hydroxyl radical (type I reactions) play a role in viral inactivation.[19,20] Protein capsid, nucleic acids and lipid-rich envelopes are potential targets for photosensitizer binding, but no generalization can be made as to the primary target. It has been shown that enveloped viruses are significantly more sensitive to photodynamic destruction than non-enveloped viruses.[19-22] It is likely that positively charged photosensitizers cause nucleic acid damage, whereas anionic photosensitizers act against the viral envelope.[23] Relatively to the non-enveloped bacteriophages some studies showed that damage in the protein capsid and in the DNA might be responsible for photodynamic inactivation by cationic porphyrins.[20,24] Other studies revealed structural changes in the protein capsid but not in DNA of the photochemically treated phages with neutral porphyrin derivatives.[19] Phage inactivation by neutral porphyrins has been also observed but required higher irradiation periods than cationic porphyrins.[25] However, it has not been reported any full effort to test the effect of the number of positive charges of cationic porphyrins on phage inactivation.

Currently, the major use of photodynamic antimicrobial chemotherapy (PACT) is in the clinical area.[7,10,26,27] A great number of bacteria of clinic importance has been efficiently inactivated by PACT, namely antibiotic-resistant pathogenic bacterial strains.[28-31] The major use of photosensitizers for viral inactivation is in the disinfection of blood and blood products.[27] Some *in vitro* studies showed that porphyrins could inhibit replication of herpes simplex viruses,[32,33] Hepatitis A viruses[25] and influenza A but not several other animal viruses.[34]

Recent studies have shown that photoinactivation of bacteria in drinking[5] and residual water[4,35] is possible under solar light irradiation. However, as far as we know, it has not been reported any attempt to inactivate environmental viruses through PACT. Viruses usually occur in domestic sewage and survive even after secondary treatment.[36] However, at concentrations found in sewage, a limited number appears to be able to produce gastroenteritis. Most important are the hepatitis A and E viruses, caliciviruses (including Norwalk virus), rotaviruses, enteroviruses and astroviruses.

The effect of photodynamic chemotherapy on bacterial viruses (bacteriophages or simply phages), frequently used as indicators of enteric viruses and public health

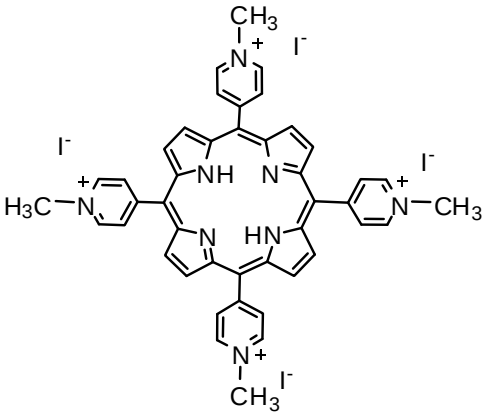
risk,[37] has been applied with success but only to collection phage and just in order to understand the mechanism of phage inactivation.[20,24,38-40]

The aim of this study was to investigate the effect of six cationic porphyrins containing between two to four charges on the photoinactivation of a somatic sewage bacteriophage of *Escherichia coli*. As far as we know, none of the tricationic and dicationic derivatives here reported has ever been used in photodynamic antimicrobial chemotherapy, although the tetracationic porphyrin is an already known compound used in bacterial and phage photoinactivation.

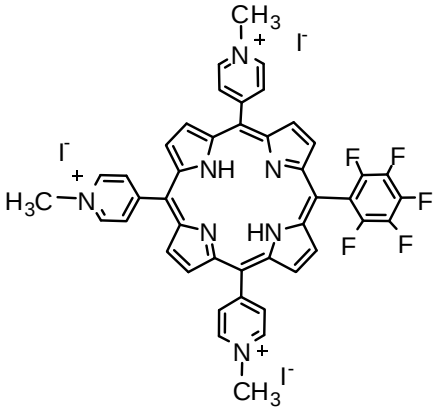
Experimental

Porphyrin synthesis

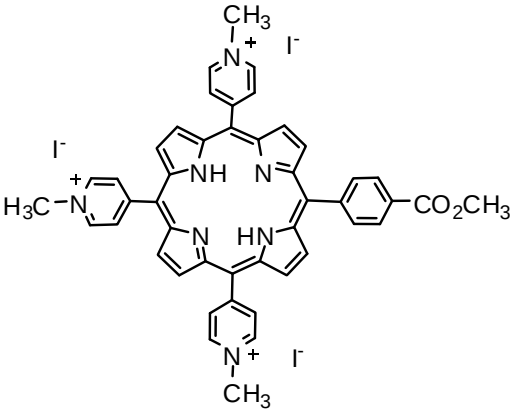
Porphyrins used in this work (Figure 1) were prepared in two steps. First, the neutral porphyrins were synthesized by the Rothemund and crossed Rothemund reactions using pyrrole and the adequate benzaldehydes (pyridine-4-carbaldehyde and pentafluorobenzaldehyde or 4-formylbenzoic acid) at reflux in acetic acid and nitrobenzene.[9,41] These reagents were purchased from Sigma-Aldrich (Madrid). The resulting porphyrins were separated by column chromatography (silica) and then the pyridyl groups were quaternized by reaction with methyl iodide. Porphyrin Tri-Py^+-Me-CO_2Me was obtained by esterification of the corresponding acid derivative with methanol/sulfuric acid followed by quaternization with methyl iodide. Porphyrins were purified by crystallization from chloroform/methanol/petroleum ether and their purities were confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy



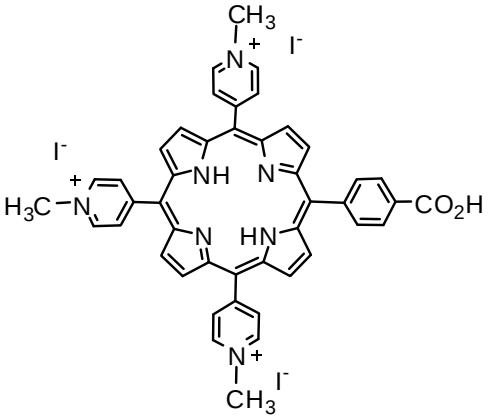
Tetra-Py$^+$-Me
5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin tetra-iodide



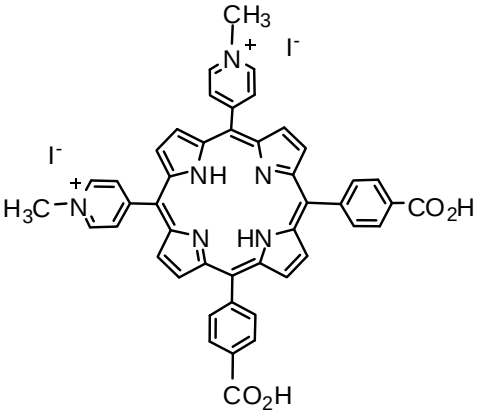
Tri-Py$^+$-Me-PF
5-(pentafluorophenyl)-10,15,20-tris(*N*-methylpyridinium-4-yl)porphyrin tri-iodide



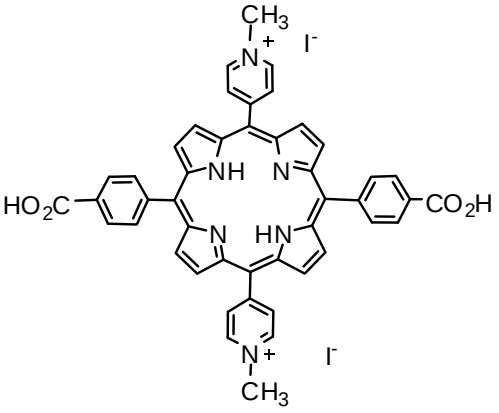
Tri-Py$^+$-Me-CO_2Me
5-(4-methoxycarbonylphenyl)-10,15,20-tris(*N*-methylpyridinium-4-yl)porphyrin tri-iodide



Tri-Py$^+$-Me-CO_2H
5-(4-carboxyphenyl)-10,15,20-tris(*N*-methylpyridinium-4-yl)porphyrin tri-iodide



Di-Py$^+$Me-Di-CO_2H-adj
5,10-bis(4-carboxyphenyl)-15,20-bis(*N*-methylpyridinium-4-yl)porphyrin di-iodide



Di-Py$^+$Me-Di-CO_2H-opp
5,15-bis(4-carboxyphenyl)-10,20-bis(*N*-methylpyridinium-4-yl)porphyrin di-iodide

Figure 1. Structure and the IUPAC name of the six porphyrin derivatives used for photoinactivation of T4-like bacteriophage.

Phage selection and quantification

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). An isolated and morphologically representative phage plaque was picked out with a Pasteur pipette, by aspiration, and was added to 50 mL of an *E. coli* culture in the exponential growth phase. The mixture was incubated with slow stirring (~100 rpm) at 37ºC, until the clarification of the medium, for about 5h. The suspension was then centrifuged at 7,000*g* (Beckman Avanti J-251 centrifuge) during 10 minutes to remove non-infected bacteria and bacterial cell residues. The supernatant, with 10^9 particles per mL, was decanted, added of 2% chloroform and kept at 4ºC. The quantification of phages was determined, in duplicate, by the agar double layer technique[42] using the aforementioned strain of *E. coli*. One millilitre of non-diluted sample or of serially diluted sample and 0.3 mL of bacterial host were added to a tube with 6 mL of soft TSA growth medium. The contents of the tube were mixed by manual rotation and then immediately poured onto a prepared confluent TSA monolayer on a Petri plate. The plates were incubated upside-down at 37ºC in the dark to avoid host bacteria inactivation. After 18 hours of incubation the number of lysis plaques was counted on the most convenient series of dilutions and the number of plaque forming units per millilitre (PFU mL^{-1}) was determined.

Bacteriophage identification

DNA extraction and purification of phage suspension was done using a standard technique.[43] DNA was extracted with phenol saturated with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) followed by extraction with a mixture of chloroform and isoamyl alcohol (24:1). The purified nucleic acid was amplified by PCR using consensus primers that amplify the central portion of capsid gene 23: Mzia 1 (5'-TGTTATIGGTATGGTICGICGTGCTAT-3') and CAP8 (5'-TGAAGTTACCTTCACCACGACCGG-3'). The conditions used for the amplification reaction with these primers involved 35 cycles consisting of 1 minute of denaturation at 95ºC, 1 minute of annealing at 58ºC and 2 minutes of extension at 72ºC. The PCR products were purified using the JETQUICK PCR Purification Spin kit from Genomed and sequenced with BigDyeTerminator v1.1 from Applied Biosystems. The phage was identified as a T4-like phage that has 82% of homology with the Enterobacteria phage RB43. The nucleotide sequence of the phage has been deposited in the GenBank

database under accession nº EU026274.

Irradiation conditions

The effect of cationic porphyrins at different concentrations (0.5, 1.0 and 5.0 μM) was evaluated by exposing a sewage somatic bacteriophage in laboratory conditions to white light (PAR radiation, 13 lamps OSRAM 21 of 18 W each one, 380-700 nm) with a fluence rate of 40 W m^{-2} at defined times. The light fluence rate was measured with a radiometer LI-COR Model LI-250.

Bacteriophage host viability test

As bacteria are sensitive to photosensitizers, the viability of the viral host was evaluated in order to confirm that the phage inactivation was not due to the inactivation of the bacteria by the photosensitizer. So, additional samples of the highest concentration of Tri-Py^{+}-Me-PF (5.0 μM), as well as light and dark control samples, were collected, in each sampling time, after irradiation, and washed by ultra-centrifugation at 28,000*g* (Beckman L8-80K ultracentrifuge, equipped with a swing-out rotor SW28) during 90 min, at room temperature, to remove the porphyrin (washed experiments). The porphyrin-free pellets of phages were re-suspended in 5 mL of PBS buffer, serially diluted and pour plated by the double layer technique. The results obtained were compared with those resulting from direct spread after irradiation (non-washed experiments). 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.

Experimental set up

The efficiency of the cationic porphyrins at different concentrations (0.5, 1.0 and 5.0 μM) was evaluated through quantification of the number of bacteriophages in laboratory conditions.

Knowing that the inactivation of bacteria by cationic porphyrins is very sensitive to the ionic strength,[44] all the experiments were performed using the same experimental conditions. The suspension of phages was diluted in phosphate buffer (PBS) until 5 x 10^{7} PFU mL^{-1} (1000 times higher than that of residual waters) and distributed in 600 mL acid-washed and sterilised glass goblets (20 mL per each of five goblets). The

photosensitizer at concentration 0.5, 1.0 and 5.0 μM (prepared from stock solutions of 500 μM in DMSO) was added to three goblets and the other two goblets were used as dark and light controls. In the light control no porphyrin was added but the goblet was exposed to the same irradiation protocol. In the dark control, the photosensitizer at the highest concentration (5.0 μM) was added to the goblet and it was covered with aluminium foil. The test goblets and the light and dark controls were exposed in parallel to white light (PAR radiation with a fluence rate of 40 W m^{-2}), at 20-25°C, during 270 minutes under stirring (100 rpm). Sub-samples of 1 mL were taken at time 0 and after 30, 60, 90, 180 and 270 minutes and analysed, in duplicate, for bacteriophage number. The Petri plates were kept on the dark immediately after spread 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 of sampling as the mean of the two duplicates in the most convenient dilution series. Viral reduction at each time was calculated by subtracting the mean number of viruses surviving at each time by the initial number (at time zero) and expressed as a $\log_{10}$ values or as a percent reduction. For each photosensitizer two independent experiments were done and the results presented are the average of the two assays.

Statistical analysis

SPSSWIN 14.0 was used for data analysis. The significance of difference in phage inactivation among the six photosensitizers was assessed using one-way ANOVA. The differences in phage inactivation during the incubation period were also evaluated by 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 photocytotoxicity action on the T4-like sewage bacteriophage, by the six cationic porphyrin derivatives (figure 1), was assessed by exposing the test assembly with different concentrations of the photosensitizer (0.5, 1.0 and 5.0 μM) to white light during different times (30, 60, 90, 180 and 270 min).

The results of the viability test (Figure 2) show that the pattern of variation of phage inactivation in non-washed and in washed samples was very similar (ANOVA, $p = 0.308$). This means that the photosensitizer does not affect the bacteria during the dark

incubation of the Petri plates and, therefore, the washing step by ultra-centrifugation, a time consuming procedure that greatly delay the assays, is not necessary. The washed/non-washed test was done at the beginning of the work and only for the most effective porphyrin at the highest concentration (5.0 μM). In the remaining experiments this step was not done, but the Petri plates were incubated in the dark.

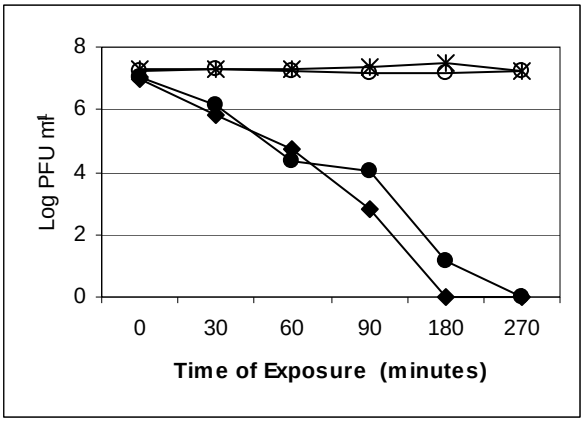


Figure 2. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation, in the presence of 5 μM of Tri-Py^{+}-Me-PF porphyrin in washed and in non-washed phages. (—o— light control, —✳— dark control, —●— washed phages, —♦— non- washed phage).

Without light (dark control), the porphyrins at the highest concentration (5.0 μM) did not exhibit activity against the phage during the 270 minutes of exposure (Figures 3 to 8). A similar trend was obtained with the phage in the absence of the porphyrins during the 270 minutes of irradiation with white light (light control) (Figures 3 to 8). It is important to note that the PAR radiation used in the experiments (380 – 700 nm) do not affect viral viability.

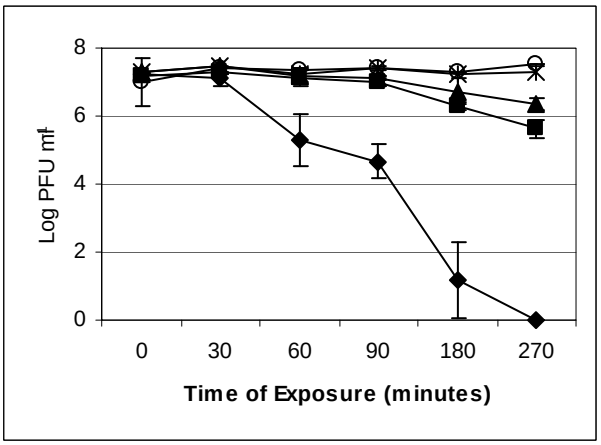


Figure 3. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation, in the presence of porphyrin Tetra-Py^{+}-Me. (—o— light control, —✳— dark control, —▲— 0.5 μM, —■— 1 μM, —♦— 5 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

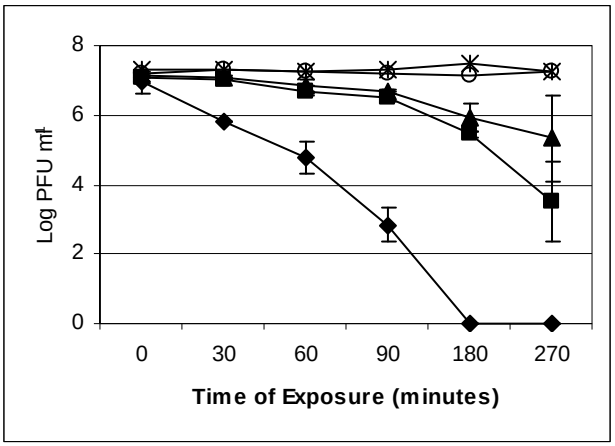


Figure 4. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation, in the presence of porphyrin Tri-Py^{+}-Me-PF. (—o— light control, —∗— dark control, —▲— 0.5 μM, —■— 1 μM, —♦— 5 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

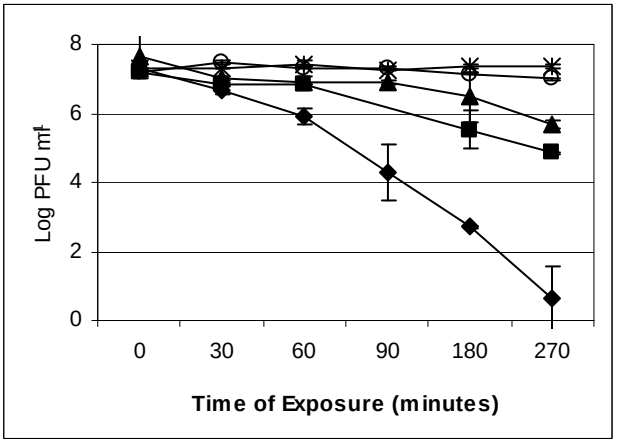


Figure 5. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation, in the presence of porphyrin Tri-Py^{+}-Me-CO_2Me. (—o— light control, —∗— dark control, —▲— 0.5 μM, —■— 1 μM, —♦— 5 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

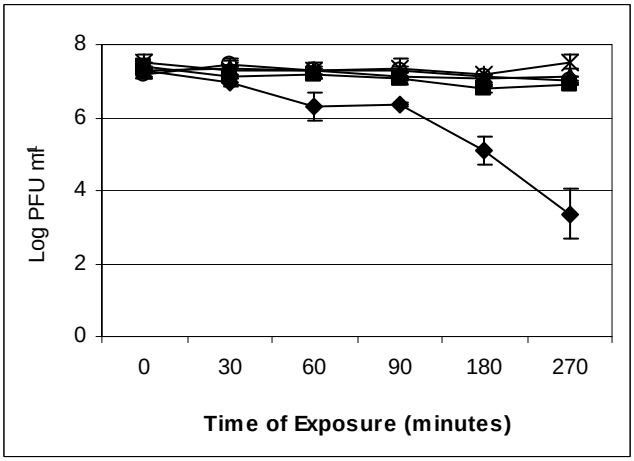


Figure 6. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation, in the presence of porphyrin Tri-Py^{+}-Me-CO_2H. (—o— light control, —∗— dark control, —▲— 0.5 μM, —■— 1 μM, —♦— 5 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

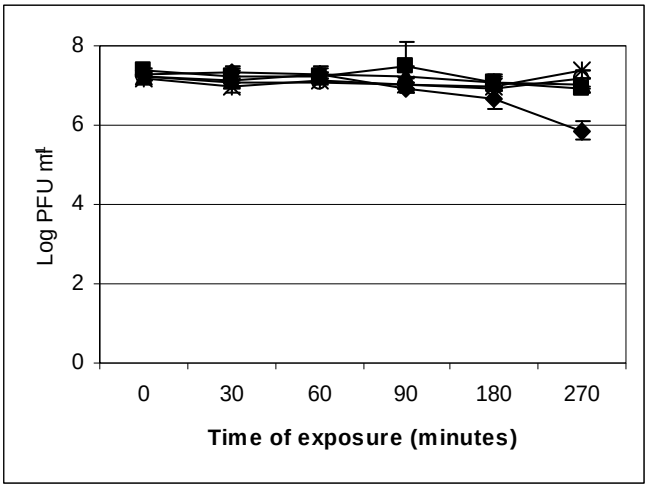


Figure 7. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation, in the presence of porphyrin Di-Py^+-Me-Di-CO_2H-adj. (—o— light control, —∗— dark control, —▲— 0.5 μM, —■— 1 μM, —♦— 5 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

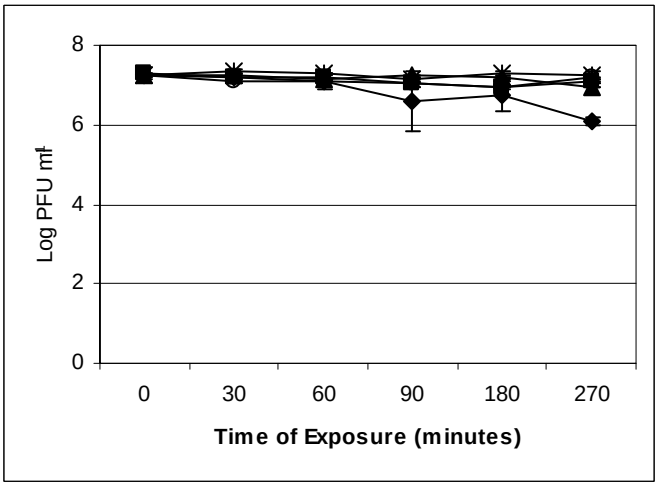


Figure 8. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation, in the presence of porphyrin Di-Py^+-Me-Di-CO_2H-opp. (—o— light control, —∗— dark control, —▲— 0.5 μM, —■— 1 μM, —♦— 5 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

The efficiency of the sewage bacteriophage inactivation by tetra-, tri- and dicationic porphyrins was different. Tetra-Py^+-Me, Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me inactivated the sewage T4-like phage by more than 7 log (Figures 3, 4 and 5), Tri-Py^+-Me-CO_2H produced a moderate phage inactivation (3.9 log) (Figure 6) while dicationic porphyrins leaded only to a small decrease in phage viability (Figures 7 and 8). The ANOVA results showed that the phage inactivation with tetra- tri- and dicationic photosensitizers was significantly different (p <0.05 for all the sensitizers) for the highest concentration (5.0 μM) at different times of exposure. However, for the other two concentrations (0.5 and 1.0 μM) only Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me

were significantly different (ANOVA, p <0.05) from the other photosensitizers. The phage viability reduction by Tri-Py^+-Me-CO_2H was significantly lower than that induced by Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me for the three concentrations (ANOVA, $p < 0.05$). Phage inactivation by Di-Py^+Me-CO_2H-adj and Di-Py^+Me-CO_2H-opp was similar for each of the three concentrations (ANOVA, p >0.05).

At the highest studied concentration (5.0 μM) of Tetra-Py^+-Me, T4-like phage was photoinactivated to the detection limit (>99.9999% of inactivation, reduction of 7.2 log) after 270 minutes of irradiation (Figure 3). With this porphyrin, at this concentration, phage inactivation was still considerable (reductions of 6.1 log) after 180 minutes of irradiation.

The tricationic derivatives Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me also led to photoinactivation to the limit of detection (>99.9999% of inactivation, 7.0 log for Tri-Py^+-Me-PF and 6.7 log (>99.9999%) for Tri-Py^+-Me-CO_2Me) at 5.0 μM. However, the inactivation was faster with Tri-Py^+-Me-PF than with Tri-Py^+-Me-CO_2Me (Figures 4 and 5). The sewage T4-like phage, in the presence of Tri-Py^+-Me-PF was inactivated to the detection limit within 180 minutes, but for Tri-Py^+-Me-CO_2Me it required irradiation for 270 minutes in order to inactivate the virus to the limit of detection. In contrast, the other tricationic derivative Tri-Py^+-Me-CO_2H did not inactivate the virus to the limit of detection. It was only observed a viral reduction of 3.9 log (inactivation of 99.99%) after 270 minutes of irradiation (Figure 6). Reductions of 1.4 log and 1.2 log were observed in the presence of the Di-Py^+Me-Di-CO_2H-adj and Di-Py^+Me-Di-CO_2H-opp porphyrins, respectively, at 5.0 μM and after 270 minutes of irradiation (figures 7 and 8).

At lower concentrations (0.5 and 1.0 μM) of the photosensitizer, the photodynamic effects also changed with porphyrin charge and were as well time dependent.

At 1.0 μM concentration and 270 minutes of irradiation, reductions of 1.5 log, 3.6 log and 2.3 log were observed for Tetra-Py^+-Me, Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me, respectively (Figures 3-5). With porphyrin Tri-Py^+-Me-CO_2H the bacteriophage reduction observed was only 0.5 log after 270 minutes (Figure 6). For Tri-Py^+-Me-PF and for Tri-Py^+-Me-CO_2Me the inactivation was still considerable after 180 minutes of exposure to white light, with a reduction of about 1.7 log and 1.6 log, respectively. Viral photoinactivation with both dicationic porphyrins at 1.0 μM was similar (ANOVA, p = 1.000) and very low (reductions of only 0.4 log and 0.2 log,

respectively) for compounds with adjacent and opposite charges, after 270 minutes of irradiation (Figures 7 and 8).

At the lowest studied concentration (0.5 μM), the inactivation was yet observable, 0.9 log, 1.8 log and 2 log for Tetra-Py^+-Me, Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me, respectively, after 270 minutes of exposure (Figures 3-5). The phage inactivation with the tricationic Tri-Py^+-Me-CO_2H porphyrin at 0.5 μM was also much lower than those observed with Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me. It was observed a reduction of less than 0.2 log after 270 minutes of irradiation. At this concentration, it was not observed any significant reduction during the 270 minutes of exposure with both dicationic porphyrins (ANOVA, $p > 0.05$ for both Di-Py^+Me-CO_2H-adj and Di-Py^+Me-CO_2H-opp) nor between them (ANOVA , $p = 1.000$) (Figures 7 and 8).

Discussion

The present study demonstrates that some cationic porphyrins, when irradiated with white light, can efficiently photoinactivate sewage non-enveloped viruses. The rate and the extent of inactivation are dependent on the photosensitizer charge and also on the lipophilic character of the *meso* substituent groups. As far as we know, this is the first report using various positively charged porphyrins in phage photoinactivation.

It is recognized that positive charges in porphyrins increase the efficiency of the photodynamic process. Cationic porphyrins have a demonstrated affinity to bacteria[9,14,31,35,45] and viruses,[24,25,33] probably due to electrostatic interactions between those positively charged porphyrins and negatively charged sites of these microorganisms. As the sewage non-enveloped T4-like phages studied in this work have a viral capsid that is negatively charged at the neutral pH of the PBS buffer solution[46] used in our experimental conditions, such behaviour also appears to apply to this study case. In fact, the Tetra-Py^+-Me and tricationic porphyrins Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me were much more efficient in phage reduction than the dicationic sensitizers, inactivating the sewage phage below the limit of detection. Therefore, the results obtained in this study show that the number of positive charges of the photosensitizer is a key factor in the photoinactivation of T4-like phages.

The tetracationic porphyrin (Tetra-Py^+-Me) used in the present study leads to complete T4-like phage inactivation (>99.9999% of reduction, 7.2 log) after 270 min of

irradiation. The efficiency of this photosensitizer is in accordance with previous studies where tetracationic porphyrins showed a high rate of viral inactivation.[25] This tetracationic porphyrin showed similar results in other studies (>7 log of reduction) for lambda phage inactivation.[38] This photosensitizer was also used with T7 phage but just to investigate the mechanism of action of its photoreaction.[24] Tetracationic *meso*-tetrapyridylporphyrins with different side-chain lengths were tested for hepatitis A virus and MS2 phage inactivation, but showed toxicity even in the absence of light.[25]

Tricationic porphyrins have never been used in phage chemotherapy. In the present study, both Tri-Py$^+$-Me-PF and Tri-Py$^+$-Me-CO_2Me sensitizers showed inactivation rates markedly different from that of Tri-Py$^+$-Me-CO_2H (ANOVA, p <0.05). While Tri-Py$^+$-Me-PF and Tri-Py$^+$-Me-CO_2Me induced a complete T4-like phage inactivation (~7 log of reduction) after 180 and 270 minutes, respectively, of exposition to white light, the inactivation with Tri-Py$^+$-Me-CO_2H was only moderate (3.9 log of reduction) after 270 minutes of irradiation. A plausible explanation for the lower activity of Tri-Py$^+$-Me-CO_2H is the presence of an acid group that can be ionized when dissolved in PBS buffer, leading to a decrease of its global charge. The ionization of the carboxylic group may result in the modification of several physical properties of the photosensitizer, namely its binding preferences, aggregation state and electronic energy levels, an important parameter for the generation of singlet oxygen, for instance. In fact, the ester derivative Tri-Py$^+$-Me-CO_2Me, where the ionization is not possible, showed a significantly higher inactivation rate for the T4-like phage than Tri-Py$^+$-Me-CO_2H. This explanation also applies to justify the low photoinactivation activity of Di-Py$^+$Me-Di-CO_2H-adj and Di-Py$^+$Me-Di-CO_2H-opp.

The results with Tri-Py$^+$-Me-PF and Tri-Py$^+$-Me-CO_2Me suggest that the presence of a lipophilic aryl group in one of the *meso* positions of the tetrapyrrolic macrocycle appears to have an important role on phage inactivation. Casteel and colleagues have also observed differences in the photoinactivation rate of hepatitis A virus and MS2 phage when they used tetracationic porphyrins with different alkyl substituent groups.[25]

In this study, the viral photoinactivation by dicationic porphyrins was low and similar for both Di-Py$^+$Me-Di-CO_2H-adj and Di-Py$^+$Me-Di-CO_2H-opp (ANOVA, p = 0.349). The different charge distribution of these two porphyrins does not seem to affect the T4-like phage inactivation. Porphyrins with opposite charged groups are more symmetric than porphyrins with adjacent charged groups. The two adjacent positive

charges in the porphyrin macrocycle should result in a molecular distortion, due to electrostatic repulsion. Actually, studies on the localization and photodynamic efficacy of two cationic porphyrins varying in charge distribution on Murine L 1210 cells showed that the efficacy of the sensitizer with the charged groups in adjacent positions was greater than that of the sensitizer with the charged groups in opposite positions.[47] Our results do not show a different kinetic profile for Di-Py^+Me-Di-CO_2H-adj and Di-Py^+Me-Di-CO_2H-opp, probably due to the low rate of phage photoinactivation. Differences in photodynamic activity due to a different charge distribution would be better detected if these two porphyrins had a higher inactivation efficacy as observed for the Tetra-Py^+-Me, Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me derivatives. The results obtained with dicationic porphyrins are not consistent with earlier studies in which even a neutral porphyrin derivative was effective for phage inactivation. Previous studies showed that neutral glycosylated tetraphenylporphyrins can significantly photoinactivate T7 phage.[19,20]

It is important to note that, under our experimental conditions, it was necessary 180 to 270 minutes to photoinactivate the viruses to the limit of detection. However, since PACT technology is to be used outdoors under solar irradiation, which is much more intense than the white light used in our experimental conditions, the photoinactivation process must be much faster. Preliminary results obtained in our laboratory with Tri-Py^+-Me-PF show that, with solar irradiation, 90 minutes is enough to inactive this bacteriophage to below the limit of detection (7 log reduction). On the other hand, this technology is to be applied in the field to disinfect secondary treated sewage in flow system conditions, which facilitate the contact between the photosensitizer and microorganisms and, consequently, it will be necessary a shorter time to inactive the viruses.

Conclusions

In conclusion, our results show that cationic porphyrins with 4 or 3 charges efficiently photoinactivate environmental non-enveloped viruses, opening the possibility to develop new technologies for wastewater treatment. As Tetra-Py^+-Me, Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me inactivated the virus to the detection limit but with Tri-Py^+-Me-PF the inactivation was faster (180 minutes), this sensitizer can be considered the most effective for the inactivation of the T4-like phage. The complete inactivation of viruses with low light intensity means that this technology can be used

even during cloudy days and winter. Photodynamic antimicrobial chemotherapy applied to wastewater disinfection under natural light conditions turns this technology cheap and accessible.

Acknowledgments

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CHAPTER 3

Effect of light intensity on sewage bacteriophage inactivation by cationic porphyrins

Liliana Costa[1], Carla M. B. Carvalho[1,2], João P. C. Tomé[2], Maria A. F. Faustino[2], Maria G. P. S. Neves[2], Augusto C. Tomé[2], José A. S. Cavaleiro[2], Ângela Cunha[1], and Adelaide Almeida[1*]

[1]CESAM and Department of Biology, University of de Aveiro, 3810-193 Aveiro, Portugal

[2]Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

* Corresponding author: aalmeida@ua.pt

Abstract

Photodynamic therapy has been used to inactivate microorganisms through the use of targeted photosensitizers. Although phototreatment is already in use, the mechanism of virus/photosensitizer interaction and viral photoinactivation is still under intensive study. In this work we tested the effect of three different sources of light with 40 W m^{-2}, 600 W m^{-2} and 1690 W m^{-2} on the photoinactivation of a sewage bacteriophage. A phage suspension of 5 x 10^7 PFU ml^{-1} was exposed to different light intensities during 270, 180 and 60 minutes for the white, solar and led light, respectively, at three concentrations of photosensitizer (0.5, 1.0 and 5.0 μM). All of the three porphyrins tested at 5.0 μM inactivated the T4-like phage to the limits of detection for each light source. At the highest light intensity (1690 W m^{-2}), the phage was completely inactivated (> 99.9999% of inactivation) after 25 minutes (reduction of 7.2 log) by Tri-Py^+-Me-PF but only after 60 minutes for both Tri-Py^+-Me-COOH and Tetra-Py^+-Me (reductions of 4.8 log and 7.2 log, respectively). With the 600 W m^{-2} of light, the phage was also completely inactivated (> 99.9999% of inactivation) after 90 minutes (7.2 log of reduction) by Tri-Py^+-Me-PF and after 180 minutes of solar light exposure (7.4 log and 7 log of reduction) by Tri-Py^+-Me-COOH and Tetra-Py^+-Me, respectively. With the

white light (40 W m^{-2}) the phage was either almost completely eradicated (> 99.9999% of inactivation) after 180 minutes (7 log of reduction) for Tri-Py^{+}-Me-PF and after 270 minutes (7.2 log and 3.9 log of reduction) for Tetra-Py^{+}-Me and Tri-Py^{+}-Me-COOH, respectively. The efficacy of bacteriophage photoinactivation varied not only with the dose of light but also with photosensitizer type and concentration. Sewage bacteriophage photoinactivation with a low intensity source can be compensated by a higher irradiation period and vice-versa, for the three sensitizers. However, a similar dose results in a higher rate of photoinactivation if it is received over a shorter period of irradiation. The complete inactivation of viruses, with solar light, means that this methodology can be applied to the disinfection of wastewater under natural irradiation conditions which turns it a less expensive, easy-applicable and an environmental friendly technology.

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 disease has been reduced by the development of good water supplies and hygienic-based procedures for a whole range of human activities, it is still important to persist in the development of novel, convenient and inexpensive methods to avoid microbial contamination.

The standard methods of disinfection include initial filtration of various sorts and UV treatment is sometimes employed in special situations, but reliance is mainly placed on chemical treatments with reactive species such as chlorine, chlorine dioxide and ozone. Chlorine is the most common method used to disinfect water and it is effective in inactivating bacteria and viruses, but the formation of potentially toxic by-products has been a concern in its use. In the process of making water drinkable apart of those traditional methods, other new technique has being recently developed (Bonnett et al, 2006), the photodynamic antimicrobial chemotherapy (PACT), that can also be applied to wastewater disinfection (Jemli et al, 2002; Carvalho et al, 2007; Alves et al, *submitted*; Costa et al, *submitted*). PACT technology combines the utilization of a photosensitizer, light and molecular oxygen. When photosensitizers are exposed to a specific wavelength of light, they produce oxygen species, like singlet oxygen and free

radicals that are cytotoxic (Rovaldi et al, 2000; Lee et al, 2004).

Photosensitizer compounds can be a promising chemical disinfectant for inactivation of pathogens as they are effective in inactivating microbial cells without formation of potentially toxic products (Jemli et al, 2002; Magaraggia et al, 2006). Photosensitizers are usually aromatic molecules that can form long-lived triplet excited states. Several lines of evidence indicate that physicochemical properties of the sensitizer have potential impact on the efficacy of photosensitization (Lukšiene, 2005). Lipophilicity, ionization, light-absorption characteristics and the efficiency of singlet oxygen and free radicals production must be included in a putative photoantimicrobial profile (Lukšiene, 2005). Photodynamic inactivation has been proven to be a powerful method for inactivating viruses, such as murine retroviral vectors (Ben-Hur et al, 1992), human immunodeficiency viruses (HIV-1 and -2) (Schagen et al, 1999; Vzorov et al, 2002), hepatitis-B (Wagner et al, 2001), vesicular stomatitis virus (Horowitz et al, 1991), herpes simplex viruses (Tomé et al, 2005), hepatitis A viruses (Casteel et al, 2004) and influenza A (Perlim et al, 1987; Lenard and Vanderoef, 1993). The effect of photodynamic chemotherapy on bacterial viruses (bacteriophages or simply phages), frequently used as indicators of enteric viruses and public health risk, has already been applied with success not only to collection phage (Kasturi and Platz, 1992; 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 to sewage bacteriophages in a study of charge effect done in our laboratory (Carvalho et al, 2007; Costa et al, *submitted*). To the best of our knowledge, no studies concerning the effect of different light intensities have already been tested with bacteriophages.

A very wide selection of light sources is available, ranging from laser technology to basic tungsten-filament lamps. PACT uses low-power light rather than the lasers used in ablative therapy: microbial photokilling is attained with milliwats rather than tens (or hundreds) of watts (Wainwright, 1998). Traditionally, lasers were considered to be superior to the conventional light sources, such as incandescent lamps (Lukšiene, 2005). However, the usage of lasers has some essential drawbacks because they are very expensive and require specially trained personnel to work with them (Lukšiene, 2005).

Accordingly to Kastury and Platz (1992), PACT depends on light intensity as well as on photosensitizer concentration. The inactivation rate increases with light intensity, indicating that the distance of the sample from the light source is a variable

which must be controlled. Increasing the duration of the irradiation will also improve the water yield treatment. Long irradiation periods can compensate for a low concentration of sensitizer, a less efficient type or a virological poor quality of water (Kastury and Platz, 1992; Jemli et al, 2002).

The aim of this study was to investigate the effect of three sources of light with different intensities on the photoinactivation of a somatic sewage bacteriophage of *Escherichia coli*, by different concentrations of three cationic photosensitizers. The relationship between irradiation time and light intensity in phage inactivation was also evaluated.

Material and Methods

Porphyrins synthesis description

Porphyrins (Figure 1) used in this work were prepared in two steps. First, the neutral porphyrins were synthesized by the Rothemund and crossed Rothemund reactions using pyrrole and the adequate benzaldehydes (pyridine-4-carbaldehyde and pentafluorophenylbenzaldehyde or 4-formylbenzoic acid) at reflux in acetic acid and nitrobenzene (Sirish et al, 2002; Tomé et al, 2004). These reagents were purchased from Sigma-Aldrich (Madrid). The resulting porphyrins were separated by column chromatography (silica) and then the pyridyl groups were quaternized by reaction with methyl iodide. Porphyrins were purified by crystallization from chloroform/methanol/petroleum ether and their purities were confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy.

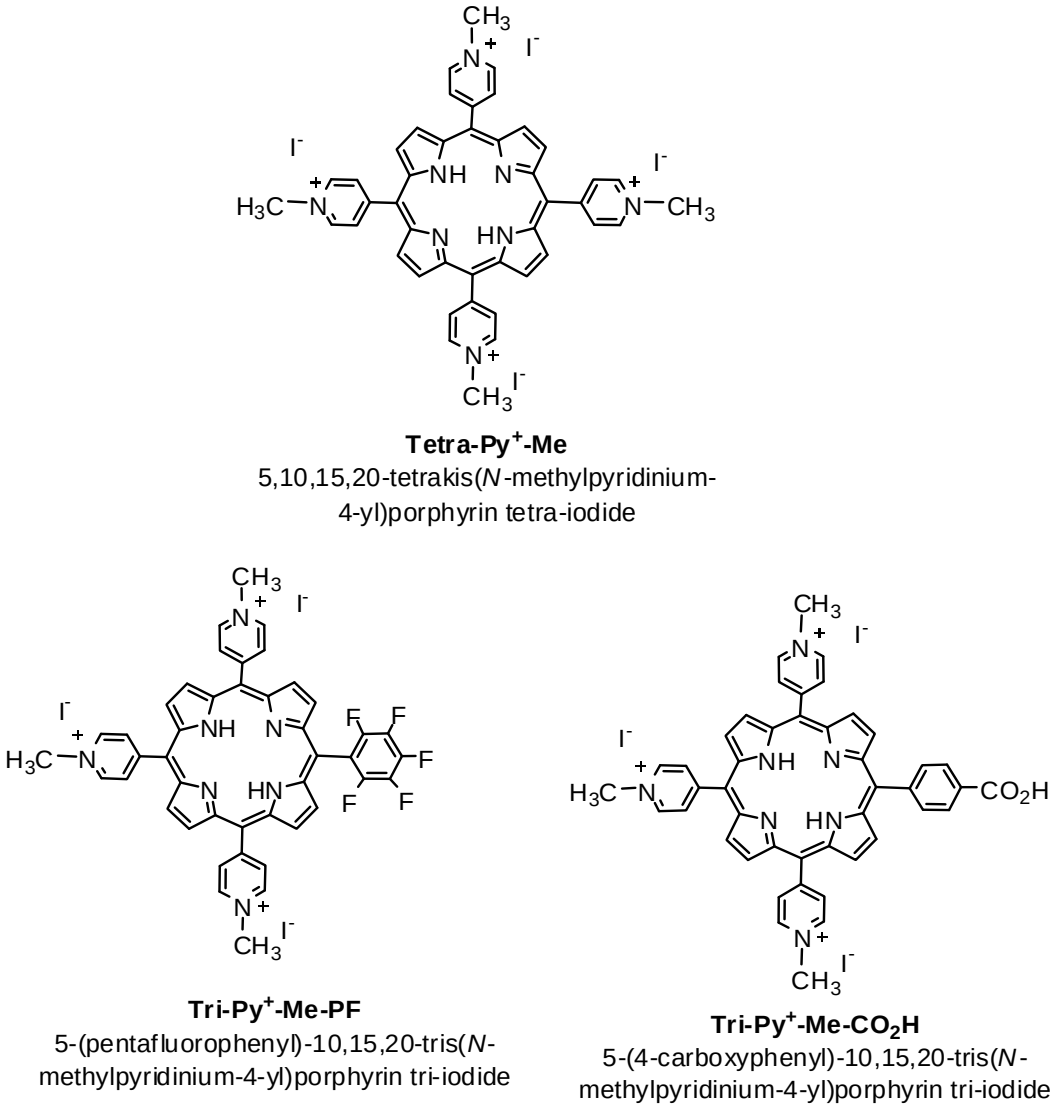

Figure 1. Structure and the IUPAC name of the three porphyrin derivatives used for photoinactivation of T4-like bacteriophage.

Phage selection and quantification

A wastewater sample from a secondary-treated sewage plant of the city of Aveiro (Portugal) was used to select the somatic bacteriophages of *E. coli* C (ATCC 13706). An isolated and morphologically representative phage plaque was picked out with a Pasteur pipette, by aspiration, and was placed in 50 mL of an *E. coli* culture in the exponential growth phase. The mixture was incubated with slow stirring (~100 rpm) at 37ºC, until the clarification of the medium, for about 5h. The suspension was then

centrifuged (Beckman Avanti J-25I centrifuge) at 7,000g during 10 minutes and the pellet was discarded. The supernatant, with 10^9 particles per millilitre, was maintained at 4ºC with 2% of chloroform. The quantification of phages was determined, in duplicate, by the agar double layer technique (Adams, 1959) using the aforementioned strain of *E. coli*. One millilitre of non-diluted sample or of serially diluted sample and 0.3 mL of bacterial host were added to a tube with 6 mL of soft TSA growth medium. The contents of the tube were mixed by manual rotation and then immediately poured onto a prepared confluent TSA monolayer on a Petri plate. The plates were incubated upside-down during 18 hours at 37ºC in the dark, to avoid host bacteria inactivation. The number of lysis plaques was counted on the most convenient series of dilutions and the results were expressed as plaque forming units per millilitre (PFU mL^{-1}).

Bacteriophage identification

DNA extraction and purification of phage suspension was done using a standard technique (Sambrook et al, 1989). DNA was extracted with phenol saturated with TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) followed by extraction with a mixture of chloroform and isoamyl alcohol (24:1). The purified nucleic acid was amplified by PCR using consensus primers that amplify the central portion of capsid gene 23: Mzia 1 (5'-TGTTATIGGTATGGTICGICGTGCTAT-3') and CAP8 (5'-TGAAGTTACCTTCACCACGACCGG-3'). The conditions used for the amplification reaction with these primers involved 35 cycles consisting of 1 minute of denaturation at 95ºC, 1 minute of annealing at 58ºC and 2 minutes of extension at 72ºC. The PCR products were purified using the JETQUICK PCR Purification Spin kit from Genomed and sequenced with BigDyeTerminator v1.1 from Applied Biosystems. The phage was identified as a T4-like phage that has 82% of homology with the Enterobacteria phage RB43. The nucleotide sequence of the phage has been deposited in the GenBank database under accession nº EU026274.

Bacteriophage host viability test

As bacteria are also sensible to the photosensitizers, the viability of the viral host was evaluated in order to prove that the phage inactivation was due to photoinactivation by the photosensitizer and not due to bacterial host inactivation by porphyrin. As at each sampling time we collected samples with the porphyrin, the sensitizer could then inactive the bacteria during the incubation period of 18 hours. Additional samples of the

highest concentration of Tri-Py^{+}-Me-PF (5.0 μM), as well as light and dark controls samples, were collected in each sampling time, after irradiation, and washed by ultra-centrifugation at 28,000*g* (Beckman L8-80K ultracentrifuge, equipped with a swing-out rotor SW28) during one hour and thirty minutes, at room temperature, to remove the porphyrin. The porphyrin-free pellets of phages were re-suspended in 5 mL of PBS buffer, serially diluted and pour plated by the double layer technique. The results obtained were compared with those resulting from direct spread after irradiation (of non-washed samples). 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 μM). In the other experiments this step was not done, but the Petri dishes were incubated in dark conditions.

Experimental set up

The effect of the cationic porphyrins at different concentrations (0.5, 1.0 and 5.0 μM) was evaluated by exposing a sewage somatic bacteriophage in laboratory conditions to white (40 W m^{-2}), solar (600 W m^{-2}) and to a led light (1690 W m^{-2}) during 270, 180 and 60 minutes, respectively.

The efficiency of the porphyrins was evaluated through quantification of the number of bacteriophage after light exposure. The suspension of phages was diluted on phosphate buffer (PBS) until 5 x 10^{7} PFU mL^{-1} (1000 times higher than that of residual waters) and distributed in 600 mL acid-washed and sterilised glass goblets (20 ml per each of 5 goblets). Three of the goblets were added of photosensitizer at 0.5, 1.0 and 5.0 μM (prepared from stock solutions of 500 μM in DMSO) and the other two were used as dark and light controls. The light control was not added of porphyrin and was exposed to light. The dark control was added of the higher concentration of the photosensitizer (5.0 μM) and was covered with aluminium foil. The test and light goblets were exposed in parallel to white, solar and led light during 270, 180 and 60 minutes, respectively, at 20-25ºC, under stirring (100 rpm).

The white light used was PAR radiation (13 lamps OSRAM 21 of 18 W each one, 380-700 nm) with an intensity of 40 W m^{-2}. The solar light used in this study was only the PAR radiation of the solar spectrum. UV radiation would inactivate the viruses and consequently would increase the phage inactivation during the exposure time to the porphyrins. The solar PAR light (measured with a radiometer LI-COR Model LI-250), during the experiment period, showed an average intensity of 600 W m^{-2}. In order to

avoid viral inactivation by UV radiation during solar exposure, the goblets were covered with a glass Petri plate. The led light used was an optical fibre illumination system (LC-122 LumaCare, London) equipped with a halogen 250 W quartz-type lamp (400-800nm) with an intensity of 1690 W m^{-2}.

Sub-samples of 1 mL were aseptically taken at time 0, 30, 60, 90, 180 and 270 minutes (0, 7.2, 14.4, 21.6, 43.2 and 64.8 J cm^{-2}) for the white light; at 0, 30, 60, 90, 120 and 180 minutes (0, 108, 216, 324, 432 and 648 J cm^{-2}) for the solar light; and at 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 45 and 60 minutes (0, 10.14, 20.28, 30.42, 40.56, 50.7, 101.4, 152.1, 202.8, 253.5, 304.2, 456.3 and 608.4 J cm^{-2}) for the led light. Sub-samples were analysed, in duplicate, for bacteriophage number. The Petri plates were kept in dark immediately after spread 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 of sampling as the mean of the two duplicates in the most convenient dilution series. Viral reductions at each time are expressed as percentage related to time zero. For each photosensitizer two experiments were done and the results presented are the average of the two assays.

Statistical analysis

SPSSWIN 14.0 was used for data analysis. The significance of difference in phage inactivation among the three 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.

Results

Bacteriophage host viability was not affected by porphyrins during the 18 hours of incubation. The pattern of phage inactivation was similar (ANOVA, $p = 0.308$) in washed and in non-washed samples (Figure 2). The dark incubation of Petri plates was sufficient to ensure the phage host viability. Consequently, the three photosensitizers were tested without the washing step, which is a time consuming procedure that would greatly delay the assays.

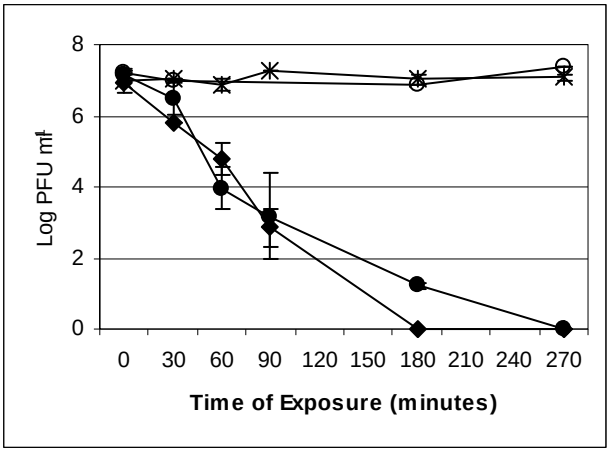

Figure 2. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation in the presence of 5 μM of Tri-Py^{+}-Me-PF porphyrin in washed and in non-washed phages. (—o— light control, —∗— dark control, —•— watched phages, —♦— non-watched phage). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

Without light (dark control), the porphyrins at the highest concentration (5.0 μM) did not exhibit activity against the phage during the all exposure time (Figures 3 to 5) (ANOVA, p>0.05). A similar trend was obtained with the phage in the absence of the porphyrins during the 270, 180 and 60 minutes of irradiation with white, solar and the led light, respectively (light control) (Figures 3 to 5) (ANOVA, p>0.05). Only when phage was incubated with the photosensitizer and irradiated with the appropriate light intensity, inactivation was observed.

The efficiency of the sewage bacteriophage inactivation by cationic porphyrins irradiated with 40 W m^{-2}, 600 W m^{-2} and 1690 W m^{-2} was different. Although all the cationic porphyrins inactivated the sewage T4-like phage to the limits of detection with all the light sources tested at 5.0 μM, photoinactivation with the led light occurred more early than with solar irradiation and much earlier than with white light (Figures 3 to 5).

At the highest studied light intensity (1690 W m^{-2}) T4-like phage was completely inactivated (> 99.9999% of inactivation) with reductions of 7.2 log after 25 and 45 minutes of irradiation, for both Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me, respectively, and of 4.9 log after 60 minutes of irradiation for Tri-Py^{+}-Me-COOH at 5.0 μM. For the concentration of 1.0 μM, the percentage of inactivation was not so high, ranging from 81.11 to 99.99%, after 30 minutes of exposure (2.2 log, 1.3 log and 0.7 log for Tri-Py^{+}-Me-PF, Tetra-Py^{+}-Me and Tri-Py^{+}-Me-COOH, respectively). For the lowest concentration of sensitizer (0.5 μM) the rate of inactivation varied between 63.19 and

89.86% (reductions of 0.5 to 1.3 log) after 30 minutes of irradiation (Figure 3). With this high light intensity the pattern of phage inactivation was similar for both Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me (ANOVA, p>0.05), but was significantly different from that of Tri-Py^{+}-Me-COOH (ANOVA p<0.05).

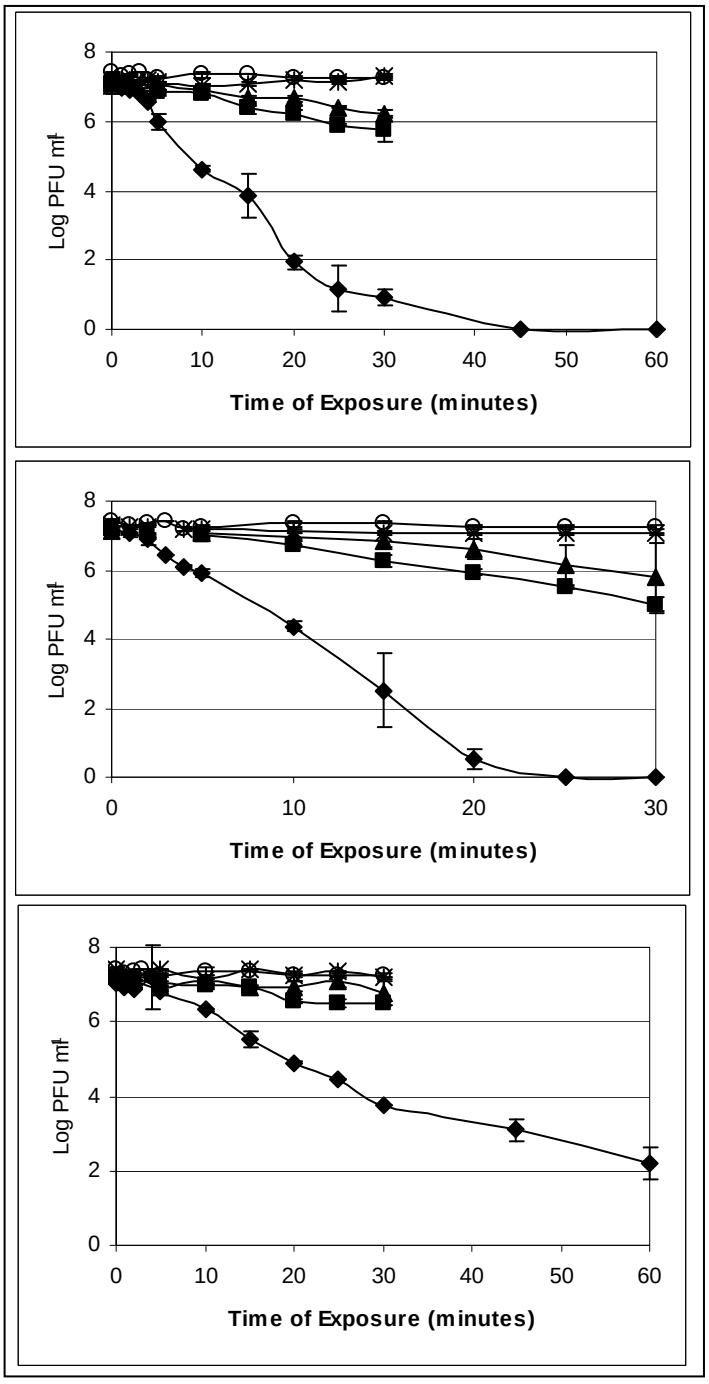

Figure 3. Density variation of the sewage bacteriophage after 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 45 and 60 minutes of irradiation with 1690 W m^{-2} in the presence of porphyrins Tetra-Py^{+}-Me, Tri-Py^{+}-Me-PF and Tri-Py^{+}-Me-COOH, respectively. (—o— light control, —∗— dark control, —▲— 0.5 μM, —■— 1

μM, —♦— 5 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

With PAR solar light (600 W m^{-2}), the phage was efficiently photoinactivated (> 99.9999% of inactivation) with reductions of 7.2 log for Tri-Py^{+}-Me-PF after 90 minutes, 7.4 log and 7 log for both Tri-Py^{+}-Me-COOH and Tetra-Py^{+}-Me, respectively, after 180 minutes of irradiation at 5.0 μM. With 1.0 μM of sensitizer, the rate of inactivation ranged from 96.07 to 99.98% giving reductions of 3.8 log (after 120 minutes), 1.8 log and 1.4 log (after 180 minutes of exposure) for Tri-Py^{+}-Me-PF, Tetra-Py^{+}-Me and Tri-Py^{+}-Me-COOH, respectively. For the concentration of 0.5 μM, the reductions observed were 2.8 log (after 120 minutes), 1.4 log and 0.8 log (after 180 minutes), corresponding to a viral inactivation of 83.97 to 99.84%, for Tri-Py^{+}-Me-PF, Tri-Py^{+}-Me-COOH and Tetra-Py^{+}-Me, respectively (Figure 4). Contrarily to the led light, the pattern of phage inactivation with solar light was similar for all the porphyrins at 5 μM (ANOVA, $p>0.05$).

Porphyrins irradiated with white light (40 W m^{-2}), at 5.0 μM, also inactivated the T4-like phage to the limits of detectable (> 99.9999% of inactivation) with reductions of 7 log (after 180 minutes of irradiation), 7.2 log and 3.9 log (after 270 minutes of irradiation) for Tri-Py^{+}-Me-PF, Tetra-Py^{+}-Me and Tri-Py^{+}-Me-COOH, respectively. For the concentration of 1.0 μM, the rates of inactivation ranged from 61.03 to 99.92%, with reductions of 3.6 log, 1.5 log and 0.5 log, respectively for Tri-Py^{+}-Me-PF, Tetra-Py^{+}-Me and Tri-Py^{+}-Me-COOH, after 270 minutes of exposure to white light. For the lowest sensitizer concentration (0.5 μM) the inactivation rate ranged from 33.08 to 93.74% with reductions of 0.2 to 1.8 log after 270 minutes (Figure 5). With white light intensity the pattern of phage inactivation was similar for Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me (ANOVA, $p>0.05$), but was significantly different from that of Tri-Py^{+}-Me-COOH (ANOVA $p<0.05$).

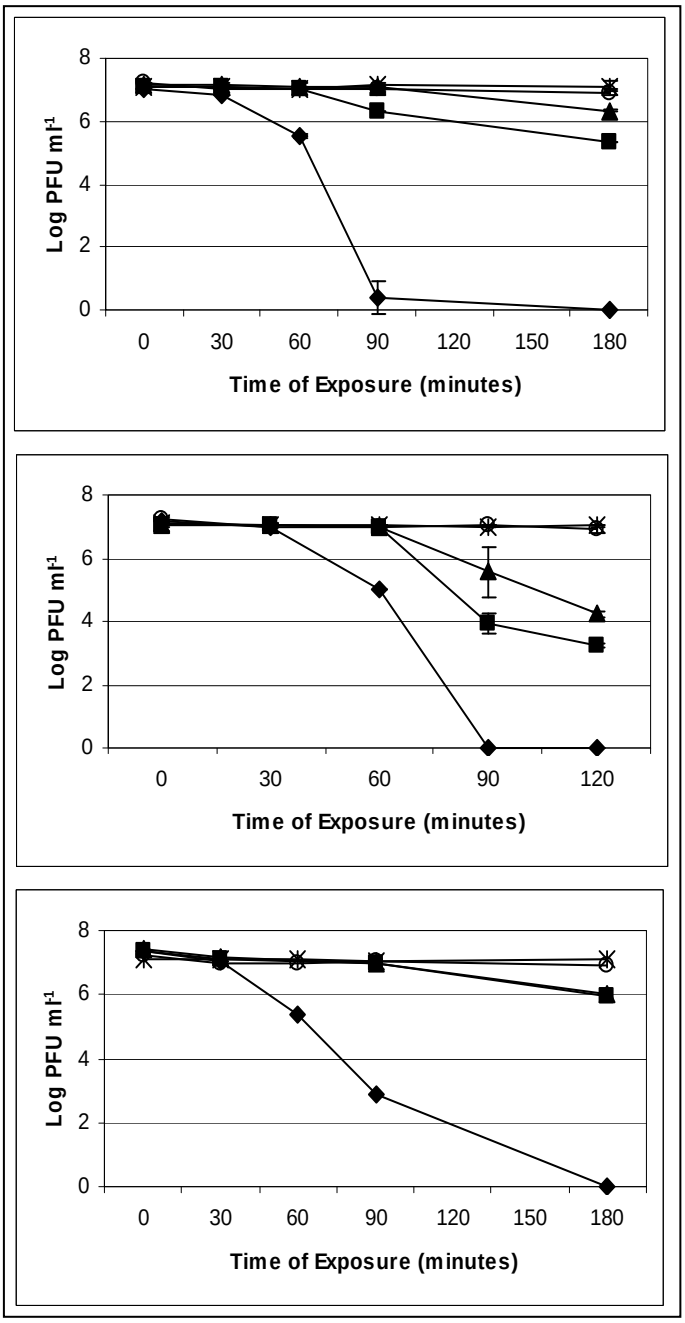

Figure 4. Density variation of the sewage bacteriophage after 30, 60, 90, 120 and 180 minutes of irradiation with solar light (600 W m^{-2}) in the presence of porphyrins Tetra-Py^{+}-Me, Tri-Py^{+}-Me-PF and Tri-Py^{+}-Me-COOH, respectively. (—o— light control, —✳— dark control, —▲— 0.5 μM, —■— 1 μM, —♦— 5 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

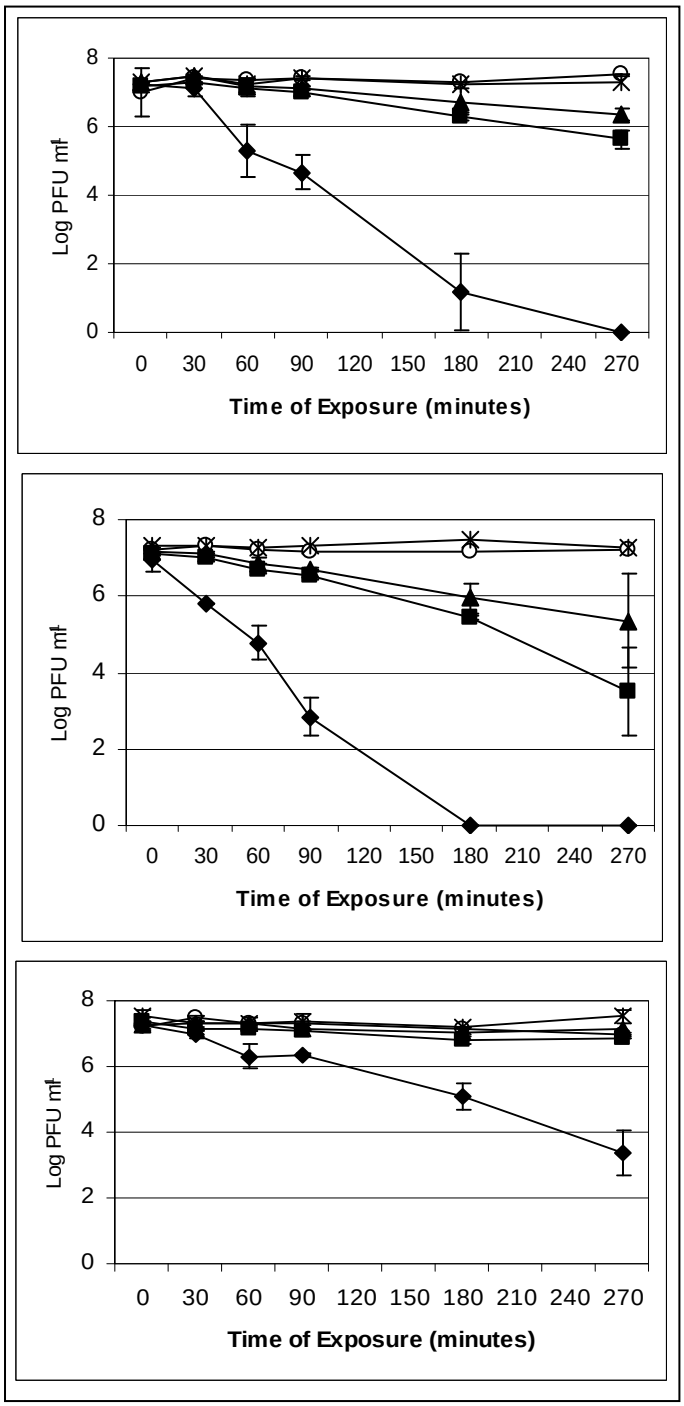

Figure 5. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation with white light (40 W m^{-2}) in the presence of porphyrins Tetra-Py^{+}-Me, Tri-Py^{+}-Me-PF and Tri-Py^{+}-Me-COOH, respectively. (—o— light control, —✳— dark control, —▲— 0.5 μM, —■— 1 μM, —♦— 5 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

Discussion

The present study demonstrates that cationic porphyrins, when irradiated with different sources of light, ranging from the white (40 W m^{-2}) and solar light (600 W m^{-2}) to a potent led light with 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. The rate and extent of inactivation was dependent on light intensity, period of irradiation and also on the structure and concentration of the photosensitizers.

For all porphyrins tested the efficiency of inactivation was proportional to the intensity of light. Higher light intensity leads to a higher rate of photoinactivation after the same irradiation time. The led light gave the earlier rate of photoinactivation when compared with the other light sources, followed by the solar light which gave a sooner inactivation when compared with the white light. The led light (1690 W m^{-2}) showed an efficient phage inactivation (until 7.2 log of reduction) at 5.0 μM, after 60 minutes for all the sensitizers. Cationic porphyrins irradiated with solar (600 W m^{-2}) and with white light (40 W m^{-2}), gave less phage inactivation (until 2.2 log, at 5.0 μM) after 60 minutes, but inactivated the phage to the limits of detection after 180-270 minutes of irradiation (reductions of about 7 log), except for porphyrin Tri-Py^{+}-Me-COOH when irradiated with white light (reduction of only 3.9 log). A similar trend was obtained by other authors with *E. coli* cells treated with 1 μM of tetracationic and tricationic porphyrins irradiated with a light intensity of 600 W m^{-2} (reductions of ~4 log and ~4.5 log after 30 minutes of irradiation, respectively) (Caminos and Durantini, 2006).

The rate of inactivation was also dependent on the duration of the irradiation period. For the highest photosensitizer concentration (5.0 μM), there were reductions of about 7 log for all the porphyrins and light intensities, but the irradiation period necessary to reach this inactivation was very different. For the three light intensities, the rate of inactivation increases with the increase of the irradiation period. It is important to note, however, that at the end of the experiments, for the lowest photosensitizer concentrations (0.5 and 1 μM), the rate of inactivation was higher with solar and white light rather then with the led light. For 1 μM, reductions in phage viability ranged from 0.7 to 2.2 log for the led light, 1.4 to 3.8 log for the solar light and 0.5 to 3.6 log for the white light. For the concentration of 0.5 μM the values ranged from 0.5 to 1.3 log, 0.8 to 2.8 log and 0.2 to 1.8 log, respectively for the led, solar and white light. This can be explained by the fact that after a longer irradiation period with solar and white light, the

light dose is higher than after a short period of irradiation with the led light. In fact, the light dose is almost two times bigger after 180 minutes of exposure to solar radiation than after 30 minutes of irradiation with the led light. It indicates that when the light intensity is lower, increasing the irradiation time will actually improve the rate of phage inactivation.

Actually, the light intensity or the illumination time can be varied for the same light dose (given in J cm^{-2}). However, some studies showed that, for a given photosensitizer concentration, a high power density over a short time period may give different results in terms of microbial inactivation to that of a low power density over a longer time even though the light dose is the same in each case (Wainwright, 2000). On the other hand, it has been shown that a similar dose results in a higher rate of inactivation if it is received over a longer time period (Gábor et al, 2001). From our results we can conclude that a high light intensity over a short period of time gives a higher rate of bacteriophage inactivation than that of a low light intensity over a longer irradiation time. For the most efficient porphyrins (Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me), the utilization of a higher light intensity produced better results in a short period of time when compared with low light intensities during a long period of time. The led light inactivated to the limits of detection the T4-like sewage bacteriophage three times earlier in time, than solar PAR light, for the same light dose. Porphyrin Tetra-Py^{+}-Me irradiated with a light dose of about 600 J cm^{-2} completely inactivated the phage (reductions of ~ 7 log) after 60 minutes with the led light, but only after 180 minutes with the solar light. For a light dose of about 300 J cm^{-2}, porphyrin Tri-Py^{+}-Me-PF completely inactivated the phage (reductions of 7.2 log) after 30 minutes with the led light and after 90 minutes with the solar light.

As observed before in our laboratory (Costa et al, *submitted*; Carvalho et al, 2007) for these porphryins and for other porphyrins in other studies (Milanesio et al, 2003; Casteel et al, 2004; Lazzeri et al, 2004; Caminos et al, 2005), the structure and concentration of the photosensitizer influenced the phage inactivation. The three porphyrins, irradiated with the same amount of light, differently inactivated the sewage T4-like phage. The tetra- (Tetra-Py^{+}-Me) and tricationic porphyrin Tri-Py^{+}-Me-PF were more efficient in phage reduction than the tricationic porphyrin Tri-Py^{+}-Me-COOH. For the concentration of 5.0 μM, the reductions observed were near 7 log for both Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me and only about 4 log for Tri-Py^{+}-Me-COOH (except for the solar light that was 7 log reduction). At this concentration, the most effective porphyrins,

Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me, showed similar rates of inactivation, although at different times, inactivating the phage to the limits of detection. However, with 1.0 and 0.5 μM the rate of inactivation was significantly lower (until 3.8 log and 2.8 log, respectively). In fact, it is recognized that increasing the concentration of sensitizer at a fixed light dose leads to increased viral inactivation as does an increase of total light exposure at a fixed concentration of sensitizer (Kasturi and Platz, 1992). Milanesio and workers (2003) also support the basic concept that the cell survival after irradiation of the cells with visible light was dependent upon both intracellular sensitizer concentration and light exposure level. Similar results were obtained by other authors with white light (Lazzeri et al, 2004; Caminos et al, 2005).

In conclusion, our results showed that cationic porphyrins irradiated with three different light intensities (40 W m^{-2}, 600 W m^{-2} and 1690 W m^{-2}) efficiently photoinactivated environmental nonenveloped viruses, opening the possibility to develop new technologies for wastewater treatment. The high rate of phage photoinactivation soon after 25 minutes, for the led light, with the most effective porphyrin, means that we can speed the destruction of bacteriophages by increasing the light intensity. For the same light dose, phage inactivation to the detection limits was sooner in time when the porphyrins were irradiated with higher light intensities. This means that we can compensate shorter periods of irradiation with bigger intensities of light. A similar dose results in a higher rate of photoinactivation if it is received over a shorter period of irradiation. The inactivation of viruses to the detection limits with solar irradiation means that photodynamic therapy can be applied to the disinfection of wastewater under natural irradiation conditions. This, associated with the recovery and re-utilization of these porphyrins when they are immobilized on solid supports (a possibility which is already being evaluated by our laboratory), makes it a less costly, easy-applicable and an environmental friendly technology which is efficient for the removal of sewage bacteriophages from wastewater.

Acknowledgments

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CHAPTER 4

Sewage bacteriophage photoinactivation by immobilized porphyrins in solid matrixes

Liliana Costa[1], Carla M. B. Carvalho[1,2], João P. C. Tomé[2], Maria A. F. Faustino[2], Maria G. P. S. Neves[2], Augusto C. Tomé[2], José A. S. Cavaleiro[2], Zhi Lin[3], José P. Rainho[3], João Rocha[3], Ângela Cunha,[1] and Adelaide Almeida[1*]

[1]CESAM and Department of Biology, University of de Aveiro, 3810-193 Aveiro, Portugal

[2]Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[3]CICECO, University of Aveiro, 3810-193 Aveiro, Portugal

* Corresponding author: aalmeida@ua.pt

Abstract

Photodynamic therapy is a platform technology which uses a combination of a photosensitizer, light and molecular oxygen to achieve selective destruction of a biological target. This methodology is already in use for the inactivation of microorganisms but there is a lack of information about photoinactivation with immobilized sensitizers, which can be removed from the environment and re-used. In this work we tested the effect of one porphyrin immobilized on two different solid matrixes (cationic and neutral magnetic supports) in the photoinactivation of a sewage bacteriophage. A phage suspension of 5 x 10^7 PFU ml^{-1} was exposed to white light during 270 minutes at three concentrations of free (0.5, 1.0 and 5.0 μM) and immobilized photosensitizers (5, 20 and 100 μM). Cationic porphyrin Tri-Py^+-Me-PF-CS supported on cationic material was the most effective, inactivating the phage to the limits of detection, as observed for the free form of this porphyrin (> 99.9999 of inactivation). Reductions of 6.9 log, 6.8 log and 6.4 log were observed after 270 minutes, respectively for the concentrations of 5, 20 and 100 μM. With the cationic porphyrin Tri-Py^+-Me-PF-NS, immobilized on the neutral support, the phage was also

almost completely inactivated (99.54 to 99.9999% of inactivation) with reductions until 6.9 log after 270 minutes of irradiation. Phage inactivation by the neutral porphyrin Tri-Py^{+}-Me-PF-NS immobilized on the cationic support was lower, reaching values of 99.76 to 99.98% (reductions until 5.2 log) after 270 minutes of irradiation. The rate and extent of bacteriophage photoinactivation seemed to vary with the solid matrixes used as supports, the sensitizer concentration and also with the porphyrin charge. Phage inactivation, to the limits of detection, with the cationic porphyrin immobilized on the cationic support, means that this immobilized sensitizer can be applied with the same efficacy of the free form to wastewater disinfection, opening the possibility of use PACT technology for residual waters treatment. The possibility of removing the magnetic supported porphyrins from the environment allows the re-utilization of the sensitizer, what turns this technology a cheap and an environmental-friendly solution for viral inactivation in polluted waters.

Introduction

The lack of safe and efficient technologies for wastewater treatment is one of the causes responsible for the reduction of water resources and the increase of environmental pollution. From the human perspective, water has an important implication in transmission of infectious diseases, being necessary to develop new technologies to get polluted waters reusable and ensure that clean waters are kept clean.

Disinfection of effluents can be achieved by a variety of methods, mostly using chlorine, hypochlorites, chlorine dioxide, ozone and UV light. However, the formation of mutagenic and carcinogenic agents in water and wastewater effluents treated with chlorine and the high cost of UV and ozone treatment has prompted research to seek alternative disinfecting methods that would minimize environmental, economical and public health impacts.

It has been known since the last century that photodynamic antimicrobial chemotherapy (PACT) can kill certain microorganisms by the combination of dyes and light *in vitro* (Hamblin and Hasan, 2004). Porphyrinic compounds associated to PACT can be a promising chemical disinfectant for the eradication of pathogens as they are effective in inactivating microbial cells without formation of potentially toxic products. The sensitivity of viruses to such photodynamic procedures was then shown in the 1930s, but only within the past two decades (and with the occurrence of AIDS) have

photodynamic techniques for the inactivation of viruses received growing attention (Käsermann and Kempf, 1998).

The majority of PACT experiments have been carried out with free photosensitizers (Kasturi and Platz, 1992; Abe et al, 1997; Lee et al, 1997; Wagner et al, 1998; Egyeki et al, 2003; Zupán et al, 2004; Embleton et al, 2005). This is far from appropriate for applications to water disinfection, where residual traces of photosensitizer in the water output would certainly not be acceptable. Free photosensitizers might not only introduce residual traces of sensitizer but would also turn this technology a high-cost one. As an inherent disadvantage of most of the sensitizers is their water solubility, which makes their removal from water extremely difficult (Käsermann and Kempf, 1998), the possibility of using these efficient photosensitizers bound to insoluble supports can be an interesting approach to inactivate pathogenic microorganisms present in water or wastewater. Suitable support materials must have some specific characteristics such as compatibility with the photosensitizer, allowing effective, easy and reproducible immobilization procedures and avoiding leaching out to water; mechanical strength and stability towards sunlight; good oxygen permeability for efficient singlet oxygen production with minimum singlet oxygen quenching; high biocompatibility, to maximize the interaction between the support and the microorganisms; and commercial availability and low cost (Jiménez-Hernández et al, 2006). Magnetic supports like microspheres, nanospheres and ferrofluids have been widely used in the field of biology and medicine and usually consist of inorganic magnetic cores and organic or polymeric shells that are either biocompatible or possessing active groups which can be conjugated to biomolecules (Liu et al, 2004). The most important features of magnetic supports are their size, size distribution, structure, hydrophobicity/hydrophilicity and density of reactive surface groups, and the superparamagnetic property (Liu et al, 2004). However, excited state lifetimes are longer in the case of cationic polymer supports. In order to have high singlet oxygen production quantum yields, sensitizers with long excited state lifetimes are required since the probability of excited state quenching by oxygen increase (Jiménez-Hernández et al, 2005).

Bonnett and colleagues (2006) have therefore proposed the use of photosensitizers immobilized on polymeric supports to inactivate bacteria in water. They designed experiments to test the idea that the photodynamic effect can be used to lower *Escherichia coli* at a level of 10^5 cells ml^{-1} in a flow of water using a sensitizer

incorporated into a cheap and simple polymeric membrane (chitosan). They concluded that the use of immobilized photosensitizers on inert solid matrixes can be a good solution for a real situation. It has been shown that the sensitizer can be covalently coupled to solid supports without impairment of its photobiological activity (Bonnett et al, 2006). Noticeable disinfection was also observed by Jiménez-Hernández and colleagues (2005) with a RDP^{2+}/silicone system, for which a high rate of cell inactivation by singlet oxygen up to 1.1 x 10^5 CFU h^{-1} L^{-1} has been measured with *E. coli* and 0.7 x 10^5 CFU h^{-1} L^{-1} with *Enterococcus faecalis*. The results obtained by other authors showed that phthalocyanines can be successfully immobilized on a silicate matrix and used for photodisinfection of microbially polluted waters (*E. coli* reductions of about 1 log after 120 minutes of exposure) (Artarsky et al, 2006). Regenerated cellulose impregnated with 5,10,15,20-tetrakis(*N*- methylpyridinium)porphyrin tetra-p-tosylate was showed to have photobactericidal activity against *Staphylococcus aureus*, *E. coli*, *Proteus vulgaris* and *Bacillus subtilis* (Bonnett et al, 1997). The support material retains both its mechanical strength and its photobactericidal properties after 50h of exposure to a xenon arc lamp (Bonnett et al, 1997). Caminos and Durantini (2006) also showed an efficient photoinactivation of *E. coli* cells (reduction of about 4 log) by cationic porphyrins immobilized on agar surfaces. On the other hand, the possibility of removing supported porphyrins from the environment allows the re-utilization of the photosensitizers, decreasing the cost, raising the advantages of using this technology for water disinfection.

As far as we know, no studies concerning the effect of photosensitizers immobilized on solid matrixes have already been tested with sewage bacteriophages. Previous studies, in our laboratory, were conducted in order to choose among six free sensitizers the most efficient free porphyrin for phage inactivation (Carvalho et al, 2007b, Costa et al, *submitted*). The best porphyrin, Tri-Py^+-Me-PF, was tested with different light intensities (Costa et al, *unpublished data*). The rate of inactivation increased with the increase of the light intensity (40, 600 and 1690 W m^{-2}). However, the phage was yet inactivated to the limits of detection even at low light intensities. The best porphyrin, Tri-Py^+-Me-PF, was then immobilized on solid matrixes.

The aim of this study was to investigate the effect of the tricationic porphyrin Tri-Py^+-Me-PF immobilized on two different solid matrixes on the photoinactivation of a somatic sewage bacteriophage of *E. coli*.

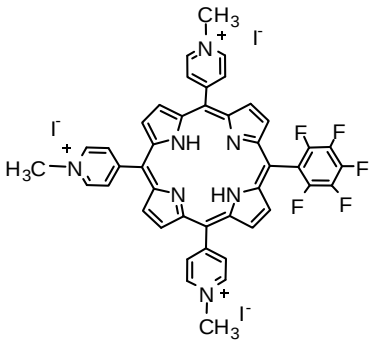
Material and Methods

Porphyrins tested

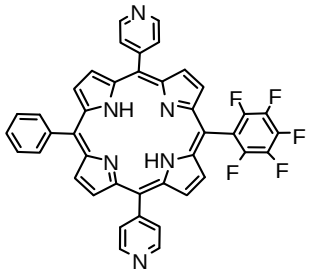
In this work, we tested the effect of one porphyrin immobilized on two different solid matrixes for bacteriophage inactivation. For that purpose, we tested one cationic porphyrin (Tri-Py$^+$-Me-PF) immobilized on a cationic solid support, Tri-Py$^+$-Me-PF-CS, and on a neutral solid support, Tri-Py$^+$-Me-PF-NS. The neutral form of the porhyrin (Tri-Py-PF) was also tested but only when immobilized on a cationic support, Tri-Py-PF-CS. The effect of these immobilized porphyrins at different concentrations (5, 20 and 100μM) was evaluated in laboratory conditions, under white light irradiation (40 Wm^{-2}). The free form of the porphyrin (Tri-Py$^+$-Me-PF) was used for comparison with the immobilized ones at 0.5, 1.0 and 5.0 μM.

Porphyrin synthesis description

The porphyrin (Figure 1) used in this work was prepared in two steps. First, the neutral porphyrin was synthsized by the Rothemund and crossed Rothemund reactions using pyrrole and the adequate benzaldehydes (pyridine-4-carbaldehyde and pentafluorophenylbenzaldehyde or 4-formylbenzoic acid) at reflux in acetic acid and nitrobenzene (Sirish et al, 2002; Tomé et al, 2004). These reagents were purchased from Sigma-Aldrich (Madrid). The resulting porphyrin was separated by column chromatography (silica) and then the pyridyl groups were quaternized by reaction with methyl iodide. Porphyrin was purified by crystallization from chloroform/methanol/petroleum ether and its purity was confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy.



Tri-Py$^+$-Me-PF
5-(pentafluorophenyl)-10,15,20-tris(*N*-methylpyridinium-4-yl)porphyrin tri-iodide



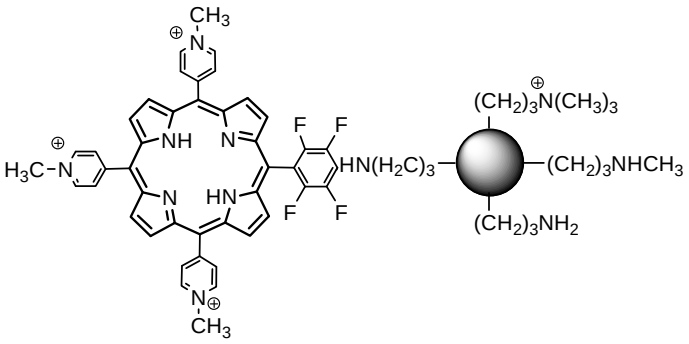
Tri-Py-PF
5-(pentafluorophenyl)-10,15,20-tris(*4*-pyridyl)porphyrin

Figure 1. Structure and the IUPAC name of the porphyrin derivative used for photoinactivation of T4-like

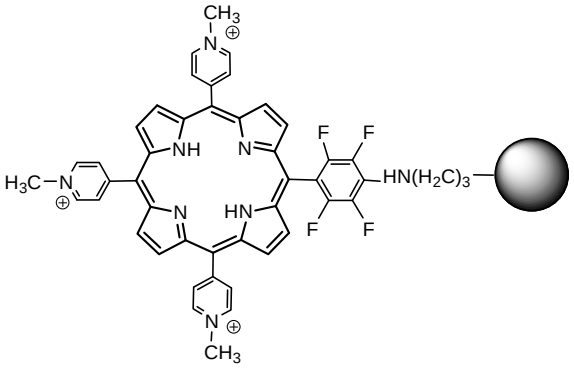
bacteriophage.

Porphyrin immobilization on solid matrixes

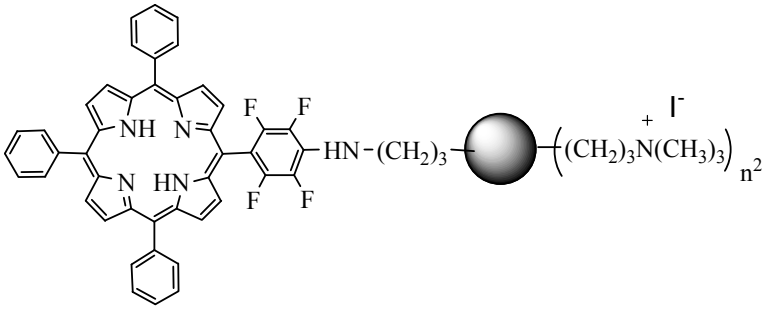
The synthesis of the nanomagnets used for porphyrin immobilization was done at three steps: synthesis of the magnetic nucleus, coating with silica and functionalization. The immobilization of the free porphyrin on solid cationic and neutral supports (figure 2) was done *via* covalent bonding between pentafluorophenyl groups of the sensitizer and the amino groups of the magnetic support.



Tri-Py^{+}-Me-PF-CS



Tri-Py^{+}-Me-PF-NS



Tri-Py-PF-CS

Figure 2. Structure of the three immobilized porphyrin derivatives used for photoinactivation of T4-like bacteriophage.

Phage selection and quantification

A wastewater sample from a secondary-treated sewage plant of the city of Aveiro (Portugal) was used to select the somatic bacteriophages of *E. coli* C (ATCC 13706). An isolated and morphologically representative phage plaque was picked out with a Pasteur pipette, by aspiration, and was placed in 50 mL of an *E. coli* culture in the exponential growth phase. The mixture was incubated with slow stirring (~100 rpm) at 37ºC, until the clarification of the medium, for about 5h. The suspension was then centrifuged at 7,000g (Beckman Avanti J-251 centrifuge) during 10 minutes. The supernatant with 10^9 particles per millilitre was decantated, added of 2% chloroform and kept at 4ºC. The quantification of phages was determined, in duplicate, by the agar double layer technique (Adams, 1959) using the aforementioned strain of *E. coli*. One millilitre of non-diluted sample or of serially diluted sample and 0.3 mL of bacterial host were added to a tube with 6 mL of soft TSA growth medium. The contents of the tube were mixed by manual rotation and then immediately poured onto a prepared confluent TSA monolayer on a petri plate. The plates were incubated upside-down during 18 hours at 37ºC in the dark, to avoid host bacteria inactivation. The number of phage plates was counted on the most convenient series of dilutions and the results were expressed as plaque forming units per millilitre (PFU ml^{-1}).

Bacteriophage identification

DNA extraction and purification of phage suspension was done using a standard technique (Sambrook et al, 1989). DNA was extracted with phenol saturated with TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) followed by extraction with a mixture of chloroform and isoamyl alcohol (24:1). The purified nucleic acid was amplified by PCR using consensus primers that amplify the central portion of capsid gene 23: Mzia 1 (5'-TGTTATIGGTATGGTICGICGTGCTAT-3') and CAP8 (5'-TGAAGTTACCTTCACCACGACCGG-3'). The conditions used for the amplification reaction with these primers involved 35 cycles consisting of 1 minute of denaturation at 95ºC, 1 minute of annealing at 58ºC and 2 minutes of extension at 72ºC. The PCR products were purified using the JETQUICK PCR Purification Spin kit from Genomed and sequenced with BigDyeTerminator v1.1 from Applied Biosystems. The phage was identified as a T4-like phage that has 82% of homology with the Enterobacteria phage RB43. The nucleotide sequence of the phage has been deposited in the GenBank database under accession nº EU026274.

Bacteriophage host viability test

As bacteria are sensible to the photosensitizers, the viability of the viral host was evaluated in order to prove that the phage inactivation was due to photoinactivation by the photosensitizer and not due to bacterial host inactivation by porphyrin. As at each sampling time we collected samples with the porphyrin, the sensitizer could then inactive the bacteria during the incubation period of 18 hours (after samples spread by the agar double layer method). Additional samples of the highest concentration (5 μM) of the free Tri-Py^{+}-Me-PF porphyrin, as well as light and dark controls samples, were collected in each sampling time, after irradiation, and washed by ultra-centrifugation at 28,000*g* (Beckman L8-80K ultracentrifuge, equipped with a swing-out rotor SW28) during one hour and thirty minutes, at room temperature, to remove the porphyrin. The porphyrin-free pellets of phages were re-suspended in 5 mL of PBS buffer, serially diluted and pour plated by the double layer technique. The results obtained were compared with those resulting from direct spread 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 μM). In the other experiments this step was not done, but the Petri plates were incubated in dark conditions.

Experimental set up

The efficiency of the Tri-Py^{+}-Me-PF porphyrin immobilized on the cationic and neutral supports and of the correspondent neutral form (Tri-Py-PF) immobilized on the cationic support was evaluated through quantification of the number of bacteriophages in laboratory conditions. The suspension of phages was diluted on phosphate buffer (PBS) until 5 x 10^{7} PFU mL^{-1} (1000 times higher than that of residual waters) and distributed in 600 mL acid-washed and sterilised glass goblets (20 ml per each of 8 goblets). Three of the goblets were added with supported photosensitizer at 5, 20 and 100 μM (Tri-Py-PF-CS was not tested for the concentration of 5 μM) and another goblet was added with 5 μM of free tricationic photosensitizer for comparison with the immobilized ones. The other four were used as dark, light and support material controls (in light and dark conditions). The light control was not added of porphyrin and was exposed to light. The dark control was added of the highest concentration of the photosensitizer (100 μM) and was covered with aluminium foil. The material controls

were added of 20 μM of support material without porphyrin and one was exposed to light (light support material control) and the other was covered with aluminium foil (dark support material control). The test and light goblets (controls) were exposed in parallel to: white light (PAR radiation, 13 lamps OSRAM 21 of 18 W each one, 380-700 nm) with an intensity of 40 W m^{-2} during 270 minutes, at 20- 25ºC, under stirring (100 rpm). Sub-samples of 1 mL were taken at time 0, 30, 60, 90, 180 and 270 minutes and analysed, in duplicate, for bacteriophage number. The Petri plates were kept in dark immediately after spread 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 of sampling as the mean of the two duplicates in the most convenient dilution series. Viral reductions were determined by subtracting the phage number at each time by the number of phages at time zero. For each photosensitizer two experiments were done and the results presented are the average of the two assays.

Statistical analysis

SPSSWIN 14.0 was used for data analysis. The significance of difference in phage inactivation among the immobilized and free 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.

Results

Bacteriophage host viability was not affected by porphyrins during the 18 hours of incubation. The pattern of phage inactivation was similar (ANOVA, $p = 0.308$) in washed and in non-washed samples (Figure 3). The dark incubation of Petri plates is sufficient to ensure the phage host viability. Consequently, all photosensitizers were tested without the washing step, which is a time consuming procedure that would greatly delay the assays.

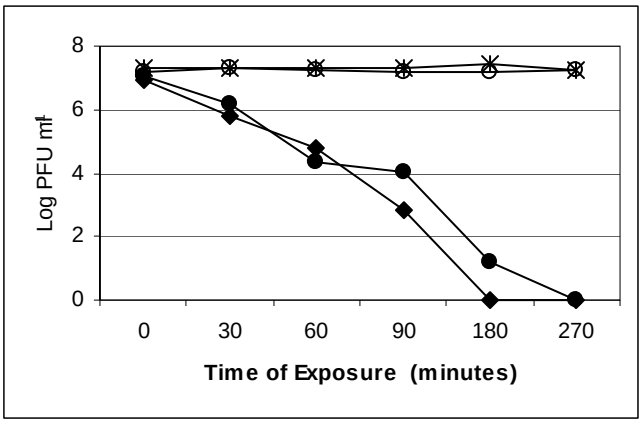


Figure 3. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation with 40 W m^{-2}, in the presence of 5 μM of porphyrin Tri-Py^{+}-Me-PF in washed and in non-washed phages. (—o— light control, —∗— dark control, —•— washed phages, —♦— non-washed phage).

The rate of phage inactivation with the cationic support, used for Tri-Py^{+}-Me-PF-CS and Tri-Py-PF-CS immobilization, was 67.9% for the concentration of 20 μM (reductions of 0.5 log), after 270 minutes of irradiation. For the neutral support used for Tri-Py^{+}-Me-PF-NS immobilization, the rate of inactivation at 20 μM was similar (83.79%), with reductions of 0.8 log (ANOVA p>0.05). The dark support controls, at 20 μM, did not show any viral activity during 270 minutes of irradiation (ANOVA p>0.05) (Figure 4).

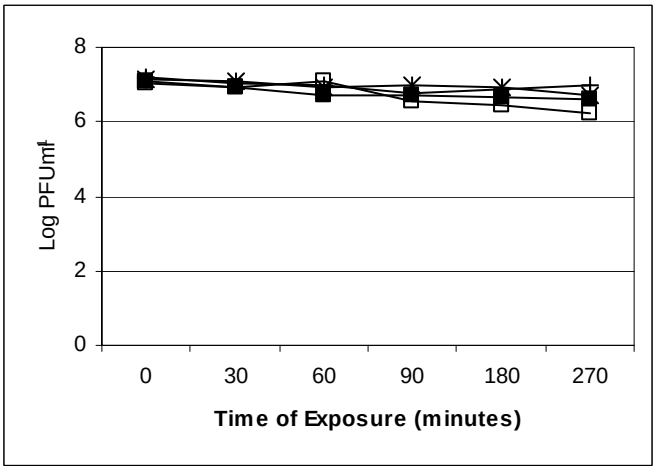


Figure 4. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation with 40 W m^{-2}, in the presence of cationic and neutral support materials. (—∗— cationic dark control, —+— neutral dark control, —■— cationic 20 μM, —□— neutral 20 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

The dark controls, which include immobilized or free porphyrins at 100 and 5 μM, respectively, did not show viral activity during the all irradiation period (ANOVA p>0.05) (Figures 5 to 8). A similar trend was observed when the phage was irradiated

with white light in the absence of the porphyrins during 270 minutes (light control) (ANOVA p>0.005) (Figures 5 to 8).

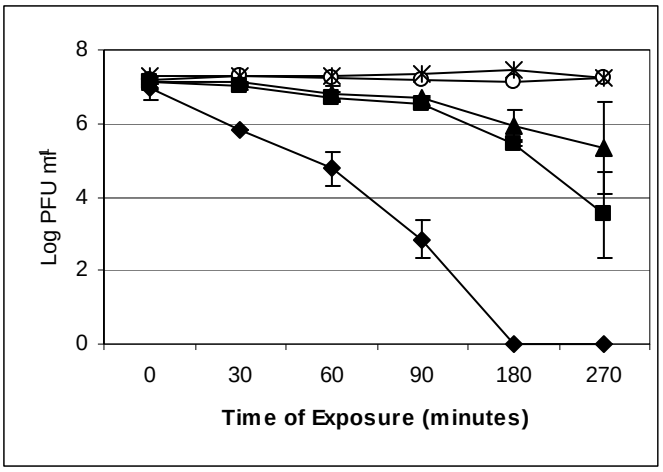


Figure 5. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation with 40 W m^{-2}, in the presence of porphyrin Tri-Py^{+}-Me-PF. (—o— light control, —∗— dark control, —●— 0.5 μM, —△— 1 μM, —▲— 5 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

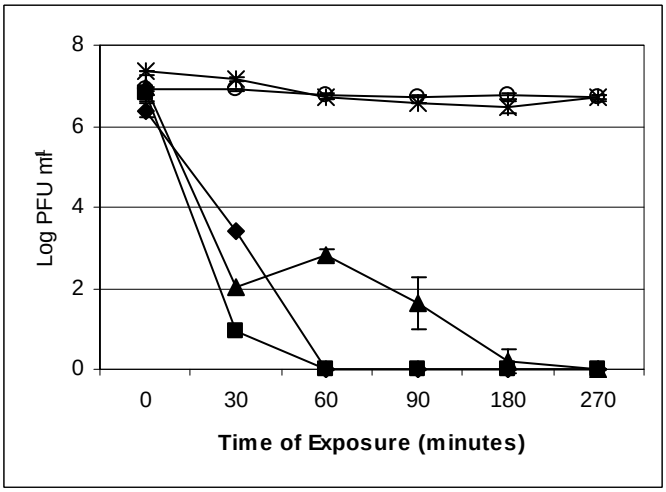


Figure 6. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation with 40 W m^{-2}, in the presence of porphyrin Tri-Py^{+}-Me-PF-CS . (—o— light control, —∗— dark control, —▲— 5 μM, —■— 20 μM, —♦— 100 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

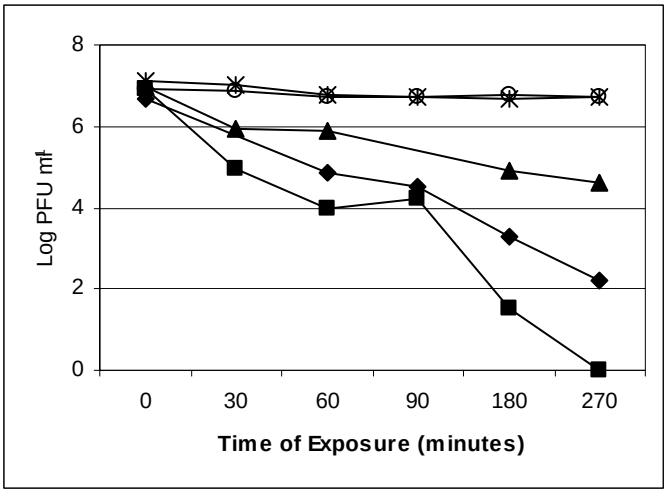


Figure 7. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation with 40 W m^{-2}, in the presence of porphyrin Tri-Py^{+}-Me-PF-NS . (—o— light control, —✳— dark control, —▲— 5 μM, —■— 20 μM, —♦— 100 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

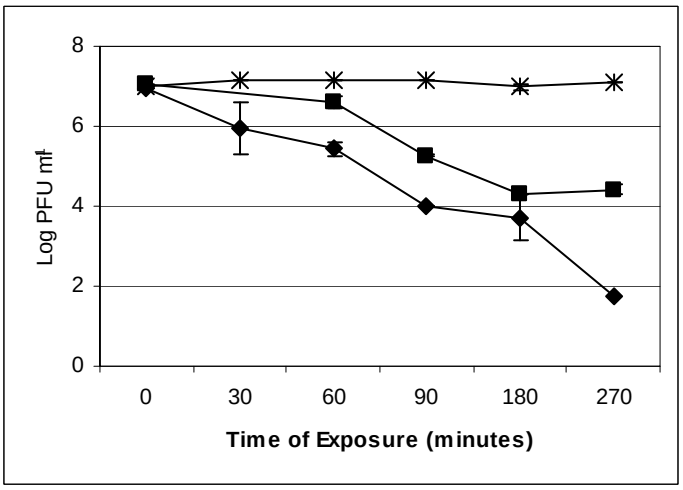


Figure 8. Density variation of the sewage bacteriophage after 30, 60, 90 and 180 minutes of irradiation with 40 W m^{-2}, in the presence of porphyrin Tri-Py-PF-CS. (—✳— dark control, —■— 20 μM, —♦— 100 μM). Each value represents mean ± standard deviation of two independent experiments, with two replicates each. Error bars represent standard deviations.

The free form of the tricationic porphyrin used in this study (Tri-Py^{+}-Me-PF) inactivated the sewage T4-like phage to the limits of detection (>99.9999% of inactivation) with reductions of 7 log, at 5 μM, after 180 minutes of irradiation. At 1 μM, it was observed a significant reduction of 3.6 log (99.92% of inactivation) after 270 minutes. At 0.5 μM, the rate of inactivation was 93.74% (reductions of 1.8 log) after 270 minutes of irradiation (Figure 5).

The efficiency of the sewage bacteriophage inactivation by supported porphyrin irradiated with 40 W m^{-2} varied with the type of support and with the concentration of the sensitizer. Cationic immobilized porphyrin Tri-Py^{+}-Me-PF-CS, supported in the

cationic support, inactivated the sewage T4-like phage to the limits of detection for all the concentrations tested, with reductions of 6.9 log, 6.8 log and 6.4 log after 270 minutes, respectively, for the concentrations of 5, 20 and 100 μM. For the highest concentrations, 20 and 100 μM, the reductions were exactly the same (6.8 and 6.4 log, respectively) soon after 60 minutes of irradiation. With the free form of this porphyrin, the rate of phage inactivation after 60 minutes was only 99.33% (reductions of 2.2 log) at the concentration 5 μM. For the immobilized cationic porphyrin Tri-Py^{+}-Me-PF-NS, supported in the neutral material, the rate of inactivation was not so high (ranging from 99.54 to 99.9999%) with reductions of 2.3 log, 6.9 log and 4.5 log, after 270 minutes, for 5, 20 and 100 μM, respectively. For the neutral porphyrin Tri-Py-PF-CS, immobilized in the cationic material, the rate of phage photoinactivation after 270 minutes was lower than those observed with the cationic porphyrin immobilized on the cationic support (ranging from 99.76% to 99.98%, with reductions of 2.6 log, for 20 μM, and 5.2 log, for 100 μM) (Figures 6 to 8).

The pattern of phage inactivation during the exposure time was different for the three immobilized sensitizers at the concentration of 100 μM (ANOVA $p<0.05$). However, for the concentration of 20 μM, the pattern of photoinactivation of Tri-Py^{+}-Me-PF-CS was considerably different from Tri-Py-PF-CS (ANOVA $p<0.05$) but similar to Tri-Py^{+}-Me-PF-NS (ANOVA $p>0.05$).

Discussion

The results of this study showed that the immobilized porphyrin Tri-Py^{+}-Me-PF, as the free form of this porphyrin, when irradiated with white light (40 W m^{-2}) can efficiently photoinactivate a sewage nonenveloped virus. The rate and extent of inactivation were, however, dependent on the support material and on photosensitizer concentration and charge. Cationic Tri-Py^{+}-Me-PF porphyrin immobilized on the cationic solid support, Tri-Py^{+}-Me-PF-CS, gave the same bacteriophage inactivation than that of the free form of the porphyrin (>99.9999%, with reductions of about 7 log) at 5 μM. It was expected that the immobilized porphyrin gave a less phage inactivation due to an eventual less availability of the sensitizer when supported on the solid matrix. The high rate of inactivation may be explained by the cationic nature of its support material. Just like cationic porphyrins, which have a demonstrated affinity to bacteria

(Polo et al, 2000; Hamblin et al, 2002; Jori and Brown, 2004) and viruses (Gábor et al, 2001; Egyeki et al, 2003; Zupán et al, 2004; Casteel et al, 2004) probably due to electrostatic interaction of positively charged porphyrins and negatively charged sites of the microorganism, the cationic support material rather than the neutral one is considered to facilitate the contact between photosensitizer and microorganism. In fact, when the tricationic porphyrin was immobilized on a neutral support, the efficiency of phage inactivation was significantly lower. On the other hand, it is well known that, in general, neutral porphyrins show low viral inactivation than cationic ones (Gábor et al, 2001; Demidova and Hamblin, 2005). However, in this study, the neutral porphyrin supported on the cationic material showed moderate phage inactivation (reduction until 5 log). This relatively high phage inactivation with the neutral immobilized porphyrin can be explained, in part, by the positive charge of its support. By this way, it can be said that for this immobilized porphyrin, the charge in the photosensitizer is not so important to photoinactivation as long as the amino groups of the support material are cationized. So, it can be said that the presence of positive charge in the support material is needed to achieve photoinactivation of the phage. This suggests that supported porphyrins can then be used for wastewater phage inactivation with a similar efficiency than of the correspondent free form, when we combine a cationic free porphyrin with a cationic support material.

As observed before in other studies for free photosensitizers (Kastury and Platz, 1992; Egyeki et al, 2003; Costa et al, *submitted*), phage inactivation with immobilized porphyrin Tri-Py^{+}-Me-PF-CS and Tri-Py-PF-CS, as well as with the free form of this porphyrin, increased with the increase of sensitizer concentration. However, for immobilized porphyrin Tri-Py^{+}-Me-PF-NS, the highest rate of inactivation was not observed for the highest photosensitizer concentration. In spite of that, with the highest concentration (100 μM) of immobilized porhyrin, the rate of bacteriophage photoinactivation was less than that of the concentration of 20 μM. According to Egyeki and colleagues (2003), a free tetraphenyl porphyrin inactivated the T7 phage in a concentration-dependent manner. However, at over 2 μM concentrations, the process was saturated. Further increase in porphyrin concentration did not lead to higher inactivation rate of T7. Similar results were obtained by Banfi and colleagues (2006). Aggregation of porphyrin derivatives in polar solvent and/or photobleaching of photosensitizer can be considered as possible reasons for such behaviour (Egyeki et al,

2003; Banfi et al, 2006). Both processes oppose the increase in number of the active molecules when the concentration is increased. Another possible explanation is that the high amount of porphyrin can make a barrier for the passage of light thus decreasing the rate of photoinactivation. The content of suspended solids can also affect the efficacy of photosensitization. Suspended solids can difficult light penetration affecting the rate of microbial inactivation. On the other hand, as viruses are colloidal particles that adsorb to suspended solids, high amount of particles in the water environment protect the viruses from the sensitizers. By this way, the photodynamic effects are greater when the experiments are carried out in clean waters (Alouini and Jemli, 2001). This fact can be used to explain the higher rate of inactivation at 20 μM than at 100 μM, when Tri-Py^{+}-Me-PF-NS porphyrin was used. The amount of support material used with 100 μM was much higher than that used with 20 μM, difficulting light penetration and protecting the microorganism. As Tri-Py^{+}-Me-PF-CS and Tri-Py-PF-CS were supported on cationic matrixes that help to achieve viral photoinactivation, these effects were not observed and, consequently, the bacteriophage was inactivated by these photosensitizers in a concentration dependent-manner.

We can consider that Tri-Py^{+}-Me-PF-CS is a very good material for the photoinactivation of the sewage phage, at 10^7 particles per millilitre, which represents significant levels of water contamination, 1000 times higher than that of residual waters. On the other hand, as the magnetic silica nanospheres used as support have a magnetic core of iron oxide, it is possible to remove them from the surrounding environment through the application of an external magnetic field, allowing their recovery and possible re-utilization. Moreover, the silica coating the nanospheres avoids oxidation and consequent degradation of the magnetic core, what is useful to prevent material aggregation (Liu et al, 2004). The nano-dimensions of these materials (~ 20nm) also allows the entrance into the phage particles (with ~ 200nm), increasing the photoinactivation of the phages.

In conclusion, our results showed that immobilized porphyrins irradiated with white light (40 W m^{-2}) efficiently photoinactivated an environmental T4-like bacteriophage, giving arise to the possibility of using this new environmental friendly technology for wastewater disinfection. One of the advantages of using immobilized sensitizers, when compared with free porphyrins, is that it avoids any residual traces of sensitizer in the water output, which would not certainly be accepted. Besides this, the

re-utilization of removed immobilized porphyrins considerably reduces the costs of PACT and makes it a simple and cheap technology for wastewater treatment. The complete eradication of viruses with this low light intensity means that this technology can be used during all year, including those dark days of winter. Consequently, photoinactivation can be applied to wastewater disinfection under natural irradiation conditions what turns it a less-costly and easily applicable method.

Acknowledgments

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CHAPTER 5

DISCUSSION

The present study demonstrates that some cationic porphyrin, when irradiated by the appropriate light, can efficiently inactivate sewage bacteriophages (reductions of about 7 log), whether they are free or immobilized on solid matrixes. The most effective free porphyrin, Tri-Py^+-Me-PF, when immobilized on a cationic support (Tri-Py^+-Me-PF-CS), inactivated the phage to the limits of detection as observed with the free form. The rate and extent of the photoinactivation are dependent on the photosensitizer charge, the charge distribution in the sensitizer, the nature of the *meso* substituent groups, the light intensity, the exposure time and on the photosensitizer concentration. Photoinactivation with immobilized porphyrins on solid matrixes is also dependent of solid supports characteristics. As far as we know, this is the first report using differently positively charged porphyrins, different light sources or different immobilized porphyrins on solid matrixes to photoinactivate sewage bacteriophages.

According to our results, the efficiency of the sewage T4-like phage inactivation by tetra-, tri- and dicationic porphryins was different (ANOVA, $p < 0.05$). For the photosensitizer concentration of 5 μM, Tetra-Py^+-Me, Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me photoinactivated the sewage to the limits of detection (>99.9999% of inactivation), with reductions of 7.2 log, 7 log and 6.7 log, respectively, after 270 minutes of irradiation. Tri-Py^+-Me-CO_2H produced a moderate decrease on phage viability, giving reductions of 3.9 log, after 270 minutes. Di-Py^+-Me-Di-CO_2H (-adj and –opp) porphyrins did not lead to a significant decrease (ANOVA, $p > 0.05$) on phage viability (reductions of 1.4 log and 1.2 log, respectively), after the 270 minutes of exposure to white light. These results clearly show that the tetra- and tricationic porphyrins were the most efficient sensitizers on phage inactivation. So, we can conclude that the number of positive charges is an important factor in the photoinactivation of sewage bacteriophages. It has been shown in other studies that the presence of one or more positively charged group plays an essential role in orientating the photosensitizer toward sites which are critical for the stability of cell organization and/or the cell metabolism (Merchat et al, 1996; Maisch et al, 2004).

The tetracationic porphyrin Tetra-Py^+-Me used in the present study leads to complete bacteriophage inactivation (> 99.9999%, 7.2 log). The efficiency of this photosensitizer is in accordance with previous studies where tetracationic porphyrins showed a high rate of viral inactivation (Casteel et al, 2004; Kastury and Platz, 1992; Zupán et al, 2004). Tetra-Py^+-Me showed similar results in other studies (reduction of > 7 log) for lambda phage inactivation (Kastury and Platz, 1992). This sensitizer was also used with T7 phage but just to investigate the mechanism of action of its photoreaction (Zupán et al, 2004). The *meso*-alkylated tetracationic porphyrin with different substituent groups was also tested for MS2 phage and hepatitis A virus inactivation and although it has been observed a lower viral inactivation (3-4 log of reduction, > 99.9% of inactivation) than with Tetra-Py^+-Me, viruses were inactivated to the limit of detection (Casteel et al, 2004). As far as we know, no other photoinactivation study was done with this sensitizer.

Tricationic porphyrins have never been used in sewage photoinactivation. In the present report, there was a marked difference between the tricationic porphyrins. Porphyrins Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me gave the best rates of phage inactivation (> 99.9999% of inactivation, with reductions of 7 and 6.7 log, respectively) when compared with the tricationic porphyrin Tri-Py^+-Me-CO_2H (reductions of 3.9 log, 99.99% of inactivation). Since Tri-Py+-Me-CO_2H has an acid group that can ionize when dissolved in PBS buffer, the global charge of the porphyrin decreases and, for that reason, diminishes the rate of inactivation. Tri-Py^+-Me-CO_2Me, by contrast, has the acid group protected and then showed a significantly higher rate of phage inactivation. These results confirm that the number of photosensitizer positive charges is a key factor in the process of phage photoinactivation. However, the rate and extent of phage inactivation with the most effective tricationic porphyrins (Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me) varied between them. Tri-Py^+-Me-PF inactivated the T4-like sewage bacteriophage early in time (after 180 minutes of irradiation) when compared with Tri-Py^+-Me-CO_2Me, which needed 270 minutes to inactivate the phage to the limits of detection. This indicates that the *meso*-substituent groups play an important role in the photoinactivation process. Similar results were obtained by Casteel and collaborators (2004) for tetracationic porphyrins with different substituent groups in MS2 phage inactivation and hepatitis A virus. As far as we know, there are no more studies about the effect of *meso*-substituent groups on viral inactivation. However,

other studies with bacteria showed similar results. Banfi and collaborators (2006) suggest that a moderate degree of lipophilicity, achievable by the introduction of aromatic hydrocarbon side chains on the pyridyl moieties, may improve photosensitizer's efficiency of Gram-negative (*E. coli* and *Pseudomonas aeruginosa)* and Gram-positive bacteria (*Staphylococcus aureus*). Lazzeri and colleagues (2004) also showed that an increase in the amphiphilic character of the photosensitizer, given by the presence of a trifluorophenyl group appears to enhance its affinity for *E. coli* cells. According to Merchat and colleagues (1996), *meso*-substituted cationic porphyrins can efficiently photosensitize the inactivation of both Gram-positive and Gram-negative bacterial strains. The authors concluded that this property appears to be independent of the number of positive charges, which are associated with the tetrapyrrolic derivative or the position of the positive charge in the *meso*-substituent. Subsequent observations have also shown that *meso*-substituted cationic porphyrins can efficiently inactivate bacteria independently of the number of positive charges (Maisch et al, 2004).

The different charge distribution of dicationic porphyrins in this study did not seem to affect the rate of phage photoinactivation. Viral inactivation by dicationic porphyrins was low and similar for both symmetric and asymmetric charged groups (ANOVA, $p > 0.005$). Porphyrins with opposite charged groups are more symmetric than porphyrins with adjacent charged groups, which should result in a molecular distortion due to electrostatic repulsion (Kessel et al, 2003). Studies of Kessel and collaborators (2003) with two cationic porphyrins varying in charge distribution on murine L 1210 cells showed that the efficacy of adjacent charged groups was greater than the opposite charged ones. Other studies showed that the asymmetric charge distribution at the peripheral position of the porphyrin produces an increase in the amphiphilic character of the structure, which can help a better accumulation in cells (Lazzeri et al, 2004). In our study, the low rate of phage inactivation observed with dicationic sensitizers (-adj and -opp) can difficult the detection of a different kinetic profile of inactivation between these two different derivative structures. Differences in photodynamic activity due to a different charge distribution will be probably better detected if these two porphyrins had a higher inactivation efficacy as observed for the Tetra-Py^+-Me, Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me derivatives.

According to our results, the rate and extent of photoinactivation are dependent on the light intensity and on the duration of the period of irradiation. All the light

sources tested lead to complete phage inactivation (> 99.9999% of reduction) although in a time- and concentration-dependent manner. For the highest photosensitizer concentration (5 μM), the led light (1690 W m^{-2}) gave the best results in phage inactivation, with reductions of 4.8 to 7.2 log after 25-60 minutes of irradiation. The solar light (600 W m^{-2}) also efficiently inactivated the phage with reductions of 7 to 7.2 log after 90-180 minutes. Cationic porphyrins irradiated with the white light (40 W m^{-2}) also showed high rates of phage inactivation (reductions of 3.9 to 7.2 log) but only after 180-270 minutes of irradiation. These results show that, for the same concentration of sensitizer, increases in light intensity will increase the rate of phage inactivation. Although there is no information about the effect of light intensity on bacteriophage inactivation, it has been shown that for bacteria higher light intensity leads to a higher rate of photoinactivation after the same irradiation time (Jemli et al, 2002; Lazzeri et al, 2004). However, for the same light intensity, the rate of photoinactivation can be increased with the increase of the irradiation time. Actually, in our study, for the lowest concentrations (0.5 and 1 μM) of the sensitizers exposed during long periods (270 minutes) to low light intensities (40 W m^{-2}), the rate of phage inactivation was higher than that obtained with the intense led light after 30 minutes of irradiation. This can be explained by the fact that after a longer irradiation period with solar and white light, the light dose is higher than after a short period of irradiation with the led light. In fact, the light dose is almost two times bigger after 180 minutes of exposure to solar radiation than after 30 minutes of irradiation with the led light. It indicates that when the light intensity is lower, increasing the irradiation time will actually improve the rate of phage inactivation. However, it has been shown that a similar dose results in a higher rate of inactivation if it is received over a longer time period (Gábor et al, 2001b). From our results we can conclude that a high light intensity over a short period of time gives a higher rate of bacteriophage inactivation than that of a low light intensity over a longer irradiation time. Porphyrin Tetra-Py^{+}-Me irradiated with a light dose of about 600 J cm^{-2} completely inactivated the phage (reductions of ~ 7 log) after 60 minutes with the led light, but only after 180 minutes with the solar light. For a light dose of about 300 J cm^{-2}, porphyrin Tri-Py^{+}-Me-PF completely inactivated the phage (reductions of 7.2 log) after 30 minutes with the led light and after 90 minutes with the solar light.

For all the free cationic porphyrins tested in this study, a progressively higher rate of phage photoinactivation was observed with the increase of the sensitizer's concentration. At the highest photosensitizer concentration (5 μM), Tetra-Py^+-Me and tricationic porphyrins (Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me) completely inactivated the phage (> 99.9999%) after 270 minutes of irradiation with white light. For the concentration of 1 μM, the photodynamic effect was not so pronounced and ranged from 61.03 to 99.92% (reductions of 0.5 to 3.6 log). For the concentration of 0.5 μM, the photoinactivation was lower but yet observable for Tetra-Py^+-Me, Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me (87.75 to 96.72% of inactivation, reductions of 0.9 to 2 log) but was considerably lower for Tri-Py^+-Me-CO_2H (reduction of 0.2 log after 270 minutes of irradiation). For the other light intensities (solar and led light), the rate of photoinactivation also increased with the increase of the concentration. For 5 μM, all porphyrins inactivated the phage to the limits of detection (> 99.9999%) for both light sources. For the other two concentrations (1 and 0.5 μM), the rates of inactivation were not so high and ranged from 1.4 to 3.8 log and 0.8 to 2.8 log, respectively, after 120-180 minutes with solar irradiation. With the led light, the values of phage inactivation varied between 0.7 and 2.2 log and between 0.5 and 1.3 log after 30 minutes, respectively for 1 and 0.5 μM.

PACT technology for wastewater disinfection, using the photosensitizer in solution, is far from being appropriate because residual traces of the sensitizer in the water output would certainly not be acceptable, and their removal from solution would become extremely difficult. So, the use of porphyrins immobilized on solid matrixes is a good purpose to overcome this situation (Käsermann and Kempf, 1998; Bonnet et al, 2006). Immobilized porphryins are, in fact, good alternatives for wastewater disinfection since they can be removed from water without leaving residual traces of sensitizer. On the other hand, they can also be re-utilized what turns PACT technology a less expensive technique for wastewater disinfection. From our results, the rate of viral photoinactivation with immobilized porphyrins is dependent on the support material, the photosensitizer concentration and also on the irradiation period as observed for free porhyrins. Immobilized porphyrin Tri-Py^+-Me-PF-CS gave the best results on phage inactivation, with reductions of 6.9 log after 270 minutes, 6.8 log and 6.4 log after 60 minutes of irradiation with white light, respectively for the concentrations of 5, 20 and 100 μM. This immobilized cationic

porphyrin, Tri-Py^{+}-Me-PF-CS , gave a similar rate of phage inactivation than that of the free form (porphyrin Tri-Py^{+}-Me-PF) when used at the same concentration (5 μM). The similar rate of phage inactivation with free and immobilized forms suggests that the covalent coupling of the sensitizer to the solid support does not impair its photobiological activity. However, part of this high rate of photoinactivation may be due to the cationic nature of its solid support. Actually, phage inactivation with the cationic porphyrin immobilized on the neutral support, Tri-Py^{+}-Me-PF-NS, was longer in time and gave lower reductions. It is important to note, however, that this cationic porphyrin immobilized on the neutral support (Tri-Py^{+}-Me-PF-NS), also efficiently inactivated the phage (until 99.9999% of inactivation), with reductions of 2.3 log, 6.9 log and 4.5 log, but only after 270 minutes, respectively for the concentrations of 5, 20 and 100 μM. The neutral porphyrin immobilized on the cationic support, Tri-Py-PF-CS, as expected, was the less efficient one, inactivating the phage with reductions of 2.6 log and 5.2 log after 270 minutes, for both 20 and 100 μM respectively. As phage inactivation by neutral porphyrin is, in general, very low (Gábor et al, 2001a; Demidova and Hamblin, 2005), the moderate inactivation (reductions of 5 log) observed with this neutral porphyrin immobilized on the cationic support can be due, in part, to the positive charge of the support.

However, for this immobilized porphyrin (Tri-Py^{+}-Me-PF-NS), the highest rate of phage inactivation was not observed for the highest concentration (100 μM) of sensitizer. In spite of that, with 100 μM of immobilized sensitizer, the rate of photoinactivation was considerably lower than at the concentration of 20 μM. Aggregation of porphyrin derivatives in polar solvent and/or photobleaching of sensitizer can be considered as possible reasons for such behaviour (Egyeki et al, 2003; Banfi et al, 2006). Both processes oppose the increase in number of the active molecules when the concentration is increased. Another possible explanation is that the high amount of immobilized porphyrin can make a barrier to the passage of light thus decreasing the rate of photoinactivation. The content of suspended solids can also affect the efficacy of photosensitization. Suspended solids can difficult light penetration affecting the rate of microbial inactivation. On the other hand, as viruses are colloidal particles that adsorb to suspended solids, high amount of particles in the water environment protect the viruses from the sensitizers. By this way, the

photodynamic effects are greater when the experiments are carried out in clean waters (Alouini and Jemli, 2001).

Our results, when compared with other study about photoinactivation in residual waters, showed a high microbial reduction (until 7 log after 270 minutes of exposure to white light) than the other (>2 log of reduction for *E. coli* cells) (Bonnett et al, 2006). Otherwise, the amount of phages used (1000 times higher than that of residual waters) turns PACT a promising technology for the disinfection of waters highly polluted.

In conclusion, our results showed that photodynamic technology with cationic sensitizers efficiently inactivated the T4-like sewage bacteriophage, opening the possibility of using this methodology for wastewater disinfection. Moreover, phototherapy approach has a potential advantage in wastewater treatment as it allows the inactivation of high concentration of microorganisms retained in solid residues during sewage treatments, as in sludges, when activated sludges are used as secondary treatment if this technology is applied to thin layers of sludges or if it is applied in a flow system. The complete inactivation of bacteriophages irradiated even with low light intensities (40 W m^{-2}), means that this therapy can be used during all year, including at those dark days of winter in which solar radiation is yet around 10 times higher than white light. Consequently, PACT can be applied under natural conditions (solar irradiation) for the wastewater treatment, becoming a cheap and accessible technology. As immobilized cationic porphyrins showed similar results for phage inactivation than that of the free form, this technology may be an environmental-friendly solution for viral inactivation in residual waters. Actually, since these porphyrins are immobilized on magnetic supports, they can be efficiently removed from water, leaving no residual traces of the sensitizer. Moreover, the possible re-utilization of these magnetic supported cationic porphyrins makes this a low-cost methodology. The high efficacy rate of bacteriophage inactivation with cationic sensitizers with three or four charges immobilized on solid matrixes, which can be recovered and re-utilized, makes PACT a very promising alternative to overcome the limitations of traditional chemical methods for wastewater disinfection.

FUTURE WORK

Although PACT is already in use, their application to wastewater disinfection is still in the initial stage. Future work will try to clear up some of the following points:

- Effectiveness of different compounds on distinct bacteriophages (DNA and RNA phages);
- Evaluation of the applicability of the photolytic treatment to the inactivation of mixed viral populations associated to treated sewage;
- Evaluation of the re-utilization of the immobilized photosensitizers;
- Evaluation of bacteriophage viability recuperation after photosensitizer exposure;
- Characterization of the mechanisms of action of free and immobilized cationic porphryins on viral photoinactivation (Type I and Type II photoprocresses).

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