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Fotoinactivação de *Escherichia coli* e de *Enterococcus faecalis* por porfirinas livres e suportadas

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Professora Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

porfirinas livres, porfirinas suportadas, terapia fotodinâmica, bactérias Gram-positivas, bactérias Gram-negativas, bactérias bioluminescentes, águas residuais

resumo

A terapia fotodinâmica antimicrobiana refere-se à combinação de uma fonte de luz, oxigénio molecular e um fotossensibilizador para inactivar células microbianas. Esta terapia tem sido considerada como uma alternativa aos métodos convencionais de desinfecção da água e recentemente demonstrado eficácia na eliminação de bactérias Gram (+) e Gram (-), usando porfirinas catiónicas como fotossensibilizadores. Tendo em conta que a introdução de resíduos porfirínicos no ambiente não é aceitável, têm sido feitos alguns estudos, com resultados promissores, para avaliar a possibilidade de imobilização dos fotossensibilizadores em matrizes sólidas. Para a monitorização do processo de fotoinactivação tornam-se necessários métodos mais rápidos de detecção microbiológica em detrimento dos métodos convencionais laboriosos de plaqueamento, incubação e contagem de colónias.

Foi objectivo deste trabalho estudar o efeito fotodinâmico sobre bactérias fecais, *Enterococcus faecalis* [Gram (+) e *Escherichia coli* Gram (-)], usando como fotossensibilizadores porfirinas catiónicas *meso*-substituídas na forma livre e imobilizadas em suportes sólidos. Foi também objectivo deste trabalho transformar *E. coli* numa estirpe indicadora bioluminescente para avaliar a eficácia de porfirinas utilizando um método rápido, sensível e rentável.

Nos ensaios com porfirinas livres, a suspensões bacterianas de 10^7 CFU mL^{-1} foram adicionadas três concentrações de porfirinas: 0.5, 1.0 e 5.0 µM. Nos ensaios com porfirinas suportadas, a suspensões bacterianas de 10^5 CFU mL^{-1} foram adicionadas duas concentrações de porfirinas: 20 µM e 200 µM. As amostras foram expostas à luz branca artificial (40 W m^{-2}) durante 270 minutos. Uma estirpe de *E. coli* foi transformada com os genes *lux*CDABE da bactéria marinha bioluminescente *Vibrio fischeri*. A estirpe recombinante resultante foi usada para avaliar, em tempo real, o efeito de três porfirinas catiónicas *meso*-substituídas na actividade metabólica das bactérias, usando luz branca artificial (40 W m^{-2}) e luz solar PAR (≈ 620 W m^{-2}).

As duas porfirinas tricatiónicas (Tri-Py^+-Me-PF e Tri-Py^+-Me-CO_2Me) foram as mais eficientes sobre as duas estirpes, originando um decréscimo na sobrevivência superior a 99,999% (≈ 7.0 log) após 270 minutos de irradiação para a concentração de 5 µM. A porfirina tetracatiónica também foi eficiente para ambas as estirpes (decréscimo na sobrevivência de 7 log com 5.0 µM após 270 minutos). As duas porfirinas dicatiónicas e a monocatiónica foram as

menos eficazes nas duas estirpes. Os híbridos Tri-Py^{+}-Me-PF-CS (fotossensibilizador catiónico imobilizado em material de suporte catiónico) e Tri-Py-Me-PF-CS (fotossensibilizador neutro imobilizado em material de suporte catiónico) foram igualmente eficientes contra as duas estirpes (decréscimo de 5 log com 20 e 200 µM na sobrevivência de *E. faecalis* após 90 minutos e decréscimo de ≈ 5 log de *E. coli* após 180 minutos de irradiação). Com o híbrido Tri-Py^{+}-Me-PF-NS (fotosensibilizador catiónico imobilizado em material de suporte neutro), praticamente não se verificou redução na viabilidade de *E. coli*, mesmo com 200 µM, mas *E. faecalis* foi inactivada após 90 minutos. A fotoinactivação da *E. coli* bioluminescente foi eficiente (> 4 log de decréscimo da bioluminescência) com as três porfirinas, sendo a porfirina tricatiónica Tri-Py^{+}-Me-PF a mais eficiente. Com a luz solar PAR, o processo de fotoinactivação é mais rápido e mais eficiente do que com a luz artificial, para as três porfirinas.

O número de cargas positivas, a distribuição das cargas na estrutura da porfirina e o carácter lipofílico dos grupos *meso*-substituintes são factores que parecem exercer efeitos diferentes na inactivação das duas estirpes. A carga no fotossensibilizador imobilizado não é um factor essencial à fotoinactivação da célula Gram (-), desde que os grupos amino do material de suporte estejam cationizados. A presença de carga positiva no material de suporte é necessária para conseguir a fotoinactivação de *E. coli*. O uso de bactérias bioluminescentes permite avaliar de forma simples e rápida a eficiência da actividade anti-metabólica destas porfirinas.

As vantagens económicas que a terapia fotodinâmica representa em termos de fonte de luz utilizada, síntese e produção de fotossensibilizadores imobilizados, possibilidade de remoção do meio após fotoinactivação e posterior reutilização, além do decréscimo significativo de sobrevivência observado, demonstram que esta metodologia poderá ser uma opção no processo de desinfecção de águas.

keywords

free porphyrins, immobilized porphyrins, photodynamic therapy, Gram-positive bacteria, Gram-negative bacteria, bioluminescent bacteria, wastewater

abstract

Photodynamic antimicrobial therapy means the combination of a light source, molecular oxygen and a photosensitizer to inactivate microbial cells. It has been considered to be a possible alternative to conventional methods of water disinfection and has recently been used to efficiently destroy Gram (+) and Gram (-) bacteria using cationic porphyrins as photosensitizers. Since the environmental contamination with porphyrinic residuals is not acceptable and in a way to overcome this water output contamination issue, some pilot studies have immobilized the photosensitizers on solid matrices, with promising results. To monitor the photoinactivation process, faster methods for microbial detection are required instead of laborious conventional plating, overnight incubation and colony count methods.

The aims of this work were to investigate the photodynamic effect on faecal bacteria, the Gram (+) *Enterococcus faecalis* and the Gram (-) *Escherichia coli*, using free and immobilized *meso*-substituted cationic porphyrins as photosensitizers. It was also an aim of this work to transform *E. coli* into an indicator bioluminescent strain in order to evaluate the efficiency of porphyrins in photoinactivation by a rapid, sensitive and cost-effective bioluminescent method.

In the experiments with porphyrins in the free form, bacterial suspensions of 10^7 CFU mL^{-1} were added with 0.5, 1.0 and 5.0 μM of porphyrin. In the experiments with immobilized porphyrins, bacterial suspensions of 10^5 CFU mL^{-1} were added with 20 μM e 200 μM of porphyrins. The samples were exposed to artificial white light (40 W m^{-2}) for 270 minutes. Then, *E. coli* cells were cloned with *lux*CDABE genes from the marine bioluminescent bacterium *Vibrio fischeri* and the recombinant bioluminescent indicator strain was used to assess, in real time, the effect of three cationic *meso*-substituted porphyrin derivatives on their metabolic activity, under artificial (40 W m^{-2}) and solar irradiation (≈ 620 W m^{-2}).

The most effective photosensitizers against both tested bacteria were the two tricationic porphyrins (Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me) when a 5.0 μM was used, leading to > 99,999% (≈ 7.0 log) photoinactivation after 270 minutes of irradiation. The tetracationic porphyrin was also a good PS against both bacteria (7 log drop with 5.0 μM after 270 minutes). Both dicationic and the monocationic were the least effective against the two strains. The Tri-Py^+-Me-PF-CS (cationic photosensitizer on cationic material) and Tri-Py-Me-PF-CS (neutral photosensitizer on cationic material) hybrids were almost equally

effective for both Gram types (5 log decrease with 20 and 200 µM on *E. faecalis* after 90 minutes and ≈ 5 log decrease on *E. coli* after 180 minutes of irradiation). With Tri-Py^{+}-Me-PF-NS (cationic photosensitizer on neutral material), almost none reduction on *E. coli* viability was observed, even with 200 µM. However, *E. faecalis* was completely inactivated after 90 minutes. The photoinactivation of bioluminescent *E. coli* was effective (> 4 log bioluminescence decrease) with the three porphyrins used, being the tricationic porphyrin Tri-Py^{+}-Me-PF the most effective. With solar irradiation, the photoinactivation process was faster and more efficient than with artificial light, for the three porphyrins.

The number of positive charges, the charge distribution in the porphyrins' structure, and the lipophilic character of the *meso*-substituent groups, seem to have different effects on the photoinactivation of both bacteria. The charge in the photosensitizer is not essential to Gram (-) cell photoinactivation, as long as the amino groups of the support material are cationized. The presence of positive charge in the support material is needed to achieve photoinactivation of *E. coli*. The use of bioluminescent bacteria allows the assessment of the efficiency of anti-metabolic action of these porphyrins.

The economical advantages this approach represents in terms of light source used, synthesis and production of the immobilized photosensitizers, possibility of removal after photoinactivation and further reuse, besides the significant photoinactivation observed, makes photodynamic antimicrobial therapy an interesting option to water disinfection.

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LIST OF ACRONYMS AND ABBREVIATIONS

µL	Microlitre
µM	Micromolar
APTES	3-aminopropyltriethoxysilane
Amp	Ampicillin
ANOVA	Analysis of variance
Ca^{2+}	Calcium ions
CFU	Colony forming units
Cm	Chloramphenicol
Da	Dalton
Di-Py^{+}-Me-Di-CO_2H *adj*	5,10-bis(4-carboxyphenyl)-15,20-bis(1-methylpiridinium-4-yl)porphyrin di-iodide
Di-Py^{+}-Me-Di-CO_2H *opp*	5,15-bis(4-carboxyphenyl)-10,20-bis(1-methylpiridinium-4-yl)porphyrin di-iodide
DMSO	Dimethyl sulfoxide
DP	Deuteroporphyrin
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EDTA	Ethylenediaminetetraacetic acid
Fe_3O_4	Ferric oxide
$FMNH_2$	Reduced riboflavin 5'-phosphate
Gram (-)	Gram-negative
Gram (+)	Gram-positive
HPD	Hematoporphyrin derivative
LB	Luria Bertani medium
LPS	Lipopolysaccharide
Mg^{2+}	Magnesium ions
mL	Millilitre
Mono-Py^{+}-Me-Tri-CO_2H	5-(1-methylpiridinium-4-yl)-10,15,20-tris(4-carboxyphenyl)porphyrin iodide
nm	Nanometre
O_2	Molecular oxygen
OD	Optical density
PACT	Photodynamic antimicrobial chemotherapy
PAR	Photosynthetically active radiation
PDI	Photodynamic inactivation
PDT	Photodynamic therapy
PI	Photoinactivation
PMBN	Polymyxin B nonapeptide
PS	Photosensitizer

rpm	Revolutions per minute
SiO_2	Silica
Tetra-Py^+-Me	5,10,15,20-tetrakis(1-methylpiridinium-4-yl)porphyrin tetra-iodide
Tri-Py^+-Me-CO_2H	5-(4-carboxyphenyl)-10,15,20-tris(1-methylpiridinium-4-yl)porphyrin tri-iodide
Tri-Py^+-Me-CO_2Me	5-phenyl-10,15,20-tris(1-methylpiridinium-4-yl)porphyrin tri-iodide
Tri-Py^+-Me-PF	5-(pentafluorophenyl)-10,15,20-tris(1-methylpiridinium-4-yl)porphyrin tri-iodide
Tri-Py-Me-PF	5-(pentafluorophenyl)-10,15,20-tris(*4*-pyridyl)porphyrin
TSA	Triptic soy agar
TSB	Triptic soy broth
V. fischeri	*Vibrio fischeri*
W	Watt

CHAPTER 1 INTRODUCTION

PHOTOTHERAPY AND PHOTODYNAMIC EFFECT

The use of light to treat disease is referred to as phototherapy (Ackroyd, Kelty et al. 2001) and remains to antiquity. In ancient Egypt, India and China, thousands of years ago, sunlight was employed to treat skin diseases (e. g., psoriasis, vitiligo and cancer) as well as rickets and even psychosis. In the past two centuries, in France, sunlight was used in the treatment of various conditions, such as tuberculosis, rickets, scurvy, rheumatism, paralysis, edema and muscle weakness. In the latter part of the twentieth century, phototherapy has been used to treat neonatal jaundice (Ackroyd, Kelty et al. 2001).

Photodynamic effect was defined by von Tappeiner and Jodlbauer in 1907 (Von Tappeiner and Jodlbauer 1907) and refers to the use of a light source (visible light of an appropriate wavelength), an oxidizing agent (molecular oxygen [O_2]) and an intermediary agent (named photosensitizer [PS]) able to absorb and transfer the energy of the light source to molecular oxygen leading to the formation of highly cytotoxic species (singlet oxygen [$^1O_2$], hydrogen peroxide [H_2O_2], superoxide [$O_2^{\bullet-}$], hydroxyl radical [$-OH^{\bullet-}$],) causing damage or destruction of living tissue (Dougherty, Gomer et al. 1998; Wainwright 1998; Shackley, Whitehurst et al. 1999; Bonnett 2000; Hamblin and Hasan 2004). The photodynamic effect was initially observed by Raab, in 1989, when he was studying the effects of acridine red dye on *Paramecium caudatum*. He discovered that the combination of acridine red and light had a lethal effect on *Paramecium caudatum* cells and demonstrated that this effect was greater than that of either acridine alone, light alone, or acridine exposed to light and then added to the protozoa (Raab 1900). At that time, that new approach was applied to the skin of patients with dermatological diseases. Jesionek and von Tappeiner, 1903, observed good results in the treatment of psoriasis, lupus vulgaris and skin cancer using a topical application of 5% eosin solution (Von Tappeiner and Jesionek 1903). However, since the middle of the last century, photodynamic antimicrobial chemotherapy (PACT) was forgotten mainly because the discovery and mass production of antibiotics (Hamblin and Hasan 2004).

PHOTODYNAMIC THERAPY

Nowadays, photodynamic therapy (PDT) is the name commonly given to photochemotherapy of cancer (Dougherty, Gomer et al. 1998) and is based on photodynamic principle. In PDT, the administered photosensitizing compound selectively accumulates in the target cells and local irradiation is employed on the lesion (Luksiene 2003; Triesscheijn, Baas et al. 2006) (visible light, normally, laser light directed via optical fiber) (Shackley, Whitehurst et al. 1999). The combination of two nontoxic elements, i. e. drug and light in the presence of molecular oxygen in the surrounding medium results in the selective destruction of tissue (Luksiene 2003) through a very localized cytotoxic effect (Shackley, Whitehurst et al. 1999).

PDT was developed in the 1960's by Lipson and Schwartz at the Mayo Clinic, USA. They observed during surgery, that injection of crude preparations of hematoporphyrin led to fluorescence of neoplastic lesions. In order to gain an optimal tumor localizing preparation, Schwartz treated hematoporphyrin with acetic acid and sulfuric acid and obtained a porphyrin mixture that he termed hematoporphyrin derivative (HPD), which was used by Lipson et al. (Dougherty and Henderson 1992) for tumor detection (Dougherty, Gomer et al. 1998). HPD contains several porphyrins, monomers, dimers and oligomers (Dougherty, Gomer et al. 1998). HPD has been partially purified, with the less-active porphyrins' monomers removed, to form Photofrin® (Dougherty 1996), the most widely used PS in clinical PDT. Because of the long-lasting skin phototoxicity of Photofrin®, several new PS have been introduced in clinical trials (Gomer 1991; Pass 1993). Photofrin® absorbs light only up to about 640 nm. Light at longer wavelengths penetrates farther into tissue and most of the new sensitizers have stronger absorbance at 650–850 nm (Dougherty, Gomer et al. 1998).

The porphyrins are generally called first generation PS. Second generation PS refer more to porphyrin derivatives or synthetics made from the late 1980's on. Third generation PS refer to available drugs modified with antibody conjugates, built in photobleaching capability (i. e., phenomenon that occurs when a fluorophore permanently loses its ability to fluoresce due to photon-induced chemical damage and covalent modification), biologic conjugates, among others (Moser 1998).

Since the 1990's, PDT has been successfully employed in the treatment of many tumors, including skin cancer, oral cavity cancer, bronchial cancer, esophageal cancer, bladder cancer, head and neck tumors in addition to nonmalignant diseases (Ackroyd, Kelty et al. 2001). Besides the cancer treatment, a very successful PDT application (FDA approved) has been in ophthalmology, as a treatment for age-related macular degeneration (Bressler and Bressler 2000).

PHOTODYNAMIC ANTIMICROBIAL CHEMOTHERAPY

Although the main use of PDT was the treatment of localized cancers, the worldwide emergence of antibiotic resistance amongst pathogenic bacteria has led to a major research effort to find alternative antibacterial therapeutics (Cassell and Mekalanos 2001) to which, it is hypothesized, bacteria will not be easily able to develop resistance. Bacteria replicate very rapidly and a mutation that helps a microbe to survive in the presence of an antibiotic drug will quickly become predominant throughout the microbial population. The inappropriate prescription of antibiotics and the failure of some patients to complete their treatment regimen also exacerbate the problem (Cassell and Mekalanos 2001). The coagulase-positive *Staphylococcus aureus* as well as both coagulase-negative *S. epidermis* and *S. hemolyticus* exhibit the capacity of developing resistance to each new generation of licensed antibiotics (Cunha 1998). Due to resistance to all ß-lactam antibiotics, vancomycin, a glycopeptid antibiotic, remained as last line of defense against Gram-positive [Gram (+) bacteria]. Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci (Murray 1997) are two resistant species that are still causing much concern (Cunha 1998). The problem is further aggravated by factors of social nature such as the inappropriate or excessive prescription of antibiotics, the widespread addition of antibiotics to livestock feedstuff, the more and more frequent transmission of microorganisms due to the global traveling and the expansion of poverty among populations in third world countries, as well as by the truly large variety of mechanisms adopted by microbial cells to increase their resistance to external insults. These include a thickening of their outer wall, encoding of new proteins which prevent the penetration of drugs, onset of mutants deficient in those porin channels allowing the

influx of externally added chemicals (Harder, Nikaido et al. 1981; Roland, Esther et al. 1994; Boyle-Vavra, Labischinski et al. 2001).

Examples of relatively novel therapies are phage therapy (Cerveny, DePaola et al. 2002), naturally occurring or synthetic antimicrobial peptides (Sajjan, Tran et al. 2001) and PACT (Wainwright 1998). All studies that have examined the inactivation of antibiotic resistant bacteria by the combination of PS and light, termed photodynamic inactivation (PDI) or photoinactivation (PI), have found them to be equally as susceptible as their naïve counterparts (Wilson and Yianni 1995; Wainwright, Phoenix et al. 1998). Because the delivery of visible light is almost by definition a localized process, PACT for infections is likely to be applied exclusively to localized disease, as opposed to systemic infections such as bacteremia (Hamblin and Hasan 2004). In contrast to PDT for cancer, where the PS is usually injected into the bloodstream and accumulates in the tumor, PACT for localized infections is thought to be carried out by local delivery of the PS into the infected area by methods such as topical application, instillation, interstitial injection or aerosol delivery (Hamblin and Hasan 2004). The key issues that need to be addressed are the effectiveness of the treatment in destroying sufficient numbers of the disease-causing pathogens; effective selectivity of the PS for the microbes, thus avoiding an unacceptable degree of PDT damage to host tissue in the area of infection; and the avoidance of regrowth of the pathogens from a few survivors following the treatment (Hamblin and Hasan 2004).

According to Jori et al., 2006, the main favorable features of PACT should be the following (Jori, Fabris et al. 2006):

- Broad spectrum of action, since one PS can act on bacteria, fungi, viruses, and parasitic protozoa;
- Efficacy independent of the antibiotic resistance pattern of the given microbial strain;
- Possibility to develop PDT protocols which lead to an extensive reduction in pathogen population with very limited damage to the host tissue;
- Lack of selection of photoresistant strains after multiple treatments;
- Small probability to promote the onset of mutagenicity;

- Availability of formulations allowing a ready and specific delivery of the PS to the infected area;
- Use of low cost light sources for activation of the photosensitizing agent.

At the moment, the main application of PACT is in the sterilization of blood and blood products, especially to be free of viral contamination. Remarkably, the human immunodeficiency virus has been inactivated *in vitro* by PACT (Wainwright 1998).

PHOTODYNAMIC ANTIMICROBIAL THERAPY AND WASTEWATER TREATMENT

Photodynamic antimicrobial therapy has been studied not only to be applied to the clinical field but also to the environmental field. This approach has been considered as a possibility to use in drinking water disinfection and in wastewater treatment plants as tertiary treatment in order to overcome the lack of water resources, mainly in densely populated areas, where wastewater may be treated and disinfected to further reuse in crop irrigation, for example (Jemli, Alouini et al. 2002; Rojas-Valencia, Orta-de-Velásquez et al. 2004).

As we know, water pollution is a major public health concern in our days. Although the improvement of sanitary conditions, at least in the urban centers, a great decrease in water resources is a reality. The effluents discharge in the environment, mostly after only secondary treatment, where no disinfection process is taken, increases the environmental contamination. Traditional water disinfection methods use chlorine, chlorine dioxide, ozone and ultraviolet radiation and are very efficient against a large range of microorganisms (Mendes and Oliveira 2004). However, those treatments involve high costs and difficulties of implementation at large scale due to operational, personnel qualification and logistic deficits (Mendes and Oliveira 2004). Ultraviolet radiation technology and ozonation are very expensive options to apply to a larger scale and disinfectants form toxic by-products, being the chlorine's by-products the most toxic (Richardson, Thruston et al. 2000). Therefore, more convenient and inexpensive technologies of water disinfection are needed (Bonnett, Buckley et al. 1994). Effectiveness of photodynamic antimicrobial therapy was observed on the destruction of faecal bacteria, viruses and helminthes eggs in wastewater (Carvalho, Costa et al.; Alouini

and Jemli 2001; Jemli, Alouini et al. 2002; Banfi, Caruso et al. 2006; Bonnett, Krysteva et al. 2006; Jiménez-Hernández 2006; Carvalho, Gomes et al. 2007; Costa, Alves et al. 2007).

PHOTODYNAMIC INACTIVATION OF BACTERIAL CELLS

Since the recovery of photodynamic antimicrobial therapy, nearly ten years ago, as an alternative to overcome the bacteria multidrug resistance problem, several studies have been carried out in order to evaluate many factors that influence the photodynamic effect on bacteria (Wainwright, Phoenix et al. 1998; Minnock, Vernon et al. 2000; Wainwright 2000; Gábor, Szocs et al. 2001; Nitzan and Ashkenazi 2001; Ashkenazi, Nitzan et al. 2003). The variables studied may be grouped according to the following:

- bacterial cell (Gram type, physiological state, cell density, and cell damage and mutagenic effects);
- photosensitizer (nature of PS, presence of positive charges, nature and number of substituents, cell binding process, overall lipophilicity and ionization, concentration used and the electronic structure of the compound);
- light source (nature of light source, light wavelength, fluence rate and irradiation time).

BACTERIAL CELL

In the 1990's, it was observed that there was a fundamental difference in susceptibility to PACT between Gram-negative [Gram (-)] and Gram (+) bacteria (Malik, Ladan et al. 1992). It was found that, in general, neutral or anionic PS molecules are efficiently bound to Gram (+) bacteria, photoinactivating them, whereas they are bound, to a greater or lesser extent, only to the outer membrane of Gram (-) bacterial cells, but do not inactivate them after illumination (Malik, Ladan et al. 1992). This difference is explained by their distinct three-dimensional architecture (Jori, Fabris et al. 2006) (Fig. 1.1).

The enhanced susceptibility of Gram (+) cells to PI is due to their thick outer wall constituted by more than a hundred peptidoglycan layers, intimately associated with lipoteichoic and negatively charged teichuronic acids. This outer cell wall displays a

relatively high degree of porosity, thus, does not act as a permeability barrier for the most commonly used PS (Jori, Fabris et al. 2006). Measurements of the penetration of polysaccharides, antimicrobial peptides and glycopeptides have shown that molecules of molecular weight in the range of 30 to 57 KDa can diffuse through the inner plasma membrane (Friedrich, Moyles et al. 2000). In general, resistance against the penetration of antibiotics is related to mechanisms concerning active efflux, changes in the target site, or inactivation (Russell 1998). Thus, in this class of bacteria, the cell wall does not act as a permeability barrier for the most commonly used PS, whose molecular weight does not generally exceed 1,500 – 1,800 Da (Jori, Fabris et al. 2006).

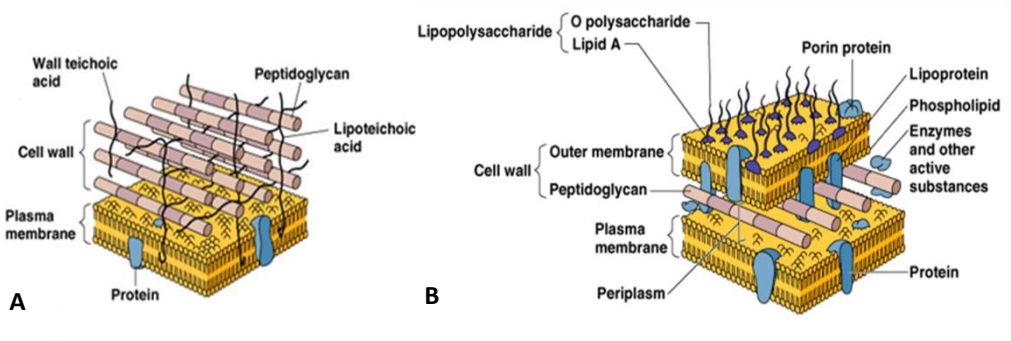

Figure 1.1 Schematic representation of the outer wall and cytoplasmic membrane structure in Gram (+) (**A**) and Gram (-) (**B**) bacteria.
Source: Jori et al. (Jori, Fabris et al. 2006)

In contrast, Gram (-) bacteria contain an additional membrane layer in the cell wall architecture, which is located outside the peptidoglycan layer and shows an asymmetric lipid structure composed by strongly negatively charged lipopolysaccharides (LPS), lipoproteins and proteins with porin function (Maisch, Szeimies et al. 2004). Hydrophilic compounds (< 600 to 700 Da for *Escherichia coli*) can diffuse through the outer membrane using the porins, which are characterized as aqueous channel-forming proteins (Nikaido and Vaara 1985; Yoshimura and Nikaido 1985). Consequently, the outer membrane acts as a very effective permeability barrier, making the Gram (-) bacteria resistant against host cellular and humoral defense factors. Furthermore, the outer membrane triggers mechanisms of resistance against many antibiotics, which are normally active in Gram (+) bacteria (Nikaido 1985; Nikaido 1994).

Photosensitivity of Gram (-) bacteria can be enhanced by the addition of biological or chemical molecules, e.g. the polycationic peptide polymyxin B nonapeptide (PMBN) or ethylenediaminetetraacetic acid (EDTA), which are known to alter the native consistence of the outer membrane, thereby enhancing its permeability and facilitating the penetration of phototoxic molecules to the cytoplasmic membrane (Malik, Ladan et al. 1992). Nitzan et al. (1992) used the PMBN, which increased the permeability of the Gram (-) outer membrane and allowed PS that are normally excluded from the cell to penetrate to a location where the reactive oxygen species generated upon illumination can cause fatal damage (Nitzan, Gutterman et al. 1992). PMBN does not release LPS from the cells, but expands the outer leaflet of the membrane, allowing PS to penetrate and permitting PDI of *E. coli* and *Pseudomonas aeruginosa* (Nitzan, Gutterman et al. 1992). Nitzan et al. (2001) demonstrated an interaction between PMBN and deuteroporphyrin (DP) in solution, and speculated that this binding assisted the penetration (Nitzan and Ashkenazi 2001). They used this method to inactivate a multi-antibiotic resistant strain of *Acinetobacter baumannii* and found that DP seemed to work much better in concert with PMBN than many other PS, including porphyrins, phthalocyanines and the merocyanine 540 dye (Nitzan and Ashkenazi 2001). The addition of EDTA removes the magnesium (Mg^{2+}) and the calcium (Ca^{2+}) ions which stabilize adjacent negative charged LPS molecules at the outer membrane of Gram (-) cells. As a consequence, electrostatic repulsion is promoted with destabilization of the native organization of the wall, inducing the release of a large fraction of the LPS into the medium (Nikaido and Vaara 1985; Jori, Fabris et al. 2006).

It was also shown that the photosensitivity of bacteria is affected by their physiological state since cells in the logarithmic phase of growth are appreciably more susceptible to photodynamic inactivation than the corresponding cells in the stationary phase (Wainwright 1998).

Another variable affecting the PI process is cell density because cells compete for binding with the available PS, as well as for reaction with photogenerated cytotoxic species (Demidova and Hamblin 2005).

There are two basic mechanisms that have been proposed to account for the lethal damage caused to bacteria by PDI: (a) DNA damage and (b) damage to the cytoplasmic membrane, allowing leakage of cellular contents or inactivation of membrane transport systems and enzymes (Fiel, Datta-Gupta et al. 1981; Capella, Coelho et al. 1996; Bertoloni, Lauro et al. 2000). There is a good deal of evidence that treatment of bacteria with various PS and light leads to DNA damage. Breaks in both single and double stranded DNA and the disappearance of the plasmid supercoiled fraction have been detected in both Gram (+) and Gram (-) species after PDI with a wide range of PS structural types (Fiel, Datta-Gupta et al. 1981; Capella, Coelho et al. 1996; Bertoloni, Lauro et al. 2000). There is some evidence that PS that can more easily intercalate into double-stranded DNA can more easily cause damage (Hass and Webb 1979; Hass and Webb 1981). Guanine residues have been shown to be the most easily oxidized (Hass and Webb 1979). The damage may able to be repaired by various DNA repairing systems (Imray and MacPhee 1973). However, various authors have concluded that although DNA damage occurs, it may not be the prime cause of bacterial cell death. As shown by Shafer et al. (1998), *Deinococcus radiodurans*, a bacterium which is known to have a very efficient DNA repair mechanism, is easily inactivated by PDI (Schafer, Schmitz et al. 1998). The alteration of cytoplasmic membrane proteins has been shown by Bertoloni et al. (Bertoloni, Rossi et al. 1990; Bertoloni, Rossi et al. 1992). Disturbance of cell wall synthesis and the appearance of a multilamellar structure near the septum of dividing cells, along with loss of potassium ions from the cells was reported by Nitzan et al. (Nitzan, Balzam-Sudakevitz et al. 1998).

PHOTOSENSITIZERS AND PHOTOPROCESSES

Photosensitizers are usually aromatic molecules which may absorb light of certain energy and may undergo an electronic transition to the singlet excited state (electron spins paired) (Wainwright 1998). Depending on its molecular structure and environment, the molecule may then lose its energy by electronic or physical processes, thus returning to the ground state, or it may undergo a transition to the triplet excited state (electron spins unpaired) (Wainwright 1998; Hamblin and Hasan 2004) which may then react further by one or both of two pathways known as the type I and type II photoprocesses, both of which require oxygen (Ochsner 1997) (Fig. 1.2).

The type I pathway involves electron-transfer reactions from the PS 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 (Athar, Mukhtar et al. 1988).

The type II pathway involves energy transfer from the PS triplet state to 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 (Redmond and Gamlin 1999). The *in situ* generation of singlet oxygen via type II pathway appears to play the central role in photodynamic cytotoxicity because of the highly efficient interaction of the $^1O_2$ species with different biomolecules (Luksiene 2003).

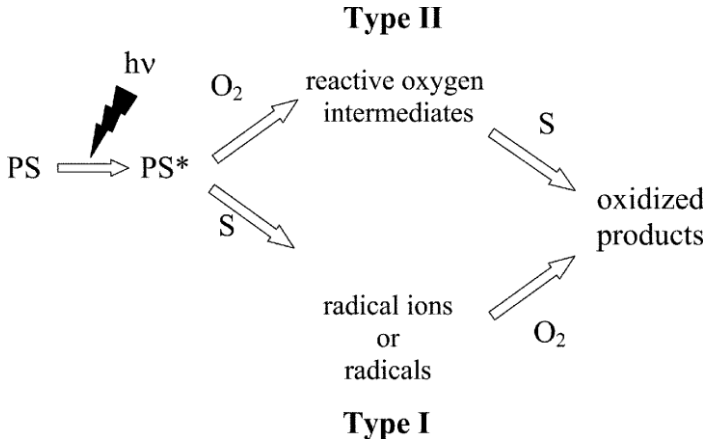

Figure 1.2 Pathway of type I and type II reaction of light absorbing photosensitizer. After light activating of the ground state of photosensitizer (PS), activated form of PS* can follow two alternative pathways via reactive singlet oxygen ($^1O_2$), hydrogen peroxide, hydroxyl radical (type II) or organic substrate (S) (type I). The intermediates react rapidly with their surroundings: cell wall, cell membrane, peptides, and nucleic acids.
Source: Maisch et al. (Maisch, Szeimies et al. 2004)

The photodynamic activity to induce cell damage or death is determined by five important photophysical/photochemical properties including (Maisch, Szeimies et al. 2004):

- an overall lipophilicity and ionization of the photoreactive PS;
- the molecular extinction coefficient *e*;
- quantum yield of the triplet state formation;

- redox potentials of the excited states (singlet and triplet) of the PS, if the reaction follows the type I pathway or
- the quantum yield of the singlet oxygen generation, if the reaction occurs by the type II pathway.

Different chemical classes of positively charged PS, including phenothiazines (methylene blue and toluidine blue), phthalocyanines and porphyrins, have been successfully tested as photoinactivating agents against Gram (+) and Gram (-) bacteria (Merchat, Bertolini et al. 1996; Minnock, Vernon et al. 1996; Wainwright 1998).

Porphyrins comprise of four pyrrole subunits linked together by four methane bridges (Fig. 1.3). This tetrapyrrole ring structure is named porphin and derivatives of porphins are named porphyrins. Tetrapyrroles are naturally occurring pigments, which are used in many biological processes and include the metallopigments heme (the prosthetic group of proteins like hemoglobin, cytochromes, catalase, peroxidase and tryptophane pyrrolase), vitamin B 12 , chlorophyll, siroheme (in nitrite and sulphite reductases) and factor F 430 (cofactor of methyl-CoM reductase). All these compounds are synthesized with uroporphyrinogen III as a common intermediate and modified to permit coordination of different metals at the ring centre, i.e. iron in heme and siroheme, magnesium in chlorophyll, cobalt in vitamin B 12 and nickel in factor F430 (Kristian 2007). These tetrapyrroles do not induce any photochemical or photobiophysical reactions in other compounds or are rapidly quenched in their normal surroundings, e.g. chlorophyll (Kristian 2007).

Synthetic porphyrins may be transformed into cationic entities through the insertion of positively charged substituents in the peripheral positions of the tetrapyrrole macrocycle (*meso* positions) which may largely affect the kinetics and extent of binding with microbial cells (Jori, Fabris et al. 2006). Cationic *meso*-substituted porphyrins have been proved to be more efficient and more photostable in water disinfection than other cationic PS as methylene blue and rose bengal (Jemli, Alouini et al. 2002).

It has been reported that cationic porphyrin derivatives are able to induce the PI of Gram (+) and Gram (-) bacteria (Merchat 1996; Merchat, Bertolini et al. 1996; Hamblin, O'Donnell et al. 2002; Jemli, Alouini et al. 2002; Banfi, Caruso et al. 2006) and some

studies compared the efficiency of synthetic *meso*-substituted cationic porphyrins with different charge distribution (tetra-, tri-, di- or monocationic) (Merchat 1996; Merchat, Bertolini et al. 1996; Lazzeri, Rovera et al. 2004; Caminos, Spesia et al. 2005; Spesia, Lazzeri et al. 2005). However, the results are not similar. Tetracationic porphyrins are efficient PS against both Gram (+) and Gram (-) bacteria on visible light (Merchat, Bertolini et al. 1996). It was also verified that some di- and tricationic porphyrins were more efficient than tetracationic ones, both against a Gram (+) strain and two Gram (-) strains (Merchat 1996). Recently, it has been shown that a dicationic porphyrin as well as two tricationic porphyrins having a trifluoromethyl group were powerful photosensitizing agents against *E. coli* (Lazzeri, Rovera et al. 2004).

An explanation for the PI of Gram (-) bacteria by porphyrins may be the impairment of cellular functions due to the localization of the molecules because of their positive charges, since *meso*-substituted but negatively charged porphyrins have not shown toxicity against Gram (-) bacteria (Merchat 1996), despite the generation of singlet oxygen (Jori 1990). It is thought that the *meso*-substitution itself is unlikely to be the relevant factor because cationic phthalocyanines, which are structurally unrelated to *meso*-porphyrins, show similar photobiological properties against Gram (+) and Gram (-) bacteria (Minnock, Vernon et al. 1996). Thus, other parameters must be relevant to explain the relative resistance of Gram (-) bacteria against photosensitization. On the other hand, it is possible to inactivate *E. coli* in the same way as Gram (+) bacteria by using ten-fold higher concentrations as well as three times longer illumination times (Merchat, Bertolini et al. 1996; Minnock, Vernon et al. 1996). A possible explanation for the cationic PS uptake by *E. coli* is the fact that in nature some cationic compounds are taken up through the so called self-promoted uptake pathway (Hancock 1984; Falla, Karunaratne et al. 1996). These molecules have a 2–4 orders of magnitude higher affinity to binding sites on surface LPS molecules than the divalent cations Ca^{2+} and Mg^{2+}, and they competitively displace these cations. The displacing of Ca^{2+} and Mg^{2+} leads to a reorganization of the outer membrane structure and permeabilization of the outer membrane to various antibiotics and hydrophobic molecules. In an early study, Ehrenberg et al. found that *E. coli* spheroplasts, which were lacking the outer membrane and cell

wall, were able to bind metal free porphyrins to the same extent as Gram (+) bacteria (Ehrenberg, Malik et al. 1985). Nevertheless, *E. coli* spheroplasts were not inactivated by these porphyrins and light. According to these findings, it is not sufficient to disturb the outer membrane structure alone but the cytoplasmic membrane must be disrupted as well to inactivate Gram (-) bacteria to the same extent as Gram (+). This observation indicates that PI of Gram (-) bacteria depends on the chemical structure of each molecule as well as its ability to penetrate the outer membrane and to reach the cytoplasmic membrane. In general, photosensitization of *S. aureus*, *E. coli* and *P. aeruginosa* has shown alterations of the ultra-structure of the cells, e. g. disordered cell wall structure; elongated cells connected together without separation of the daughter cells and different

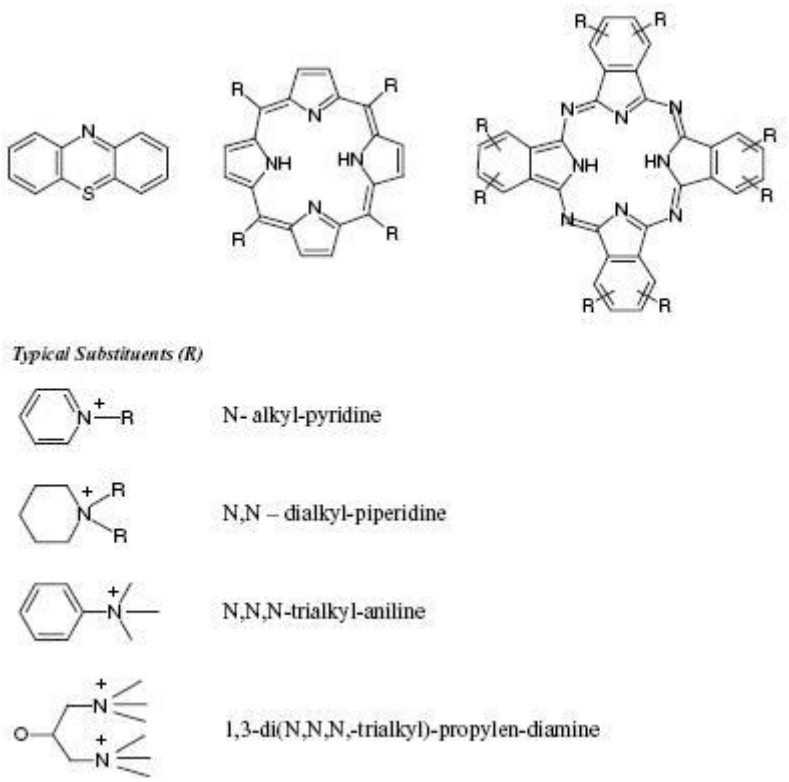

Figure 1.3. Basic chemical structure of phenothiazine, porphyrin, phthalocyanine photosensitizers and typical peripheral substituents (**R**) giving the photosensitizer a cationic character and enhancing the antimicrobial photosensitising efficiency.
Source: Jori et al. (Jori, Fabris et al. 2006)

low density areas in the cytoplasm (Ehrenberg, Malik et al. 1985; Malik, Faraggi et al. 1992; Nitzan, Gutterman et al. 1992). The nature and distribution of the functional groups in the molecule tells us whether the molecule is hydrophobic, hydrophilic or amphiphilic.

Information of these properties is important because they affect crucially the photophysical parameters and efficacy of the PS (Nyman and Hynninen 2004). A chemical compound is amphiphilic if possesses both hydrophilic and hydrophobic properties (Lincoln 2006). In the transport system of cell membranes, the lipid bilayer of the membrane allows for passive transport of hydrophobic molecules. This means that molecules that repel water may diffuse across the cellular membrane without the need of an active transport system, like that of adenosine 5'-triphosphate. Therefore, by making the PS hydrophobic, it can more easily diffuse across the cell membrane and improve the efficiency of photodynamic effect. At the same time PS must be in solution, so they need to be hydrophilic. Therefore, the ideal PS will demonstrate both hydrophilic and hydrophobic properties, making it amphiphilic (Lincoln 2006). There are some factors which increase the amphiphilic character of the porphyrins: the asymmetric charge distribution at their peripheral positions, cationic charges combined in different patterns with highly lipophilic groups (e. g., trifluoromethyl groups), introduction of aromatic hydrocarbon side groups and modulation of the number of positive charges on the PS (Boyle and Dolphin 1996; Ando and Kumadaki 1999; Grancho, Pereira et al. 2002; Caminos, Spesia et al. 2005; Spesia, Lazzeri et al. 2005; Banfi, Caruso et al. 2006; Caminos and Durantini 2006). The increase in the amphiphilic character of the PS seems to enhance its affinity for bacteria which helps a better accumulation in the cells (Boyle and Dolphin 1996; Lazzeri, Rovera et al. 2004) accompanied by an increase in the photocytotoxic activity (Caminos, Spesia et al. 2005).

As previously referred, the driving force for binding of the cationic PS to the negatively charged functional groups on the cell surface is of electrostatic nature, hence the binding process is completed within a very short time period. Several independent reports indicate that extending the pre-irradiation incubation from 5 minutes to 1–2 hours has no effect on the amount of PS bound to the microbial cells (Wainwright 1998; Jori and Brown 2004).

LIGHT SOURCE

In PI studies related to water disinfection, artificial white light (halogen or xenon lamps) and sunlight have been used in order to achieve microbial destruction. The light

fluence varied between mW m^{-2} and KW m^{-2} and since the experimental conditions described are very different, the results cannot be directly compared. One should note that the light fluence, the irradiation time or both can be varied to give the same light dose. However, a high light fluence over a short time period may give different results, in terms of microbial inactivation, from those of a low fluence rate over a longer time even though the light dose is the same in each case (Wainwright 1998). In general, PI of microorganisms is more extensive at a higher light fluence and longer treatment duration (Alouini and Jemli 2001). Increasing duration of irradiation will improve the wastewater yield treatment (Jemli, Alouini et al. 2002). It can compensate for a low concentration of sensitizer, a less efficient sensitizer type or a mediocre quality wastewater (Jemli, Alouini et al. 2002).

PORPHYRIN IMMOBILIZATION ON SOLID SUPPORTS

Despite the proven efficiency of cationic *meso*-substituted porphyrins as PS, their application in wastewater treatment plants would only be possible if they were immobilized on solid supports, in order to allow the PI process and subsequent removal of the sensitizer after photodynamic action to avoid the release of the PS to the water output (Bonnett, Krysteva et al. 2006; Jiménez-Hernández 2006). To this extent, few studies have developed PS immobilized on solid supports and the PI against faecal bacteria was tested (Bonnett, Evans et al. 1997; Artarsky, Dimitrova et al. 2006; Bonnett, Krysteva et al. 2006; Caminos and Durantini 2006; Jiménez-Hernández 2006). Bonnett et al. (2006) used a phthalocyanine immobilized on a polymeric membrane of chitosan in a model reactor of water disinfection (Bonnett, Krysteva et al. 2006). They used an *E. coli* suspension of 10^5 cell mL^{-1} representing the significant levels of water contamination. After 160 minutes of irradiation, a bacterial inactivation of > 2 logs was achieved. Also, after the dyed membrane was stored in the dark for 9 months, the photodynamic action was still detectable, demonstrating the thermodynamic stability of the PS system. They concluded that with that model system, the PI with immobilized PS can be used to lower microbial levels in water flow systems and that might also have applications to water detoxification (Bonnett, Krysteva et al. 2006). Jímenez-Hernandez et al. (2006) have used

polymer-supported Ru(II) sensitizers in a homemade microreactor with a solar simulator source for laboratory-scale water disinfection assays using a water sample containing *E. coli* and *E. faecalis* (2 x 10^3 CFU mL^{-1}). They observed noticeable disinfection with the RDP^{2+}/silicone system, for which has been measured a rate of cell inactivation by $^1O_2$ up to 1.1 x 10^5 CFU h^{-1} L^{-1} with *E. coli* and 0.7 x 10^5 CFU h^{-1} L^{-1} with *E. faecalis*. They concluded that photodisinfection with visible light was significant against both microorganisms (Jiménez-Hernández 2006). Artarsky et al. (2006) immobilized zinc phthalocyanines in a silicate matrix to test their photobactericidal properties on *E. coli* in model aqueous media. They obtained with the zinc phthalocyanine tetrasulfonic acid conjugate, a log kill of 1.32 and with the tetra tertiary butyl zinc phthalocyanine conjugate, a 0.98 log kill, in each case after 120 min. They concluded that phthalocyanines can be immobilized successfully in a silicate matrix and used for photodisinfection of microbially polluted waters (Artarsky, Dimitrova et al. 2006).

Suitable polymers must have specific characteristics such as compatibility with the PS, allowing easy and reproducible immobilization procedures and avoiding leaching out to water; mechanical strength and stability towards sunlight; good oxygen permeability for efficient $^1O_2$ production with minimum quenching; high biocompatibility to maximize the interaction between the support and the microorganisms and commercial availability and low cost (Jiménez-Hernández 2006).

Besides polymeric supports, magnetic supports as microspheres, nanospheres and ferrofluids have been widely used in biomedicine and bioengineering, such as in protein and enzyme immobilization, immunoassay, RNA and DNA purification, cell isolation and target drug (Zhang, Xing et al. 2007). These magnetic supports usually consist of inorganic magnetic cores (e. g., magnetite, haematite, nickel, alloys of cobalt) and organic or polymeric shells that are either biocompatible or possessing active groups which can be conjugated to biomolecules such as proteins and antibodies (Zhang, Xing et al. 2007). According to the target substances and their application system, different property requirements and evaluation standards should be taken in account about magnetic carriers: the size and its distribution of particles, magnetic response capability (reaction to external magnetic field, providing convenient separation from the surrounding medium),

surface functional groups (-CHO, -OH, -NH_2 and -COOH can be introduced onto the surface of magnetic microsphere by surface modification and therefore bioactive matters such as drugs and other molecules can be easily coupled to the surface of magnetic carriers), and biocompatibility (Zhang, Xing et al. 2007).

The first step of the preparation of magnetic carriers is to select the material of magnetic core. The magnetism, diameter and surface property of the magnetic core should be considered thoroughly (Zhang, Xing et al. 2007). The commonly used magnetic cores are inorganic nanoparticles with superparamagnetism such as metal (Fe, Co, Ni or alloy), ferrite [($CoFe_2O_4$, $(Mn,Zn)Fe_2O_4$, etc.], chromium-dioxide (CrO_2) and nitrided iron (Fe_4N). Ferric oxide (Fe_3O_4) is most often used in biomedicine because it is a good antioxidant and it has low toxicity (Zhang, Xing et al. 2007). The methods to prepare Fe_3O_4 include physical, chemical and biological origin. Among the different chemical methods, the coprecipitation method (Vayssieres, Chanei et al. 1998; Liu, Ma et al. 2004) is the most widely used. It is simple, convenient and appropriate for large scale preparation (Zhang, Xing et al. 2007). Magnetite nanoparticles with average diameter of 10 nm (Fig. 1.4) may be prepared by coprecipitation of ferrous and ferric iron ions (Fe^{2+} and Fe^{3+}, respectively) with the addition of alkaline precipitator, ammonium hydroxide (NH_4OH), and then coated with silica on the surface. Silica (SiO_2) is precipitated from sodium silicate solution with the addition of hydrochloric acid and then deposited on the magnetite to form a SiO_2 coating layer. The magnetite nanoparticles after being coated with SiO_2 will be useful to prevent the aggregation and even the partial exposure of naked magnetite once the naked magnetite will damage the activity of biological substances. Well-dispersed magnetic silica nanospheres can be further derivatized to which a large amount of substances may be immobilized, such as porphyrin derivatives (Liu, Ma et al. 2004; Zhang, Xing et al. 2007).

RAPID METHODS TO MONITOR THE BACTERIAL PHOTOINACTIVATION

To monitor the bacterial PI process, faster methods for microbial detection are required instead of laborious conventional methods of plating, overnight incubation and time consuming counting of colony forming units (CFU) (Vesterlund, Paltta et al. 2004;

Demidova and Hamblin 2005). New approaches to study potential PS *in vitro* are essential to accelerate the development of photodynamic antimicrobial therapy in drinking and residual waters treatment. To this end, the bacterial bioluminescence method is considered to be a rapid (Hamblin, O'Donnell et al. 2002), sensitive (Francis, Yu et al. 2001) and cost-effective option (Vesterlund, Paltta et al. 2004). Is also allows only living or viable cells to be detected and doesn't need exogenous administration of substrates (Rocchetta, Boylan et al. 2001) to obtain light emission.

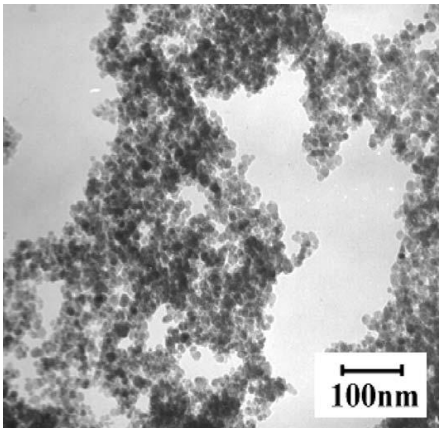

Figure 1.4 Transmission electronic microscopy of uncoated magnetite (Fe_3O_4).
Source: Liu et al. (Liu, Ma et al. 2004)

Bioluminescence refers to the process of visible light emission by living organisms (Meighen 1993) and this production is directly dependent on metabolic activity of the organism (Vesterlund, Paltta et al. 2004), once an inhibition of cellular activity results in a decrease in the respiration rate and consequently a decrease in the bioluminescence rate.

Among the light-emitting species are bacteria, dinoflagellates, fungi, fish, insects, shrimp and squid. This set of organisms includes terrestrial, freshwater and marine species (Harveyi 1952). Luminous bacteria are the most abundant and widely distributed of the light-emitting organisms and are found in marine, freshwater and terrestrial environments as free-living species, saprophytes, symbionts, or parasites (Hastings 1986). These bacteria are all Gram (-) motile rods and can function as facultative anaerobes (Nealson and Hastings 1979; Baumann, Baumann et al. 1983). Light production by symbiotic bacteria living in association with higher organisms may serve to attract prey, for intraspecies communication or to escape from predators (Nealson and Hastings 1979). The enzymes that catalyze the bioluminescent reaction are called luciferases and the

substrates are often referred to as luciferins. However, the light-emitting reactions are quite distinct for different organisms, with the only common component being molecular oxygen. Luciferase, a mixed function oxidase consisting of α and β subunits, catalyzes the emission of light (Ziegler and Baldwin 1981).

The light-emitting reaction in bacteria involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long chain fatty aldehyde with the emission of blue-green light (Rodriguez, Nabi et al. 1985; Meighen 1993) (Fig. 1.5).

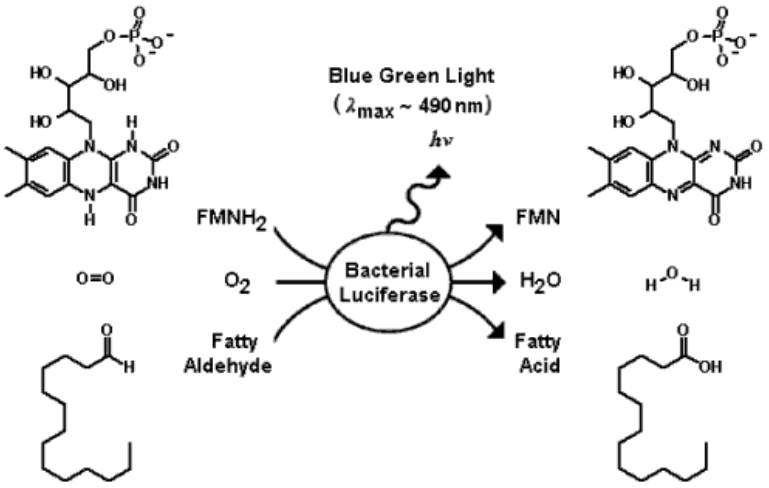

Figure 1.5 The net chemical equation of the bacterial luciferase catalyzed reaction.
Source: Lin and Meighen (Lin and Meighen 2004)

In both marine and terrestrial bioluminescent bacteria, a five-gene operon (*luxCDABE*) encodes the luciferase and biosynthetic enzymes (for the synthesis of the aldehyde substrate) necessary for light production. *luxA* and *luxB* genes encode the α and β subunits of the luciferase, and *luxC*, *luxD* and *luxE* encode proteins for aldehyde production (Meighen 1991). Although a number of additional *lux* genes in bioluminescent bacteria have been identified, only *luxCDABE* is essential for the biosynthesis of light (Meighen 1993; Meighen 1994). In marine bioluminescent bacteria light emission occurs preferentially at temperatures above 30°C (Hill, Rees et al. 1993).

Nearly two decades ago, the isolation of the genes responsible from bioluminescence in bacteria and the ability to transfer these genes into prokaryotic and eukaryotic organisms have greatly extended the capacity and potential uses of bacterial bioluminescence (Meighen 1993) therefore allowing the detection of antimicrobial

activity in real time (Vesterlund, Paltta et al. 2004). Amongst the applications of these recombinant bacteria are clinical research (Contag, Jenkins et al. 2000; Rocchetta, Boylan et al. 2001; Jawhara and Mordon 2004; Demidova, Gad et al. 2005; Doyle, Nawotka et al. 2006), environmental research (Burlage, Sayler et al. 1990; Ptitsyn, Horneck et al. 1997; Verschaeve 1999; Johnson 2005; Grande, Pietro et al. 2007) and biotechnology (Maoz, Mayr et al. 2002; Kadurugamuwa, Sin et al. 2003). In the clinical studies, PDT is applied to laboratory animal models inoculated with genetically engineered bioluminescent bacteria, in order to assess *in vivo* and in real time the progress of infectious disease by optical detection and monitoring of bioluminescence through sensitive imaging cameras (Contag, Contag et al. 1995 ; Francis, Joh et al. 2000; Francis, Yu et al. 2001; Hamblin, O'Donnell et al. 2002; Demidova, Gad et al. 2005; Doyle, Nawotka et al. 2006) (Fig. 1.6).

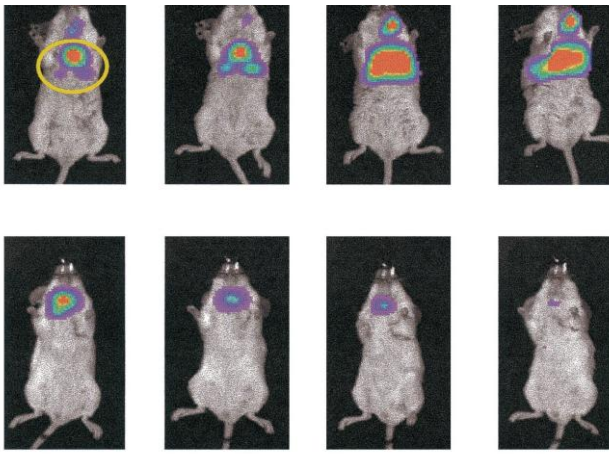

Figure 1.6 Bioluminescent *S. pneumoniae* A66.1 Xen 10 in a mouse pneumococcal lung model, without and with antibiotic treatment. The first row of animals (first four images) were left untreated as controls. The second row represents animals treated with amoxicillin at 1 mg/kg given subcutaneously at 0, 18, 24, and 42 h postinfection.
Source: Francis et al. (Francis, Yu et al. 2001)

Light output from these bioluminescent bacteria is a highly sensitive reporter of metabolic activity (Marincs 2000) and can therefore be used to monitor the real time effects of antimicrobials on bacterial metabolism (Salisbury, Pfoestl et al. 1999; Rocchetta, Boylan et al. 2001). Furthermore, in experimental systems in which a strong correlation between bioluminescence and viable counts can be demonstrated,

measurement of bioluminescence offers a rapid, alternative method for monitoring bacterial viability (Marincs 2000; Rocchetta, Boylan et al. 2001).

Light output is noncumulative, reflecting actual metabolic rate, and can be measured directly, continuously and nondestructively in high-throughput screening or continuous-culture models (Beard, Salisbury et al. 2002). Thus, the transformation of pathogenic bacteria into indicator bioluminescent strains allows using a rapid, sensitive and cost-effective methodology (Simon, Fremaux et al. 2001; Beard, Salisbury et al. 2002; Hamblin, O'Donnell et al. 2002; Vesterlund, Paltta et al. 2004; Jawhara and Mordon 2006) to evaluate the efficiency of PI.

THESIS OUTLINE

The aims of this thesis are to investigate the photodynamic effect on two faecal pollution indicator strains: a Gram positive bacterium, *Enterococcus faecalis*, and a Gram negative bacterium, *Escherichia coli* using free and immobilized *meso*-substituted cationic porphyrin derivatives as PS, artificial white light and sunlight as light sources and molecular oxygen dissolved in water as oxidizing agent, in a static water system. Is also an aim to transform *Escherichia coli* into an indicator bioluminescent strain in order to evaluate the efficiency of PI by porphyrins through a rapid, sensitive and cost-effective bioluminescent method instead of conventional laborious plating and counting methods.

In a first step, will be investigated the kinetics of photodynamic inactivation of seven *meso*-substituted cationic porphyrins derivatives taking in account their charge number (one to four positive charges), charge distribution (adjacent and opposite) and *meso*-substituent groups (carboxyl, pentafluorophenyl and carboxymethyl) varying the concentration of PS. Artificial white light will be used (40 W m^{-2} of photosynthetically active radiation [PAR]). The best PS will be chosen for immobilization. Chapter 2 describes this work.

Chapter 3 describes the preliminary assessment of the photodynamic inactivation of immobilized porphyrins on solid supports, using artificial white light (40 W m^{-2} of PAR

radiation). Magnetic silica nanospheres were used as support materials and were covalently coupled to porphyrins (work done by the Organic Chemistry group from Chemistry Department of University of Aveiro).

In the Chapter 4, a recombinant bioluminescent strain of *Escherichia coli* will be constructed using the *lux* operon from marine bacterium *Vibrio fischeri*. Then, the anti-metabolic activity of three cationic non-immobilized *meso*-substituted cationic porphyrins will be assessed using both artificial white light (40 W m^{-2} of PAR radiation) and solar PAR radiation (≈ 620 W m^{-2}).

Chapter 5 discusses the obtained results and presents the main conclusions and suggestions for future work.

CHAPTER 2 CHARGE EFFECT ON THE PHOTOINACTIVATION OF GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA BY CATIONIC PORPHYRINS

ABSTRACT

Photodynamic antimicrobial therapy has recently been used to efficiently destroy Gram-positive and Gram-negative bacteria using cationic porphyrins as photosensitizers. Although there is an increasing interest on this approach, little is known about the effect of the number of positive charges on the porphyrin, their distribution at peripheral positions as well as the *meso*-substituent groups present, on the bacterial photoinactivation process. We used seven synthetic cationic *meso*-substituted porphyrins to photoinactivate a Gram-positive bacterium (*Enterococcus faecalis*) and a Gram-negative bacterium (*Escherichia coli*). Samples consisting of bacterial suspension ($\approx 10^7$ CFU mL^{-1}) with different concentrations of porphyrins (0.5, 1.0 and 5.0 μM) where exposed to white light (40 W m^{-2}) for 270 minutes. The most effective photosensitizers against both tested bacteria are the two tricationic porphyrins (Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me) when a 5.0 μM is used, leading to > 99,999% (≈ 7.0 log) photoinactivation after 270 minutes of irradiation. The tetracationic porphyrin is also a good PS against both bacteria (7 log drop with 5.0 μM after 270 minutes) and for *E. coli* is more effective than the tricationic porphyrin with a carboxyphenyl group for 0.5 and 5.0 μM. Both di-cationic and the monocationic are the least effective against the two strains. The number of positive charges, the charge distribution in the porphyrins' structure, and the lipophilic character of the *meso*-substituent groups, seem to have different effects on the photoinactivation of both bacteria and need to be further investigated. The low cost associated with the efficient inactivation of pathogenic microorganisms using tri- and tetracationic photosensitizers under natural light conditions and immobilized on solid supports indicate photodynamic antimicrobial therapy as a promising possibility for wastewater disinfection.

Keywords: porphyrin, photodynamic therapy, Gram-positive bacteria, Gram-negative bacteria, charge effect, wastewater

INTRODUCTION

The environmental contamination due to domestic and industrial waste discharges has become a major public health concern. Wastewater treatment process includes a final disinfection stage which eliminates pathogenic microorganisms (bacteria, yeasts, virus and protozoa). The water disinfection may be achieved using chlorine, chlorine dioxide, hypochlorite, ozone or ultraviolet radiation. Although very efficient against a large range of microorganisms, the implementation of these solutions for wastewater treatment has been limited by environmental factors, namely the formation of toxic by-products from chorine (Richardson, Thruston et al. 2000), or by economic factors, as ultraviolet radiation technology and ozonation are very expensive options to apply to a larger scale. Thus, as water reuse may be a way to cope with low water resources (Jemli,

Alouini et al. 2002) in densely populated areas, more convenient and inexpensive technologies of water disinfection are needed (Bonnett, Buckley et al. 1994).

Photodynamic antimicrobial therapy has recently been used to efficiently destroy microorganisms. This methodology combines a photosensitizer (PS) typically a porphyrin or a phthalocyanine derivative, light and oxygen (Wainwright 1998) leading to the formation of cytotoxic species (singlet oxygen and free radicals) that destroy those cells (Wainwright 1998). This methodology has been shown to be effective *in vitro* against bacteria (including drug-resistant strains), yeasts, viruses and protozoa (Wainwright 1998; Wainwright, Phoenix et al. 1998; Alouini and Jemli 2001; Makowski and Wardas 2001; Nitzan and Ashkenazi 2001; Ashkenazi, Nitzan et al. 2003; Wainwright 2004; Caminos, Spesia et al. 2005; Lambrechts, Aalders et al. 2005; Banfi, Caruso et al. 2006; Caminos and Durantini 2006; Drábková, álek et al. 2007). Recent studies have shown that photoinactivation (PI) of bacteria in drinking (Bonnett, Krysteva et al. 2006) and residual waters (Jemli, Alouini et al. 2002; Carvalho, Gomes et al. 2007) is possible under solar irradiation. Bonnett et al. (2006) used a porphyrin and a phthalocyanine immobilized on a polymeric membrane of chitosan in a model reactor of water disinfection (Bonnett, Krysteva et al. 2006). The recovery and reuse of immobilized PS opens the possibility to apply the photodynamic process in the real context, avoiding the PS release and contamination of water output (Bonnett, Krysteva et al. 2006; Carvalho, Gomes et al. 2007).

In the last decade, several studies have used tetrapyrrolic derivatives as PS in order to assess the PI efficiency against Gram-negative [Gram (-)] and Gram-positive [Gram (+)] bacteria (Jemli, Alouini et al. 2002; Spesia, Lazzeri et al. 2005). It is well documented that neutral PS (porphyrins and phthalocyanines), efficiently destroy Gram (+) bacteria but are not able to photoinactivate Gram (-) bacteria (Perria, Carai et al. 1988; Dahl, Midden et al. 1989; Bonnett, Buckley et al. 1993; Hamblin, O'Donnell et al. 2002). However many of these PS can become effective against Gram (-) bacteria if they are co-administrated with outer membrane disrupting agents such as calcium chloride ($CaCl_2$), ethylenediaminetetraacetic acid (EDTA) or polymixin B nonapeptide (Ehrenberg, Malik et al. 1993; Jori and Brown 2004) which are able to promote electrostatic repulsion with

destabilization of the native organization of the cell wall. This allows significant concentrations of the PS to penetrate into the cytoplasmic membrane, which can be photosensitized after light activation of the PS (Bertoloni, Rossi et al. 1990; Malik, Ladan et al. 1992; Nitzan, Gutterman et al. 1992).

Porphyrins can be transformed into cationic entities through the insertion of positively charged substituents in the peripheral positions of the tetrapyrrole macrocycle which may largely affect the kinetics and extent of binding with microbial cells (Jori, Fabris et al. 2006). The hydrophobicity degree of porphyrins can be modulated by either the number of cationic moieties (up to four in *meso*-substituted porphyrins) or the introduction of hydrocarbon chains of different length on the amino nitrogens (Jori, Fabris et al. 2006). It has been reported that cationic porphyrin derivatives are able to induce the PI of Gram (+) and Gram (-) bacteria (Merchat 1996; Merchat, Bertolini et al. 1996; Hamblin, O'Donnell et al. 2002; Jemli, Alouini et al. 2002; Banfi, Caruso et al. 2006) and some studies compared the efficiency of synthetic *meso*-substituted cationic porphyrins with different charge distribution (tetra-, tri-, di- or monocationic) (Merchat 1996; Merchat, Bertolini et al. 1996; Lazzeri, Rovera et al. 2004; Caminos, Spesia et al. 2005; Spesia, Lazzeri et al. 2005). However, the results are not similar. Tetracationic porphyrins are efficient PS against both Gram (+) and Gram (-) bacteria on visible light (Merchat, Bertolini et al. 1996). It was also verified that some di- and tricationic porphyrins were more efficient than tetracationic ones, both against a Gram (+) strain and two Gram (-) strains (Merchat 1996). Recently, it has been shown that a dicationic porphyrin as well as two tricationic porphyrins having a trifluoromethyl group were powerful photosensitizing agents against *Escherichia coli* (Lazzeri, Rovera et al. 2004).

Reviewing the literature, it can be said that there are some factors which increase the amphiphilic character of the porphyrins: the asymmetric charge distribution at the peripheral position of the porphyrin, cationic charges combined in different patterns with highly lipophilic groups (e.g., trifluoromethyl groups), introduction of aromatic hydrocarbon side groups and modulation of the number of positive charges on the PS (Boyle and Dolphin 1996; Ando and Kumadaki 1999; Grancho, Pereira et al. 2002; Caminos, Spesia et al. 2005; Spesia, Lazzeri et al. 2005; Banfi, Caruso et al. 2006; Caminos

and Durantini 2006). This increase in the amphiphilic character of the PS seems to enhance its affinity for bacteria which helps a better accumulation in the cells (Boyle and Dolphin 1996; Lazzeri, Rovera et al. 2004) accompanied by an increase in the photocytotoxic activity (Caminos, Spesia et al. 2005).

The aim of this study was to investigate the bactericidal effect of seven *meso*-substituted cationic porphyrins according to charge number (one tetracationic, three tricationic, two dicationic and one monocationic), charge distribution (adjacent and opposite) and *meso*-substituent groups (pentafluorophenyl, carboxyl and carboxymethyl) against a Gram (+) bacterium (*Enterococcus faecalis*) and a Gram (-) bacterium (*Escherichia coli*). As far as we know, none of these tricationic, dicationic or monocationic derivatives have been used in photodynamic antimicrobial therapy, although the tetracationic porphyrin has been used in bacterial and viral PI.

MATERIALS AND METHODS

PHOTOSENSITIZERS

Porphyrins 5,10,15,20-tetrakis(1-methylpiridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^+-Me), 5-(4-carboxyphenyl)-10,15,20-tris(1-methylpiridinium-4-yl)porphyrin tri-iodide (Tri-Py^+-Me-CO_2H), 5-(pentafluorophenyl)-10,15,20-tris(1-methylpiridinium-4-yl)porphyrin tri-iodide (Tri-Py^+-Me-PF), 5-metoxicarbonilphenyl-10,15,20-tris(1-methylpiridinium-4-yl)porphyrin tri-iodide (Tri-Py^+-Me-CO_2Me), 5,10-bis(4-carboxyphenyl)-15,20-bis(1-methylpiridinium-4-yl)porphyrin di-iodide (Di-Py^+-Me-Di-CO_2H *adj*), 5,15-bis(4-carboxyphenyl)-10,20-bis(1-methylpiridinium-4-yl)porphyrin di-iodide (Di-Py^+-Me-Di-CO_2H *opp*) and 5-(1-methylpiridinium-4-yl)-10,15,20-tris(4-carboxyphenyl)porphyrin iodide (Mono-Py^+-Me-Tri-CO_2H) (Fig. 2.1) were prepared in two steps. First, the neutral porphyrins were obtained from the Rothemund and crossed Rothemund reactions using pyrrole and the appropriate benzaldehydes (pyridine-4-carbaldehyde and pentafluorophenylbenzaldehyde or 4-formylbenzoic acid) at reflux in acetic acid and nitrobenzene (Sirish, Chertkov et al. 2002; Tome, Neves et al. 2004). After being separated by column chromatography (silica), the pyridyl groups of each porphyrin 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. All porhyrins were kindly provided by the Organic Chemistry group from Chemistry Department of University of Aveiro.

BACTERIAL STRAINS AND GROWTH CONDITIONS

Escherichia coli ATCC® 13706 (USA) and *Enterococcus faecalis* ATCC® 51299 (USA) were stored at 4°C in triptic soy agar (TSA, Merck). Before each assay the strains were grown aerobically for 24 hours at 37°C in 30 mL of triptic soy broth (TSB, Merck). Then, an aliquot of this culture (240 µL) was aseptically transferred to 30 mL of fresh TSB medium and grew overnight at 37°C to reach an optical density (OD_{600}) of ≈ 1.3, corresponding to ≈ 10^8 cells mL^{-1}.

EXPERIMENTAL SETUP

Bacterial suspensions were prepared from bacterial cultures (≈ 10^8 cells mL^{-1}) which were diluted ten-fold in phosphate buffered saline, pH 7.4, to a final concentration of ≈ 10^7 colony forming units (CFU) mL^{-1}. In all the experiments 49.5 mL of bacterial suspension were aseptically distributed in 600 mL acid-washed and sterilised glass beakers and the PS was added from a stock solution (500 µM in DMSO) to achieve final concentrations of 0.5, 1.0 and 5.0 µM. After the addition of the appropriate volume of porphyrin, all beakers (total volume of 50 mL) were protected with aluminium foil to avoid accidental light exposure and followed a pre-irradiation period where they incubated during 10 minutes under 100 rpm stirring at 20-25°C.

Light and dark control experiments were carried out simultaneously. The light controls consisted only of bacterial suspension and were exposed to light. The dark controls were bacterial suspensions added with the higher concentration of PS (5.0 µM) and were covered with aluminium foil to protect from light exposure. The controls also followed the pre-irradiation incubation protocol.

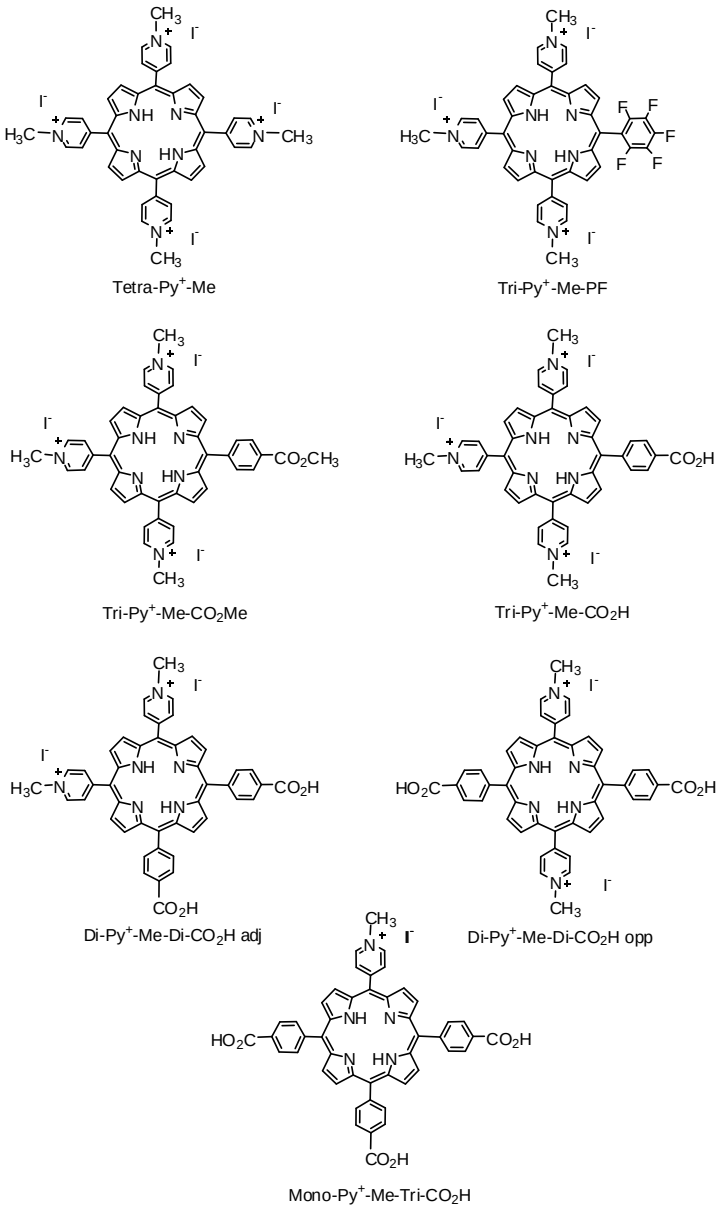

Figure 2.1 Structure of the seven cationic *meso*-substituted porphyrin derivatives used for photoinactivation of *E. faecalis* and *E. coli*

This photosensitization procedure was used for each of the seven PS tested and for both bacterial strains under investigation.

IRRADIATION CONDITIONS

Following the pre-irradiation incubation period, all samples were exposed, in parallel, to white light (PAR radiation, 13 OSRAM 21 lamps of 18 W each, 380-700 nm)

with a fluence rate of 40 W m^{-2} (measured with a light meter LI-COR Model LI-250, Li-Cor Inc., USA), at 20-25°C for 270 minutes, under 100 rpm mechanical stirring.

BACTERIAL QUANTIFICATION

A standard volume (1 mL) of undiluted and serially diluted irradiated and control samples were plated in duplicate in TSA medium at time 0 and after 15, 30, 60, 90, 180 and 270 minutes of light exposure. After 24 hours of incubation at 37°C in the dark, the number of colonies was counted on the most convenient series of dilution. The dark control plates were kept on the dark immediately after plating and during the incubation period. The assays for each concentration of each porphyrin and for each bacterial strain were done in duplicate and averaged. Data are presented by survival curves plotted as logarithmic bacterial reduction (in log CFU mL^{-1}) *versus* time of irradiation (in minutes).

STATISTICAL ANALYSIS

Statistical analysis was performed by using SPSS (SPSS 15.0 for Windows, SPSS Inc., USA). Normal distributions were assessed by Kolmogorov-Smirnov test. The significance of both porphyrin derivatives and irradiation time on bacterial inactivation was assessed by two-way univariate analysis of variance (ANOVA) model with the Bonferroni *post-hoc* test. A value of $p < 0.05$ was considered significant.

RESULTS

In this study, seven *meso*-substituted cationic porphyrin derivatives varying in charge number, charge distribution and *meso*-substituent groups were tested for photocytotoxicity against *E. coli* and *E. faecalis* based on the determination of the number of CFU per millilitre during 270 minutes of irradiation.

Light controls and dark controls results show that the viability of *E. coli* and *E. faecalis* is not affected by irradiation itself nor by any of the PS tested in the dark with 5.0 µM of porphyrin (≈ 7.2 log CFU mL^{-1} maintained during all irradiation period). This indicates that the reduction obtained on cell viability after irradiation of the treated samples is due to the photosensitizing effect of the porphyrin.

The three tricationic porphyrins were the most efficient PS against *E. faecalis* (≈ 7 log survivors reduction with 5.0 µM after 270 minutes of irradiation) and, in general, no significant difference exists among them on the PI of this strain (p > 0.05, ANOVA). However, Tri-Py^{+}-Me-PF showed the most rapid decrease on *E. faecalis* survival causing a drop of ≈ 6.80 log (>99.999%), after 60 minutes of irradiation (p > 0.05, ANOVA), for the three concentrations used (Fig. 2.2A). Against *E. coli*, the most efficient PS were Tri-Py^{+}-Me-PF and Tri-Py^{+}-Me-CO_2Me (p > 0.05, ANOVA) causing more than a 7 log survivors reduction with 5.0 µM after 270 minutes of irradiation (Fig. 2.2B and 2.3B).

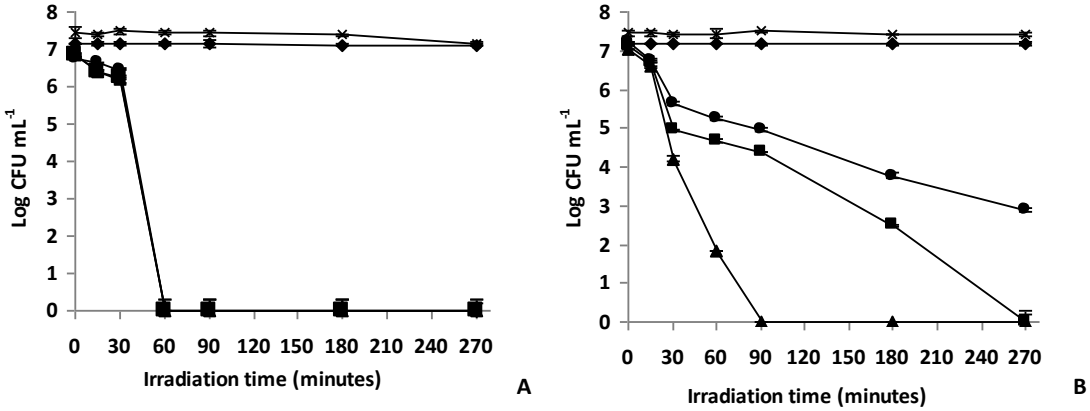

Figure 2.2 Survival curves of *E. faecalis* (**A**) and *E. coli* (**B**) (≈ 10^7 CFU mL^{-1}) incubated with porphyrin Tri-Py^{+}-Me-PF after 15, 30, 60, 90, 180 and 270 minutes of irradiation. (✕ light control, ◆ dark control, ● 0.5 µM, ■ 1.0 µM, ▲ 5.0 µM). Values represent means of two independent experiments and vertical bars represent the standard deviation of the mean.

Tetra-Py^{+}-Me was also a good PS against both bacteria, however, not as good as tricationic porphyrins ($p < 0.05$, ANOVA), at least for *E. faecalis*. It caused a drop of 7.35 logs after 60 minutes of irradiation with 5.0 µM (Fig. 2.4A). With lower concentrations, 1.0 µM and 0.5 µM, it was observed a 7.33 log (99.77%) and a 5.07 log (93.23%) reduction after 270 minutes of irradiation, respectively. Against *E. coli*, this PS caused a 7.50 log survivors reduction after 270 minutes of irradiation with 5.0 µM, being the third most effective PS against this strain (Fig. 2.4B).

Tri-Py^{+}-Me-CO_2H was less effective for *E. coli* than the other two tricationic porphyrins ($p < 0.05$, ANOVA), except for 5.0 µM (p = 1.000, ANOVA), as it was observed a 5.18 log (>99.99%) reduction after 270 minutes (Fig. 2.5B). This PS was even less effective than Tetra-Py^{+}-Me ($p < 0.05$, ANOVA), except for 1.0 µM (p = 0.128, ANOVA).

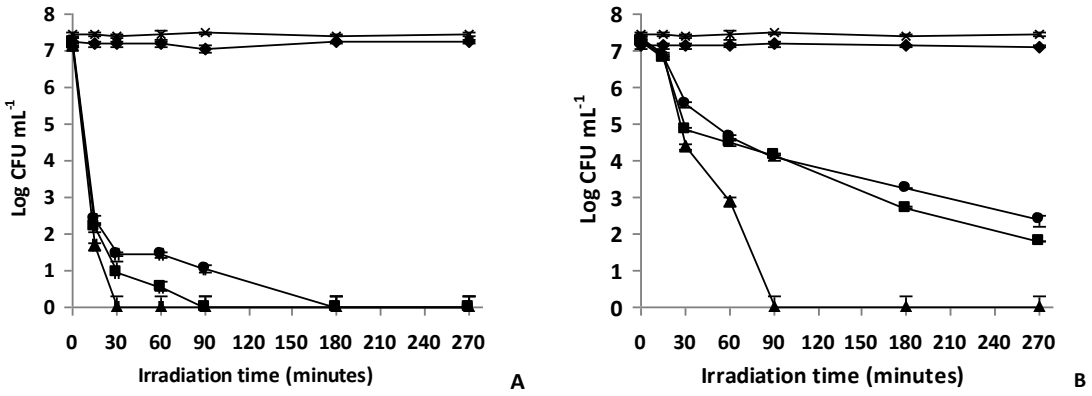

Figure 2.3 Survival curves of *E. faecalis* (**A**) and *E. coli* (**B**) (≈ 10^7 CFU mL^{-1}) incubated with porphyrin Tri-Py^+-Me-CO_2Me after 15, 30, 60, 90, 180 and 270 minutes of irradiation. (× light control, ◆ dark control, ● 0.5 µM, ■ 1.0 µM, ▲ 5.0 µM). Values represent means of two independent experiments and vertical bars represent the standard deviation of the mean.

Photoinactivation pattern for both dicationic porphyrins was not considered significantly different on *E. faecalis* for 1.0 and 5.0 µM ($p > 0.05$, ANOVA). However, with 0.5 µM concentration there was a 7.03 log reduction with Di-Py^+-Me-Di-CO_2H *adj* compared with a 0.88 log reduction with Di-Py^+-Me-Di-CO_2H *opp* after 270 minutes (Fig. 2.6). Statistic test demonstrates that Di-Py^+-Me-Di-CO_2H *adj* was much more effective than Di-Py^+-Me-Di-CO_2H *opp* at that concentration of PS ($p = 0.000$, ANOVA). These dicationic porphyrins showed significant differences on the PI pattern against *E. coli* both with 0.5 µM and 5.0 µM ($p < 0.05$, ANOVA), being the Di-Py^+-Me-Di-CO_2H *adj* more efficient. With 5.0 µM Di-Py^+-Me-Di-CO_2H *adj* and Di-Py^+-Me-Di-CO_2H *opp* caused > 3.0 log (> 99.9%) survivors reduction after 270 minutes (Fig. 2.7).

Overall, the PI pattern against *E. faecalis* with Mono-Py^+-Me-Tri-CO_2H was not significantly different from Di-Py^+-Me-Di-CO_2H *adj* nor from Di-Py^+-Me-Di-CO_2H *opp* ($p > 0.05$, ANOVA), although there is an exception. Comparing Mono-Py^+-Me-Tri-CO_2H with Di-Py^+-Me-Di-CO_2H *opp*, it can be seen that with 0.5 µM, Mono-Py^+-Me-Tri-CO_2H showed more PI activity than Di-Py^+-Me-Di-CO_2H *opp*: 2.16 log (99.31%) survivors reduction versus 0.88 log (86.25%) survivors reduction, respectively ($p = 0.000$, ANOVA) (Fig. 2.8A and 2.6A1).

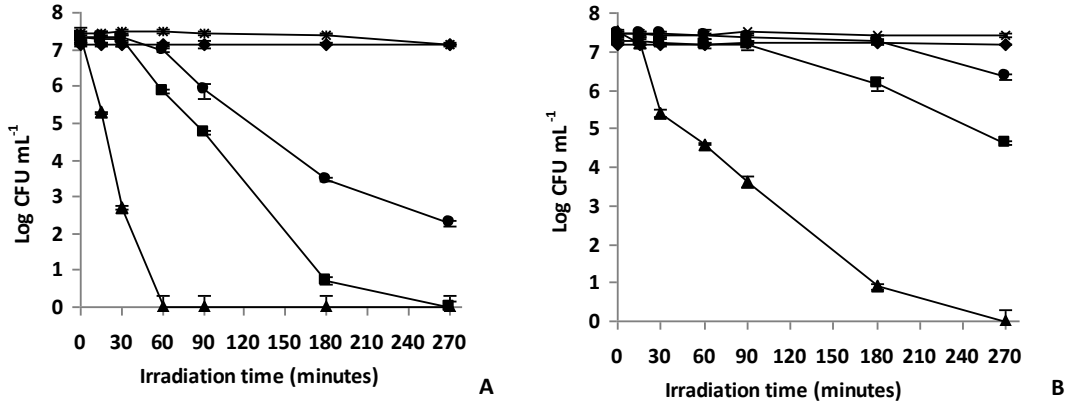

Figure 2.4 Survival curves of *E. faecalis* (**A**) and *E. coli* (**B**) (≈ 10^7 CFU mL^{-1}) incubated with porphyrin Tetra-Py^+-Me after 15, 30, 60, 90, 180 and 270 minutes of irradiation. (× light control, ◆ dark control, • 0.5 μM, ■ 1.0 μM, ▲ 5.0 μM). Values represent means of two independent experiments and vertical bars represent the standard deviation of the mean.

This means that the monocationic porphyrin is more effective than the dicationic *opp* porphyrin, if the lower concentration of PS is used. Against *E. coli*, this monocationic porphyrin was not significantly different from Di-Py^+-Me-Di-CO_2H *opp* (p = 0.000, ANOVA), except for 0.5 μM (Fig. 2.8B), similarly to what was shown for *E. faecalis*.

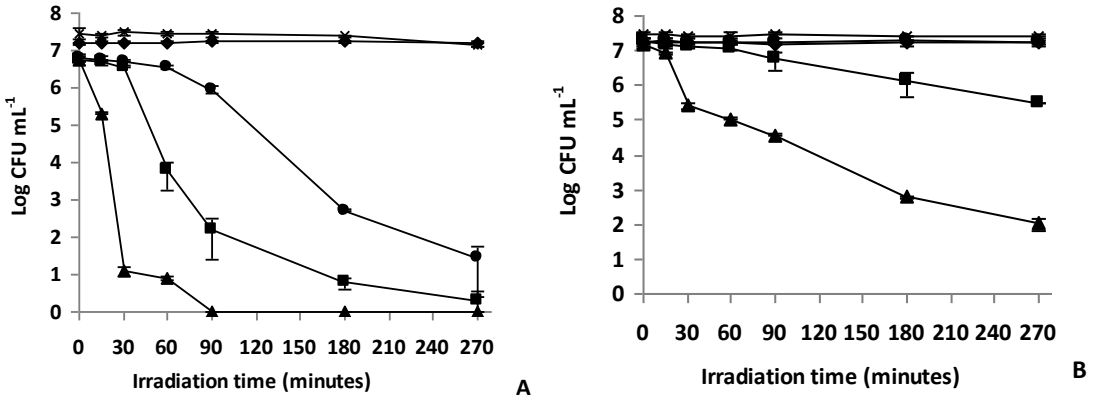

Figure 2.5 Survival curves of *E. faecalis* (**A**) and *E. coli* (**B**) (≈ 10^7 CFU mL^{-1}) incubated with porphyrin Tri-Py^+-Me-CO_2H after 15, 30, 60, 90, 180 and 270 minutes of irradiation. (× light control, ◆ dark control, • 0.5 μM, ■ 1.0 μM, ▲ 5.0 μM). Values represent means of two independent experiments and vertical bars represent the standard deviation of the mean.

Mono-Py^+-Me-Tri-CO_2H caused a major inactivation with 5.0 μM only after 270 minutes (3.28 log, 99.93%).

DISCUSSION

According to obtained results, we can say that bacterial PI process with the seven *meso*-substituted cationic porphyrins studied varies with porphyrin charge but also with the lipophilic character of the *meso*-substituent groups and charge distribution for both Gram (+) and Gram (-) bacteria. As far as we know, this is the most complete report using different positively charged porphyrins in the PI of both bacterial Gram types, with respect to charge effect.

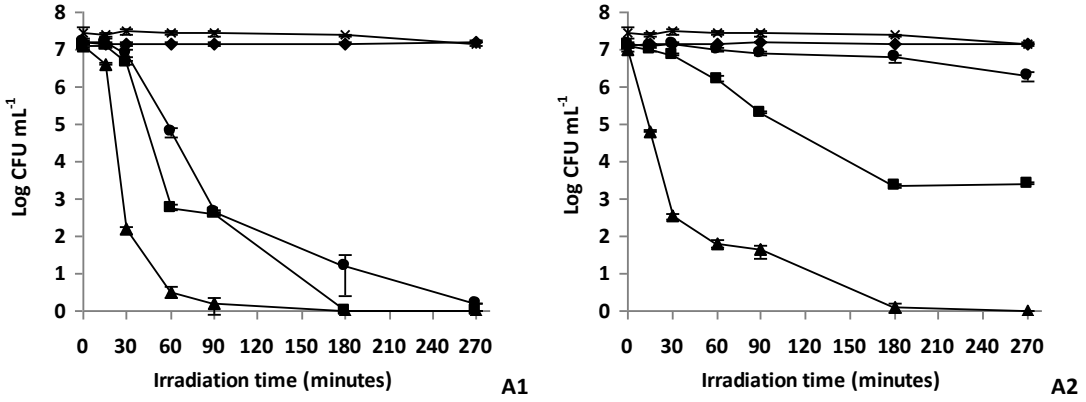

Figure 2.6 Survival curves of *E. faecalis* (≈ 10^7 CFU mL^{-1}) incubated with porphyrin Di-Py^+-Me-Di-CO_2H *adj* (**A1**) and Di-Py^+-Me-Di-CO_2H *opp* (**A2**) after 15, 30, 60, 90, 180 and 270 minutes of irradiation. (× light control, ◆ dark control, ● 0.5 μM, ■ 1.0 μM, ▲ 5.0 μM). Values represent means of two independent experiments and vertical bars represent the standard deviation of the mean.

Cationic porphyrin derivatives induce direct PI of Gram (+) and also of Gram (-) bacteria. This porphyrins type allows the absence of additives to inactivate Gram (-) cells because the positive charge on the PS molecule promote a tight electrostatic interaction with negatively charged sites at the outer surface of the bacterial cells, which increases the efficiency of the PI process (Merchat 1996; Merchat, Bertolini et al. 1996; Nitzan, Balzam-Sudakevitz et al. 1998). All porphyrins in this study are effective PS against Gram (+) strain *E. faecalis* achieving more than 99.999% (≈ 7 logs) reduction on cell survival after 270 minutes of light exposure with 5.0 μM concentration. The PI process against the Gram (-) strain, *E. coli*, is efficient after 270 minutes of light exposure with 5.0 μM concentration as it was observed more than 99.999% (≈ 7.50 log) reduction with Tri-Py^+-Me-PF, Tri-Py^+-Me-CO_2Me, Tetra-Py^+-Me and Tri-Py^+-Me-CO_2H. The reduction on cell survival, for that time of light exposure and concentration value, is much lower with Di-

Py^+-Me-Di-CO_2H *opp* (3.77 log, 99.98%), Di-Py^+-Me-Di-CO_2H *adj* (3.40 log, 99.96%) and Mono-Py^+-Me-Tri-CO_2H (3.28, 99.93%).

The PI pattern of both bacterial strains with all seven porphyrins is different. In general, against *E. faecalis*, the efficiency of PS follows the order: Tri-Py^+-Me-PF = Tri-Py^+-Me-CO_2Me = Tri-Py^+-Me-CO_2H > Tetra-Py^+-Me > Di-Py^+-Me-Di-CO_2H *adj* > Mono-Py^+-Me-Tri-CO_2H >Di-Py^+-Me-Di-CO_2H *opp*. Against *E. coli*, the order is Tri-Py^+-Me-PF = Tri-Py^+-Me-CO_2Me > Tetra-Py^+-Me > Tri-Py^+-Me-CO_2H > Di-Py^+-Me-Di-CO_2H *adj* > Di-Py^+-Me-Di-CO_2H *opp* > Mono-Py^+-Me-Tri-CO_2H. The porphyrins with three and four positive charges are the most effective PS against both bacterial strains.

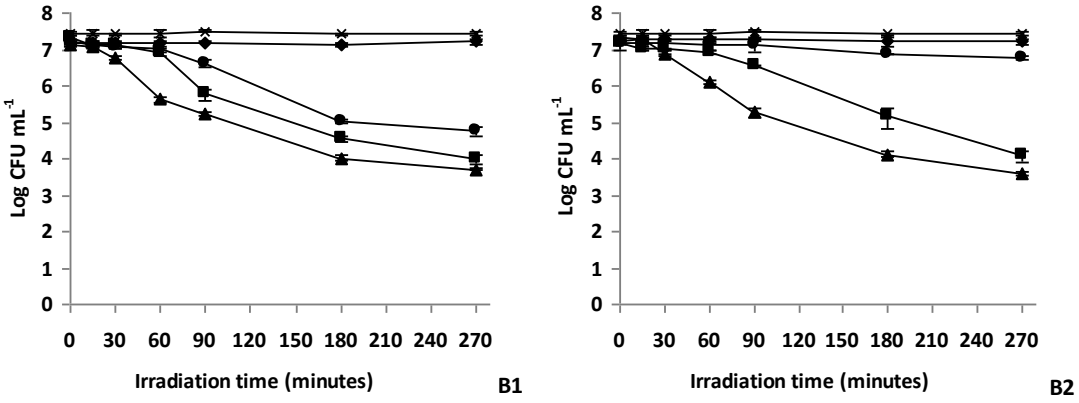

Figure 2.7 Survival curves of *E. coli* (≈ 10^7 CFU mL^{-1}) incubated with porphyrin Di-Py^+-Me-Di-CO_2H *adj* (**B1**) and Di-Py^+-Me-Di-CO_2H *opp* (**B2**) after 15, 30, 60, 90, 180 and 270 minutes of irradiation. (✕ light control, ◆ dark control, ● 0.5 µM, ■ 1.0 µM, ▲ 5.0 µM). Values represent means of two independent experiments and vertical bars represent the standard deviation of the mean.

However, Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me are even more efficient than Tetra-Py^+-Me. It was expected that, increasing the number of positive charges, the reduction on cell viability should also increase. In fact, some studies showed a high rate of bacterial inactivation with tri- and tetracationic porphyrins compared with di- and monocationic ones (Lazzeri, Rovera et al. 2004; Caminos, Spesia et al. 2005). However, other studies report on contradicting results. Merchat et al. (1996) concluded that the number of charges does not affect the activity of the PS against both bacterial Gram types (Merchat 1996). Caminos et al. (2005) showed that the photodynamic activity of a tricationic porphyrin which cationic charges were combined in different patterns with trifluoromethyl groups, in solution or immobilized on agar surfaces, was higher for an *E.*

coli strain than that of a tetracationic porphyrin. The binding of that *meso*-substituted tricationic porphyrin to cells was over two times higher than those for other cationic porphyrins (tetra-, di- and mono-) (Caminos, Spesia et al. 2005). Banfi et al. (2006) also concluded that a dicationic porphyrin was more efficient than that of the corresponding tetracationic derivatives against Gram (+) *Staphylococcus aureus,* and Gram (-) *E. coli* and *Pseudomonas aeruginosa* (Banfi, Caruso et al. 2006).

According to our results, we can conclude that the number of positive charges affects the PI process, as tetra- and tricationic PS are the most efficient. Nevertheless, the higher inactivation rate observed with Tri-Py^{+}-Me-PF and Tri-Py^{+}-Me-CO_2Me than with tetracationic PS suggests that a high number of positive charges can decrease the PI efficiency as already shown in other studies (Jori, personal communication) and/or that the *meso*-substituent groups can also play an important role on bacterial PI process. In fact, it has been shown that positive charges combined with highly lipophilic groups might increase the amphiphilic character of the PS, enhancing its affinity to bacteria (Boyle and Dolphin 1996; Lazzeri, Rovera et al. 2004), thus increasing the photocytotoxic activity (Caminos, Spesia et al. 2005).

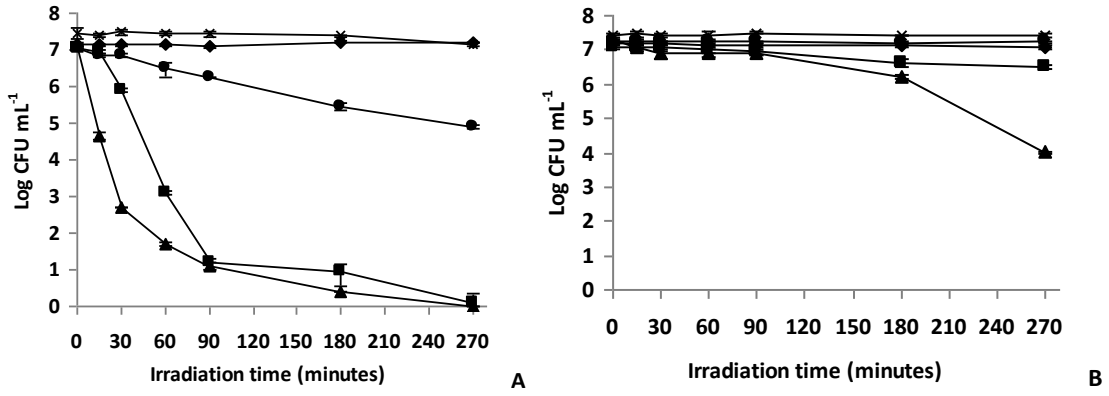

Figure 2.8 Survival curves of *E. faecalis* (**A**) and *E. coli* (**B**) (≈ 10^7 CFU mL^{-1}) incubated with porphyrin Mono-Py^{+}-Me-Tri-CO_2H after 15, 30, 60, 90, 180 and 270 minutes of irradiation. (× light control, ◆ dark control, ● 0.5 μM, ■ 1.0 μM, ▲ 5.0 μM). Values represent means of two independent experiments and vertical bars represent the standard deviation of the mean.

In this study, the results obtained with Tri-Py^{+}-Me-PF and Tri-Py^{+}-Me-CO_2Me on both bacterial strains were significantly different ($p < 0.000$, ANOVA) from that obtained

with Tri-Py^+-Me-CO_2H against *E. coli*. Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me causes a reduction below the detectable limits (≈ 7 log) after 90 minutes on *E. coli* while Tri-Py^+-Me-CO_2H causes a ≈ 5 log reduction after 270 minutes on *E. coli*. The difference observed in the PI pattern with Tri-Py^+-Me-CO_2H derivative may be explained by the presence of an acid group that can be ionized when dissolved in PBS, 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 PS, namely its binding preferences, aggregation state and electronic energy levels, an important parameter for the generation of singlet oxygen, for instance. On the other hand, the Tri-Py^+-Me-CO_2Me, an ester derivative that has the acid group protected which doesn´t allow ionization, showed a significantly higher ($p < 0.000$, ANOVA) inactivation rate for *E. coli* than Tri-Py^+-Me-CO_2H. The obtained 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 bacterial inactivation as already observed on similar studies (Boyle and Dolphin 1996; Lazzeri, Rovera et al. 2004; Caminos, Spesia et al. 2005).

The distribution of the charges on the sensitizer is another factor that influences the efficiency of the PI process. In this study, the pattern of inactivation by symmetric and asymmetric dicationic porphyrins was significantly different. Di-Py^+-Me-Di-CO_2H *adj* shows much more efficiency than Di-Py^+-Me-Di-CO_2H *opp* on *E. coli* with the lower (0.5 µM) and the higher (5.0 µM) concentrations. On *E. faecalis*, Di-Py^+-Me-Di-CO_2H *adj* is significantly different from Di-Py^+-Me-Di-CO_2H *opp* only with the lower concentration (0.5 µM). According to Kessel et al. (2003) who studied the cell localization and photodynamic efficacy of two dicationic porphyrins varying in charge distribution on Murine L 1210 cells, the dicationic adjacent (10 µM) PS was five-fold more efficient than the dicationic opposite (10 µM) PS (Kessel, Luguya et al. 2003). The two adjacent positive charges in the porphyrin macrocycle should result in a molecular distortion due to electrostatic repulsion. In contrast, the porphyrin with the two opposite positive charges is a much more symmetric molecule. The affinity of these asymmetric cationic molecules for cells structures was not yet established but it is thought to be a function of hydrophobicity factors, charge distribution or both (Kessel, Luguya et al. 2003).

The PI with the monocationic PS is the most inefficient against *E. coli*, causing a 3.28 log reduction on this strain only after 270 minutes. This result is in agreement with previous results for monocationic sensitizer against Gram (-) bacteria (Merchat 1996; Caminos, Spesia et al. 2005).

The results obtained in this study show that the cationic porphyrins having three and four charges are highly efficient PS against both bacterial strains. The distinct *meso*-substituent groups in the porphyrin structure seem to have different effects on PI with Tri-Py^{+}-Me-PF porphyrin providing the highest log reduction on cell survival. These results represent a new insight in the development of novel technologies for wastewater treatment and need to be further investigated. Furthermore, photodynamic antimicrobial therapy represents a potential advantage in environmental management, as it allows for the inactivation of high concentrations of microorganisms retained in solid residues during sewage treatments, as in sludges, when activated sludges are used as secondary treatment if this technology would be applied to a thin layer of sludges or if the sludge treatment would be done on a flow system.

The PI of both bacteria was achieved with low light fluence (40 W m^{-2}). In temperate climates, as in Portugal where this work was done, solar irradiation reaches much higher fluence rates, reaching for case in point, values of 200 W m^{-2} in winter and of 710 W m^{-2} in summer in the 2006-2007 period. The light fluence we used in our study is lower than solar irradiation about fifteen times in winter and nearly fifty times lower in summer. This means that if this technology is applied to the real context of a flow system and if the sunlight is used as the light source, the time needed for the photodynamic inactivation to occur would be shorter than the 4.5 hours observed in our study and that it would also work on dark days even during winter. Therefore, this photodynamic approach applied to wastewater treatment under natural light conditions makes this technology cheap and accessible, referring to the light source.

One of the major concerns about the applicability of this approach to wastewater treatment is the contamination of the water output by porphyrins release. One way to overcome this issue is to immobilize the PS, as already showed by Banfi et al. (Banfi,

Caruso et al. 2006) and also in our laboratory for the Tri-Py^{+}-Me-PF porphyrin (data not shown).

The presented study is a preliminary research on the efficiency of seven *meso*-substituted cationic porphyrins that can be immobilized on solid supports to be tested in a water flow system model, under solar irradiation, and possibly reused for subsequent disinfection treatment.

In conclusion, the economic advantages associated with the efficient inactivation of pathogenic microorganisms by tri- and tetracationic PS indicates photodynamic antimicrobial therapy as a very promising possibility to wastewater disinfection.

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CHAPTER 3 PHOTOINACTIVATION OF FAECAL BACTERIA BY CATIONIC PORPHYRINS IMMOBILIZED ON MAGNETIC SILICA NANOSPHERES

ABSTRACT

Photodynamic antimicrobial therapy has been considered to be a possible alternative to conventional methods of water disinfection. To overcome the water output contamination issue, some pilot studies have immobilized the photosensitizers on solid matrices, as silica, or polymers, as chitosan, with encouraging results. We carried out a preliminary study of photomicrobicidal activity against two faecal pollution indicator strains (*Escherichia coli* and *Enterococcus faecalis*) using porphyrins immobilized on magnetic silica nanospheres (Tri-Py^{+}-Me-PF-CS, Tri-Py^{+}-Me-PF-NS and Tri-Py-Me-PF-CS hybrids). Bacterial suspensions (10^{5} CFU mL^{-1}) were added with 20 µM and 200 µM of immobilized porphyrins and the samples were irradiated during 270 minutes, with 40 W m^{-2} artificial white light. The Tri-Py^{+}-Me-PF-CS (cationic photosensitizer on cationic material) and Tri-Py-Me-PF-CS (neutral photosensitizer on cationic material) hybrids were almost equally effective for both Gram types (5 log decrease with 20 and 200 µM on *E. faecalis* after 90 minutes and ≈ 5 log decrease on *E. coli* after 180 minutes of irradiation). With Tri-Py^{+}-Me-PF-NS (cationic photosensitizer on neutral material), almost none reduction on *E. coli* viability was observed, even with 200 µM. However, *E. faecalis* was completely inactivated after 90 minutes. We observed that the charge in the photosensitizer is not essential to Gram (-) cell photoinactivation as long as the amino groups of the support material are cationized. Also, the presence of positive charge in the support material is needed to achieve photoinactivation of *E. coli*. The complete inactivation of bacteria with this low fluence means that this technology can be used with solar light. The economical advantages that this approach represents in terms of light source (solar light is freely available), synthesis and production of these immobilized photosensitizers, possibility to their removal after photoinactivation and further reuse, besides the significant photoinactivation observed, makes photodynamic antimicrobial therapy an interesting option to water disinfection.

Keywords: immobilized porphyrin, magnetic nanoparticles, photodynamic therapy, *Escherichia coli*, *Enterococcus faecalis*, water disinfection

INTRODUCTION

Water pollution is a major public health concern in our days. Although the improvement of sanitary conditions, at least in the urban centers, a great decrease in water resources is a reality. The effluents discharge in the environment, mostly after only secondary treatment, where no disinfection process is taken, increases the environmental contamination. Traditional methods of water disinfection use chlorine, chlorine dioxide, ozone and ultraviolet radiation and are very efficient against a large range of microorganisms. However, those treatments involve high costs and difficulties of implementation at large scale due to operational, personnel qualification and logistic deficits (Mendes and Oliveira 2004). Ultraviolet radiation technology and ozonation are very expensive options to apply to a larger scale and disinfectants form toxic by-products,

being the chlorine's by-products the most toxic (Richardson, Thruston et al. 2000). The water reuse might be a possibility to cope with low water resources in densely populated areas, e.g., for agricultural use (Jemli, Alouini et al. 2002), therefore more convenient and inexpensive technologies of water disinfection are needed (Bonnett, Buckley et al. 1994).

Photodynamic antimicrobial therapy has been considered to be a promising approach on the destruction of faecal bacteria, viruses and helminthes eggs in wastewater (Alouini and Jemli 2001; Jemli, Alouini et al. 2002; Banfi, Caruso et al. 2006; Bonnett, Krysteva et al. 2006; Jiménez-Hernández 2006; Carvalho, Gomes et al. 2007; Costa, Alves et al. 2007). This approach refers to the combination of a photosensitizer (PS), a light source (visible or ultraviolet light) and molecular oxygen to achieve selective destruction of microorganisms normally via oxidative damage (Wainwright 1998). In the photodynamic process, the PS molecule, after absorbing light of an appropriate wavelength, is able to transfer energy to molecular oxygen, leading to the formation of highly reactive and cytotoxic species, such as singlet oxygen ($^1O_2$) and free radicals (Wainwright 1998).

It is well known that cationic PS cause effective photoinactivation (PI) against Gram-positive [Gram (+)] and Gram-negative [Gram (-)] bacteria (Merchat, Bertolini et al. 1996). The PI is enhanced in Gram (+) bacteria due to their thick peptidoglican outer wall which displays a relatively high degree of porosity, thus, does not act as a permeability barrier for the most commonly used PS (Jori, Fabris et al. 2006). In Gram (-) bacteria, the cell destruction is possible since the positively charged PS cause the removal of the magnesium and the calcium ions which neutralize the superficial negative charges of Gram (-) cells and, as a consequence, electrostatic repulsion is promoted with destabilization of the native organization of the wall, inducing the release of a large fraction of the lipopolysaccharides into the medium (Jori, Fabris et al. 2006).

Porphyrins are organic compounds with four pyrrole rings and a metal cofactor found in biomolecules such as hemoglobin, chlorophyll and certain enzymes. Synthetic porphyrins may be transformed into cationic entities through the insertion of positively charged substituents in the peripheral positions of the tetrapyrrole macrocycle (*meso* positions) which may largely affect the kinetics and extent of binding with microbial cells

(Jori, Fabris et al. 2006). Cationic *meso*-subtituted porphyrins have been proved to be more efficient and more photostable in water disinfection than other cationic PS as methylene blue and rose bengal (Jemli, Alouini et al. 2002). There are some factors which increase the amphiphilic character of the porphyrins: the asymmetric charge distribution at their peripheral positions, cationic charges combined in different patterns with highly lipophilic groups (e.g., trifluoromethyl groups), introduction of aromatic hydrocarbon side groups and modulation of the number of positive charges on the PS (Boyle and Dolphin 1996; Ando and Kumadaki 1999; Grancho, Pereira et al. 2002; Caminos, Spesia et al. 2005; Spesia, Lazzeri et al. 2005; Banfi, Caruso et al. 2006; Caminos and Durantini 2006). The increase in the amphiphilic character of the PS seems to enhance its affinity for bacteria which helps a better accumulation in the cells (Boyle and Dolphin 1996; Lazzeri, Rovera et al. 2004) accompanied by an increase in the photocytotoxic activity (Caminos, Spesia et al. 2005).

Despite the efficiency of these molecules, their application in wastewater treatment plants will only be possible if they are immobilized on solid supports, in order to allow the PI process and subsequent removal of the sensitizer after photodynamic action to avoid the release of the PS to the water output (Bonnett, Krysteva et al. 2006; Jiménez-Hernández 2006). To this extent, few studies have developed PS immobilized on solid supports and the PI against faecal bacteria was tested (Bonnett, Evans et al. 1997; Artarsky, Dimitrova et al. 2006; Bonnett, Krysteva et al. 2006; Caminos and Durantini 2006; Jiménez-Hernández 2006). Bonnett et al. (2006) used a phthalocyanine immobilized on a polymeric membrane of chitosan in a model reactor of water disinfection (Bonnett, Krysteva et al. 2006). They used an *E. coli* suspension of 10^5 cell mL^{-1} representing the significant levels of water contamination. After 160 minutes of irradiation, a bacterial inactivation of > 2 logs was achieved. Also, when the dyed membrane was stored in the dark for 9 months, the photodynamic action was still detectable, demonstrating the thermodynamic stability of the PS system. They concluded that with that model system, the PI with immobilized PS can be used to lower microbial levels in water flow systems and that might also have applications to water detoxification (Bonnett, Krysteva et al. 2006). Jímenez-Hernandez et al. (2006) have used polymer-supported Ru(II) sensitizers in

a homemade microreactor with a solar simulator source for laboratory-scale water disinfection assays using a water sample containing *E. coli* (2 x 10^3 CFU mL^{-1}) and *E. faecalis*. They observed noticeable disinfection with the RDP^{2+}/silicone system, for which has been measured a rate of cell inactivation by $^1O_2$ up to 1.1 x 10^5 CFU h^{-1} L^{-1} with *E. coli* and 0.7 x 10^5 CFU h^{-1} L^{-1} with *E. faecalis*. They concluded that photodisinfection with visible light was significant against both microorganisms (Jiménez-Hernández 2006). Artarsky et al. (2006) immobilized zinc phthalocyanines on a silicate matrix to test their photobactericidal properties on *E. coli* in model aqueous media. They obtained with the zinc phthalocyanine tetrasulfonic acid conjugate, a log kill of 1.32 and with the tetra tertiary butyl zinc phthalocyanine conjugate, a 0.98 log kill, in each case after 120 min, concluding that phthalocyanines can be immobilized successfully in a silicate matrix and used for photodisinfection of microbially polluted waters (Artarsky, Dimitrova et al. 2006).

Suitable polymers must have specific characteristics such as compatibility with the PS, allowing easy and reproducible immobilization procedures and avoiding leaching out to water; mechanical strength and stability towards sunlight; good oxygen permeability for efficient $^1O_2$ production with minimum quenching; high biocompatibility to maximize the interaction between the support and the microorganisms, and commercial availability and low cost (Jiménez-Hernández 2006). The immobilization of PS on silicate matrix, the mostly tested one, has some advantages in comparison with other organic matrices once it is insoluble in water, resistant towards microorganisms, easy to fabricate and might be developed successfully for the photodisinfection of water as in swimming pools and in other open water reservoirs (Artarsky, Dimitrova et al. 2006). Besides the advantages already mentioned, the immobilization of PS also allows the porphyrins reuse due to their stability (Bonnett, Krysteva et al. 2006), making photodynamic antimicrobial therapy a cost effective approach.

Besides polymeric supports, magnetic supports as microspheres, nanospheres and ferrofluids have been widely used in biomedicine and bioengineering, such as in protein and enzyme immobilization, immunoassay, RNA and DNA purification, cell isolation and target drug (Bonnett, Krysteva et al. 2006). These magnetic supports usually consist of inorganic magnetic cores (e.g., magnetite, haematite, nickel, alloys of cobalt) and organic

or polymeric shells that are either biocompatible or possessing active groups which can be conjugated to biomolecules such as proteins and antibodies (Bonnett, Krysteva et al. 2006). According to the target substances and their application system, different property requirements and evaluation standards should be taken in account about magnetic carriers: the size and its distribution of particles, magnetic response capability (reaction to external magnetic field, providing convenient separation from the surrounding medium), surface functional groups (-CHO, -OH, $-NH_2$ and -COOH can be introduced onto the surface of magnetic microsphere by surface modification and therefore bioactive matters such as drugs and other molecules can be easily coupled to the surface of magnetic carriers), and biocompatibility (Zhang, Xing et al. 2007).

In this study, three different magnetic silica nanospheres/porphyrin hybrids will be used as PS to preliminarily assess their photomicrobicidal activity against *Escherichia coli* and *Enterococcus faecalis* in a static water system.

MATERIALS AND METHODS

SYNTHESIS OF MESO-TRISUBSTITUTED PORPHYRIN

The *meso*-trisubstituted porphyrin 5-pentafluorophenyl-10,15,20-triphenylporphyrin (Tri-Ph-PF) was prepared by condensation of pyrrole and appropriate aromatic aldehydes. The condensation takes place in refluxing acetic or propionic acids in the presence of nitrobenzene. Under these conditions the cyclic porphyrinogen intermediates are oxidized by oxygen or nitrobenzene to the corresponding porphyrins (Gonçalves, Varejão et al. 1991). This porphyrin has groups which can be derivatized in order to perform couplings with other molecules (Fig. 3.1A).

PREPARATION OF MAGNETIC SILICA NANOSPHERES AND PHOTOSENSITIZER IMMOBILIZATION

The magnetite nanoparticles were prepared in three steps: synthesis of iron oxide magnetic core, coating with silica and functionalization with 3-aminopropyltriethoxysilane (APTES). The magnetite nanoparticles were prepared by the conventional co-precipitation method (Guan, Liu et al. 1998) with some modifications. $NH_3.H_2O$ was added to an aqueous solution of $FeNH_4(SO_4)_2$ and $(NH_4)_2Fe(SO_4)_2$. The colour of bulk solution turned

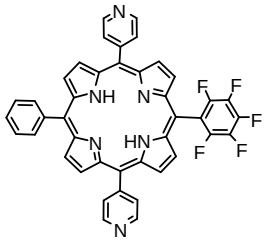
from orange to black immediately (pH=6). The magnetite precipitates were washed several times with deionised water. Sodium silicate was dissolved in deionised water and the pH value of the solution was adjusted to 12–13 by hydrochloric acid. The sodium silicate solution and Fe_3O_4 nanoparticles prepared in the above were poured into a beaker equipped with a mechanical stirrer. The mixture was ultrasonicated for 30 min. Then, the temperature of the mixture was increased to 80°C. After the mixture has reached room temperature, hydrochloric acid was added dropwise to adjust the pH value to 6–7. The precipitates were washed several times with deionised water by magnetic decantation and then dispersed in ethanol (600 mL). Four millilitres of APTES were added to the suspension and the mixture was kept in magnetic stirring at room temperature till 24 hours. The suspension was filtered and washed again with ethanol (Liu, Ma et al. 2004) (Fig. 3.1A and 3.1B). The porphyrin derivatives were immobilized on the surface of the magnetite nanoparticles via covalent bonding (Fig. 3.1). Support material and hybrids were kindly provided by the Organic Chemistry group from Chemistry Department of University of Aveiro.

MICROORGANISMS AND GROWTH CONDITIONS

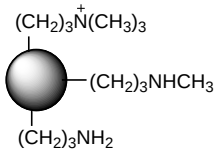
The Gram (-) strain *Escherichia coli* ATCC® 13706 (USA) and the Gram (+) strain *Enterococcus faecalis* ATCC® 51299 (USA) were stored at 4°C in triptic soy agar (TSA, Merck). Before each assay the strains were grown aerobically for 24 hours at 37°C in 30 mL of triptic soy broth (TSB, Merck). Then, an aliquot of this culture (200 µL) was aseptically subcultured to 30 mL of fresh TSB medium and grew overnight at 37°C to reach an optical density (OD_{600}) of ≈ 1.3, corresponding to ≈ 10^8 cells mL^{-1}.

PHOTOINACTIVATION METHOD

The efficiency of the PS both in free and in immobilized forms was assessed using bacterial suspensions. Bacterial cultures grown overnight were diluted one thousand fold in phosphate buffered saline, pH 7.4, to a final concentration of ≈ 10^5 colony forming units (CFU) mL^{-1} and equally distributed in 600 mL acid-washed and sterilised glass beakers (20 mL in each beaker).



A Tri-Py-Me-PF



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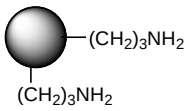

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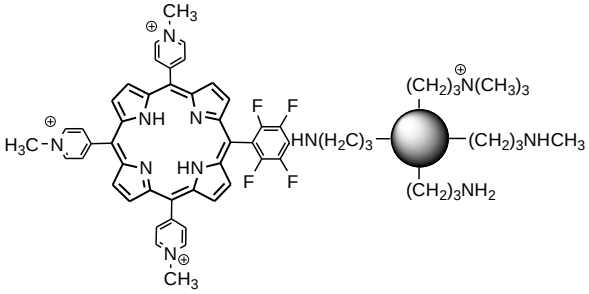
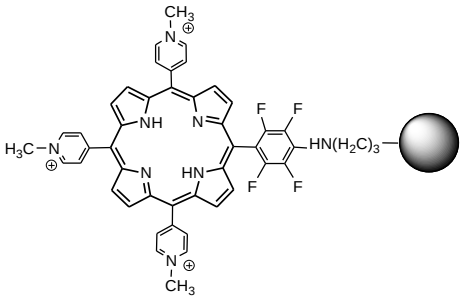
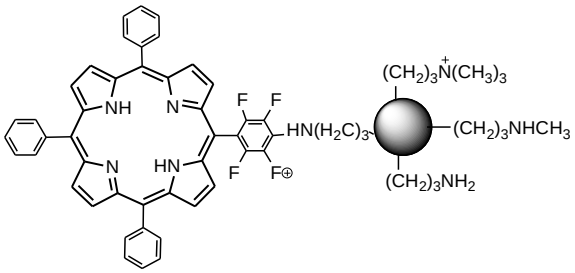


D Tri-Py^{+}-Me-PF-CS



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



F Tri-Ph-PF-CS

Figure 3.1 Structure of the *meso*-trisubstituted porphyrin, support materials and the magnetic silica nanospheres/porphyrin hybrids used for photoinactivation of *E. coli* and *E. faecalis.* **A***:* Tri-Py-Me-PF; **B**: Cationic support material; **C**: Neutral support material; **D**: Cationic porphyrin immobilized on cationic support material (Tri-Py^{+}-Me-PF-CS); **E**: Cationic porphyrin, immobilized on neutral support material (Tri-Py^{+}-Me-PF-NS); **F**: Neutral porphyrin immobilized on cationic support material (Tri-Ph-PF-CS).

EXPERIMENTS WITH FREE TRI-ME^{+}-PY-PF

In the experiments with porphyrin in the free form, five samples were prepared. After the addition of bacterial suspension to the respective beaker, three of the samples were added with appropriate quantities of PS to achieve final concentrations of 0.5 µM, 1.0 µM and 5.0 µM and the other two microcosms were used as dark and light controls (final volume was 20 mL in the beaker). The light control sample was not added

with porphyrin and was exposed to light. The dark control sample was added with the higher concentration of PS (5.0 μM) and was protected from light with aluminium foil.

EXPERIMENTS WITH HYBRIDS

In the experiments with immobilized porphyrins, five samples were also prepared. Following the addition of bacterial suspension to the respective beaker, one beaker was added with appropriate quantity of PS in the free form to reach 5.0 μM (the most effective concentration to inactivate both bacteria), two beakers were added with immobilized porphyrin to achieve final concentrations of 20 μM and 200 μM respectively and the other two beakers were used as dark and light controls. The light control sample was not added with porphyrin and was exposed to light. The dark control sample was added with 20 μM of immobilized PS and was protected from light with aluminium foil. Before each assay, the immobilized porphyrins were sonicated for 45 minutes to promote the materials disaggregation.

CONTROL EXPERIMENTS WITH SUPPORT MATERIALS

In addition to light and dark control samples, other four control samples were carried out for both bacterial strains. One control sample with bacterial suspension was added with 20 μM neutral solid support and exposed to light (light control of neutral material), one control sample with bacterial suspension was added with 20 μM neutral solid support and protected from light (dark control of neutral material), one control sample with bacterial suspension was added with 20 μM cationic solid support and exposed to light (light control of cationic material) and one control sample with bacterial suspension was added with 20 μM cationic solid support and protected from light (dark control of cationic material).

IRRADIATION CONDITIONS

The samples were exposed in parallel to white light (PAR radiation, 13 OSRAM 21 lamps of 18 W each, 380-700 nm) with a fluence rate of 40 W m^{-2}, for 270 minutes, under 100 rpm stirring (mechanic stirring when using free form PS and magnetic stirring when using immobilized PS).

BACTERIAL QUANTIFICATION

Aliquots of undiluted and serially diluted treated and control samples were pour plated in duplicate in TSA medium at time 0 and after 90, 180 and 270 minutes of light exposure. After 24 hours of dark incubation at 37°C, the number of colonies was counted on the most convenient series of dilution. The dark control plates were kept on the dark immediately after plating and during the incubation period. The assays with the porphyrin in the free form, with Tri-Ph-PF-CS and with support materials were done in duplicate and averaged for each concentration and for each bacterial strain. For hybrids Tri-Py^{+}-Me-PF-CS and Tri-Py^{+}-Me-PF-NS, only one assay was done for each concentration and for each strain. Data are presented by survival curves plotted as logarithmic bacterial reduction in log CFU mL^{-1} *versus* time of irradiation in minutes. Bacterial PI was calculated from the initial concentration of bacteria ($\approx 10^5$ CFU mL^{-1}) at 0 minutes of irradiation, for each one of the samples.

STATISTICAL ANALYSIS

Statistics were performed by using SPSS (SPSS 15.0 for Windows, SPSS Inc., USA). Normal distributions were assessed by Kolmogorov-Smirnov test. The significance among porphyrin derivatives on bacterial inactivation was assessed by one-way analysis of variance (one-way ANOVA) model with the Bonferroni *post-hoc* test. A value of $p < 0.05$ was considered significant.

RESULTS

EXPERIMENTS WITH FREE TRI-PY^{+}-ME-PF

The experiments with the porphyrin Tri-Py^{+}-Me-PF in the free form show equal effectiveness against *E. coli* [Gram (-)] and *E. faecalis* [Gram (+)], once after 270 minutes of irradiation a 5.0 log reduction on cell viability was observed for 0.5, 1.0 and 5.0 µM (Fig. 3.2). However, the PI process for *E. faecalis* is faster than for *E. coli* and not dose-dependent ($p > 0.05$, ANOVA) when bacteria inactivation with the three concentrations was compared (Fig. 3.2B). As we can see, the major reduction on *E. faecalis* viability was achieved after 90 minutes of irradiation with the three concentrations of porphyrin. On

the other hand, a 5.0 log reduction on *E. coli* viability was obtained after 180 minutes of irradiation with 1.0 and 5.0 µM and after 270 minutes with 0.5 µM (Fig. 3.2A). Results for control samples show no variability ($p > 0.05$, ANOVA) on cell viability during all irradiation time (≈ 5.0 log CFU mL^{-1}) when the bacterial suspension is exposed to light without PS and when the bacterial suspension is added with PS but protected from light.

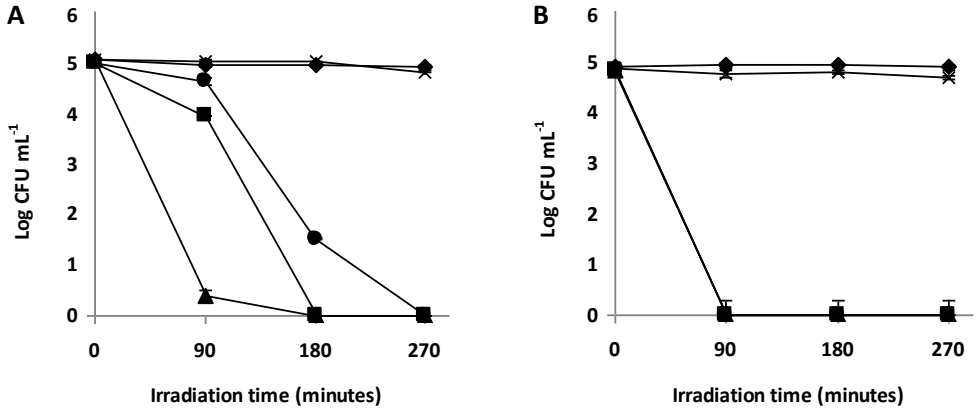


Figure 3.2 Logarithmic reductions of *E. coli* (**A**) and *E. faecalis* (**B**) treated with porphyrin Tri-Py^{+}-Me-PF at 0.5 µM (●), 1.0 µM (■) and 5.0 µM (▲) after 90, 180 and 270 minutes of 40 W m^{-2} irradiation. Light control (×) was not added with porphyrin and dark control (◆) was added with 5.0 µM of porphyrin. Values represent means of two independent experiments and vertical bars represent the standard deviation of the mean.

EXPERIMENTS WITH HYBRIDS

The results of the experiments with the immobilized PS show that, as with the free form, *E. faecalis* is more easily photoinactivated than *E. coli*. For all three hybrids, the PI patterns of *E. faecalis* were similar, showing a 5.0 log drop on cell viability after 90 minutes of irradiation with 20 µM and 200 µM ($p > 0.05$, ANOVA) (Fig. 3.3B, 3.4B and 3.5B). Experiments with hybrid Tri-Ph-PF-CS showed, after 270 minutes, a decrease on *E. coli* viability of 2.90 log (99.88%) with 20 µM and 4.59 log (≈ 99.999%) with 200 µM (Fig. 3.4A). Hybrid Tri-Py^{+}-Me-PF-NS was the weakest PS against *E. coli* as it caused ≈ 0.25 log (≈ 44.0%) decrease on cell viability after 270 minutes of irradiation with both 20 µM and 200 µM (Fig. 3.5A) and these results are significantly different from those obtained with the other two immobilized PS ($p < 0.05$, ANOVA).

As already seen in the experiments with the free porphyrin, with 5.0 µM concentration, the major PI is observed only after 90 minutes of irradiation for *E. coli* and for *E. faecalis*.

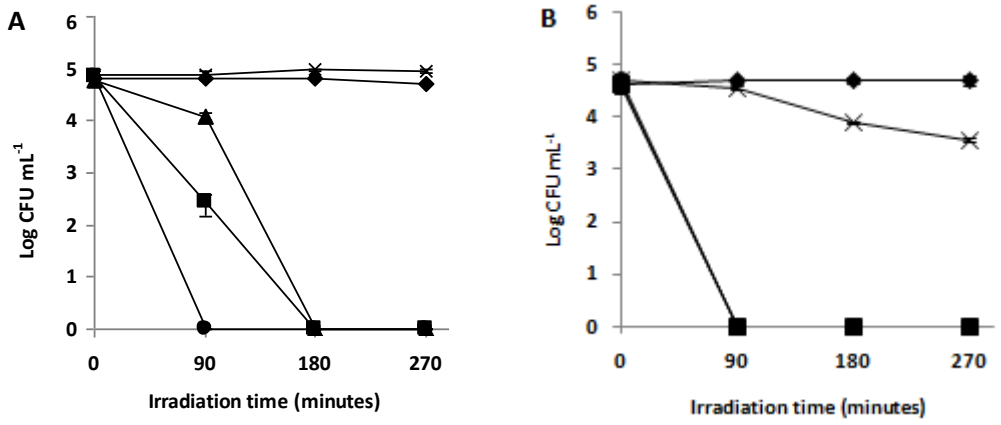


Figure 3.3 Logarithmic reductions of *E. coli* (**A**) and *E. faecalis* (**B**) treated with free porphyrin Tri-Py^{+}-Me-PF at 5 µM (●), and with Tri-Py^{+}-Me-PF-CS at 20 µM (■) and 200 µM (▲) after 90, 180 and 270 minutes of 40 W m^{-2} irradiation. Light control (×) was not added with porphyrin and dark control (◆) was added with 20 µM of Tri-Py^{+}-Me-PF-CS. Values represent results of one single experiment and vertical bars represent the standard deviation of the two replicas.

CONTROL EXPERIMENTS WITH SUPPORT MATERIALS

Viability of *E. coli* and *E. faecalis* with 20 µM of neutral and cationic support materials were not significantly different, both upon light and dark conditions ($p > 0.05$, ANOVA). With neutral support material, viability values varied between 1.77 x 10^5 and 2.06 x 10^5 CFU mL^{-1} for *E. coli* and between 1.46 x 10^5 and 2.02 x 10^5 CFU mL^{-1} for *E. faecalis* (Fig. 3.6). With cationic support material, bacterial viability was about 1.84 x 10^5 – 2.16 x 10^5 CFU mL^{-1} for *E. coli* and 1.36 x 10^5 – 1.93 x 10^5 CFU mL^{-1} for *E. faecalis* (Fig. 3.6).

DISCUSSION

A previous study done in our laboratory about the bactericidal effect of porphyrins varying the number of charges, *meso*-substituent groups and charge distribution (adjacent and opposite) on *E. faecalis* and *E. coli* has shown that the Tri-Py^{+}- Me-PF was the most efficient PS (data not shown). As a result, this PS was chosen among six other cationic porphyrins to be covalently immobilized in nanomagnetic support materials

covered with silica. In addition, the pentafluorophenyl group being *meso* positioned allows a better covalent bond to the nanomagnetic support (Liu, Ma et al. 2004).

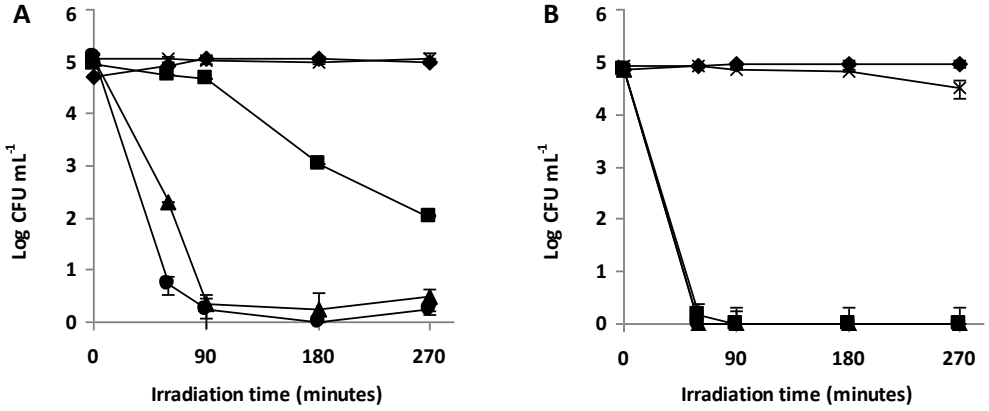


Figure 3.4 Logarithmic reductions of *E. coli* (**A**) and *E. faecalis* (**B**) treated with free porphyrin Tri-Py^{+}-Me-PF at 5 μM (●), and with Tri-Ph-PF-CS at 20 μM (■) and 200 μM (▲) after 90, 180 and 270 minutes of 40 W m^{-2} irradiation. Light control (×) was not added with porphyrin and dark control (◆) was added with 20 μM of Tri-Ph-PF-CS. Values represent means of two independent experiments and vertical bars represent the standard deviation of the mean.

In this study, three different magnetic silica nanospheres/porphyrin hybrids were tested for photomicrobicidal effect against the mentioned strains: cationic PS coupled to cationic material (Tri-Py^{+}-Me-PF-CS), cationic PS coupled to neutral material (Tri-Py^{+}-Me-PF-NS) and neutral PS coupled to cationic material (Tri-Ph-PF-CS). Obtained results showed that the Tri-Py^{+}-Me-PF-CS hybrid was the most effective for both Gram types. It caused a 5 log decrease with both concentrations (20 and 200 μM) on *E. faecalis* after 90 minutes and ≈ 5 log decrease on *E. coli* after 180 minutes of irradiation. Tri-Ph-PF-CS was the second most effective hybrid. Again, the PI pattern was similar for *E. faecalis*: 5 log reduction after 90 minutes with both concentrations (20 and 200 μM) and 4.82 log for *E. coli* after 180 minutes. The Tri-Py^{+}-Me-PF-NS was also effective against the Gram (+) strain: 5 log reduction after 90 minutes with both concentrations (20 and 200 μM). However, for *E. coli*, almost none cell viability reduction was observed, even with the highest concentration (≈ 0.25 log after 270 minutes).

In general, it can be said that for *E. faecalis* strain, the three hybrids tested are good, achieving more than 3 log reduction on cell viability (bactericidal effect) after 90 minutes of mild fluence rate. As already stated, Gram (+) bacteria are easily photoinactivated, not only with cationic PS but also with neutral and anionic ones what is

explained by the mode of PS interaction with the outer cell surface (Jori, Fabris et al. 2006).

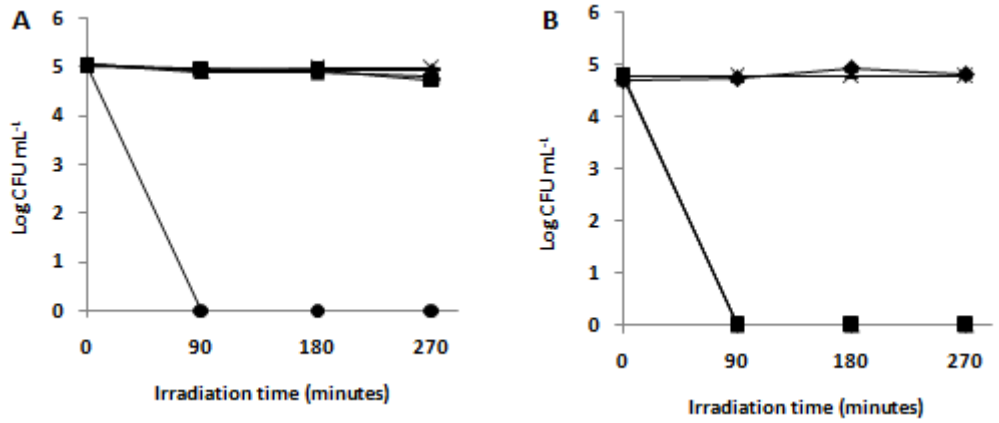


Figure 3.5 Logarithmic reductions of *E. coli* (**A**) and *E. faecalis* (**B**) treated with free porphyrin Tri-Py^{+}-Me-PF at 5 µM (●), and with Tri-Py^{+}-Me-PF-NS at 20 µM (■) and 200 µM (▲) after 90, 180 and 270 minutes of 40 W m^{-2} irradiation. Light control (×) was not added with porphyrin and dark control (◆) was added with 20 µM of Tri-Py^{+}-Me-PF-NS. Values represent results of one single experiment and vertical bars represent the standard deviation of the two replicas.

Against *E. coli*, results with Tri-Ph-PF-CS (neutral PS coupled to cationic material) were not significantly different from Tri-Py^{+}-Me-PF-CS (cationic PS coupled to cationic material). In this manner, it can be said that the charge in the PS is not essential to Gram (-) cell PI, as long as the amino groups of the support material are cationized. Comparing *E. coli* results between hybrid Tri-Py^{+}-Me-PF-CS (cationic PS coupled to cationic material) and Tri-Py^{+}-Me-PF-NS (cationic PS coupled to neutral material), it can be said that the presence of positive charge in the support material is needed to achieve PI of this strain. The charge in the material might have two important roles: adhesion to bacterial membrane with consequent disruption and also stabilization among magnetic nanoparticles through cationic charges repulsion, allowing PS availability to photodynamic process.

We can consider both Tri-Py^{+}-Me-PF-CS and Tri-Ph-PF-CS as very good hybrids to the PI of the tested strains at 10^{5} CFU mL^{-1}, which represents significant levels of water contamination (Bonnett, Krysteva et al. 2006), leading to a bactericidal effect (Knapp and Moody 1992). In other studies, where phthalocyanines were used as PS and were immobilized in silica or chitosan matrices, the results of bacterial PI were considered good

to be applied to photodisinfection of microbially polluted waters (Artarsky, Dimitrova et al. 2006) with lower microbial levels in water flow systems (Bonnett, Krysteva et al. 2006).

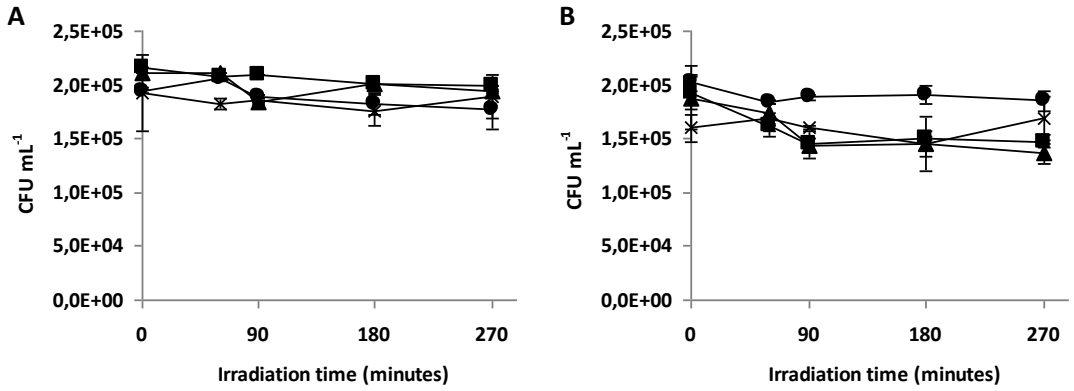


Figure 3.6 Logarithmic reductions of *E. coli* (**A**) and *E. faecalis* (**B**) treated with 20 μM of neutral and cationic support materials after 90, 180 and 270 minutes. Light controls were exposed to 40 W m^{-2} irradiation and dark controls were protected from light. Light control of neutral material (×), dark control of neutral material (•), light control of cationic material (■) and dark control of cationic material (▲). Values represent means of two independent experiments and vertical bars represent the standard deviation of the mean.

In those studies, a maximum of almost 3 log kill was achieved. In our study, we used a porphyrin derivative coupled to magnetic silica nanoparticles, and for the same initial bacterial level, we achieved a 5 log cell kill. The magnetic silica nanospheres have the following advantages: the magnetic core of iron oxide allows the removal from the surrounding medium, applying an external magnetic field (Lu, Yin et al. 2002) allowing their recovery and reuse; the silica coating avoids oxidation and consequent degradation of the magnetic core; and is also useful to prevent the aggregation and even the partial exposure of naked magnetite (Liu, Ma et al. 2004). They may also be derivatized with organic groups that promote the covalent coupling to different synthetic porphyrin derivatives as well as they promote easy access of the PS to microorganisms surface, once the nano dimension allows the entrance trough the cell wall (Zhang, Xing et al. 2007). Also, the method of producing the nanomagnetic particles and the porphyrin synthesis allows for a large scale production of these materials, becoming a commercially viable process.

We used a low artificial light fluence (40 W m^{-2}) to photoinactivate both bacteria. In temperate climates, as in Portugal where this work was done, solar PAR radiation reaches much higher fluence rates, about 400 W m^{-2} in winter and of 620 W m^{-2} in

summer (values obtained in the 2006-2007 period). The light fluence we used in our study is lower than solar PAR radiation about ten times in winter and nearly sixteen times lower in summer. This means that if this technology is applied to the real context of a flow system and if the sunlight is used as the light source, the time needed for the photodynamic inactivation to occur would be shorter than the 4.5 hours observed in our study and that it would also work on dark days even during winter. Therefore, this photodynamic approach applied to wastewater treatment under natural light conditions makes this technology cheap and accessible, referring to the light source. Moreover, the immobilization of porphyrin in magnetic materials allows the removal of the sensitizer after photodynamic action, avoiding the release of the PS to the water output and allows also the PS reuse, due to their stability (Bonnett, Krysteva et al. 2006).

This is a preliminary study on the PI of faecal pollution indicator strains using synthetic porphyrins as PS immobilized in magnetic silica nanospheres in a static model. These results seem to be promising in the development of photodynamic antimicrobial therapy applied to wastewater treatment and need to be further investigated. In the future, we aim to carry out experiments with the best hybrids, in a pilot plant, under different meteorological conditions of light and temperature and further reuse on bacterial inactivation.

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CHAPTER 4 PHOTODYNAMIC INACTIVATION OF RECOMBINANT BIOLUMINESCENT *ESCHERICHIA COLI* BY CATIONIC PORPHYRINS UNDER ARTIFICIAL AND SOLAR IRRADIATION

ABSTRACT

Photodynamic antimicrobial therapy means the combination of a light source, molecular oxygen and a photosensitizer to originate microbial cell death. This new approach has been studied to further application in wastewater treatment using different light sources and fluences, and different photosensitizers to study different microorganisms. To monitor the photoinactivation process, faster methods for microbial detection are required instead of laborious conventional plating, overnight incubation and colony counting methods. *Escherichia coli* cells were cloned with *luxCDABE* genes from the marine bioluminescent bacterium *Vibrio fischeri* and the recombinant bioluminescent indicator strain was used to assess, in real time, the effect of three cationic *meso*-substituted porphyrin derivatives on their metabolic activity, under artificial (40 W m^{-2}) and solar irradiation (≈ 620 W m^{-2}). The photoinactivation of bioluminescent *E. coli* was effective (> 4 log bioluminescence decrease) with the three porphyrins used, being the tricationic porphyrin Tri-Py^{+}-Me-PF the most effective. With solar irradiation, the photoinactivation process is faster and more efficient than with artificial light, for the three porphyrins. The use of bioluminescent bacteria allows the assessment of the efficiency of anti-metabolic action of these porphyrins. This method is effective and sensitive besides that is simpler, faster, cheaper and much less laborious than conventional methods and can be used as a screening method for bacterial photoinactivation studies *in vitro*.

Keywords: porphyrin, photodynamic therapy, bioluminescence, *Escherichia coli*, solar irradiation, water disinfection

INTRODUCTION

The growing reduction of water resources due to environmental pollution has become a major public health concern. Thus, wastewater treatment and disinfection is even more necessary at large scale. The inactivation of pathogenic microorganisms, in the last stage of wastewater treatment, can be achieved by several techniques as chlorination, ozonation and ultraviolet radiation. In a way to overcome the high costs and the difficulty in implementing these techniques due to operational, personnel qualification and logistic deficits, alternative physical-chemical methods have been studied, as photodynamic antimicrobial therapy (Alouini and Jemli 2001; Jemli, Alouini et al. 2002; Bonnett, Krysteva et al. 2006). So far, with major application in the clinical field, namely for whole blood and blood products disinfection, this somewhat new approach uses a light source (as sunlight or artificial light), an oxidizing agent (molecular oxygen

dissolved in water) and an intermediary agent (named photosensitizer [PS], able to absorb and transfer the energy of light source to molecular oxygen in water leading to the formation of highly cytotoxic species ($^1O_2$, $OH^{\bullet}$, O_2^-, H_2O_2) (Wainwright 1998). Such species are able to irreversibly alter the cells' vital constituents resulting in oxidative lethal damage (DeRosa and Crutchley 2002). The potential use of this approach for water disinfection has already been studied, showing that photoinactivation (PI) of bacteria in drinking (Bonnett, Krysteva et al. 2006) and residual waters (Jemli, Alouini et al. 2002; Carvalho, Gomes et al. 2007) is possible under solar irradiation. In those studies, the experimental conditions were different, namely in light source and fluence used, the microorganisms tested, as well as in the intermediary agents (dyes as methylene blue, rose Bengal and porphyrins) (Jiménez-Hernández 2005; Bonnett, Krysteva et al. 2006). The results were promising, namely with porphyrins. Porphyrins can be transformed into cationic entities through the insertion of positively charged substituents in the peripheral positions of the tetrapyrrole macrocycle (*meso* positions) which may largely affect the kinetics and extent of binding with microbial cells (Jori, Fabris et al. 2006). Cationic *meso*-substituted porphyrins are known to efficiently destroy Gram-negative and Gram-positive bacteria (Merchat 1996; Merchat, Bertolini et al. 1996; Ashkenazi, Nitzan et al. 2003; Lazzeri, Rovera et al. 2004; Caminos, Spesia et al. 2005; Banfi, Caruso et al. 2006). The combination of hydrophobic and hydrophilic substituents in the sensitizer structure results in an intramolecular polarity axis, which can facilitate membrane penetration and produces a better accumulation in subcellular compartments, enhancing the effective photosensitization (Milanesio, Alvarez et al. 2003).

To monitor the bacterial PI process, faster methods are required instead of laborious conventional methods of plating, overnight incubation and time consuming counting of colony forming units (CFU) (Vesterlund, Paltta et al. 2004; Demidova and Hamblin 2005). New approaches to study potential PS *in vitro* are essential to accelerate the development of photodynamic antimicrobial therapy in drinking and residual waters treatment. To this end, the bacterial bioluminescence method is considered to be a rapid (Hamblin, O'Donnell et al. 2002), sensitive (Francis, Yu et al. 2001) and cost-effective option (Vesterlund, Paltta et al. 2004). It also allows only living or viable cells to be

detected and doesn't need exogenous administration of substrates (Rocchetta, Boylan et al. 2001) to obtain light emission. Bioluminescence refers to the process of visible light emission by living organisms (Meighen 1993) and this production is directly dependent on metabolic activity of the organism (Vesterlund, Paltta et al. 2004), once an inhibition of cellular activity results in a decrease on the respiration rate and consequently a decrease in the bioluminescence rate. The light-emitting reaction in bacteria involves the oxidation of reduced riboflavin 5'-phosphate ($FMNH_2$) and a long chain fatty aldehyde with the emission of blue-green light (Rodriguez, Nabi et al. 1985; Meighen 1993). In both marine and terrestrial bioluminescent bacteria, a five-gene operon (*luxCDABE*) encodes the luciferase and biosynthetic enzymes (for the synthesis of the aldehyde substrate) necessary for light production. *luxA* and *luxB* genes encode the α and β subunits of the luciferase, and *luxC*, *luxD* and *luxE* encode proteins for aldehyde production (Meighen 1991). Although a number of additional *lux* genes in bioluminescent bacteria have been identified, only *luxCDABE* is essential for the biosynthesis of light (Meighen 1993; Meighen 1994).

Nearly two decades ago, the isolation of the genes responsible from bioluminescence in bacteria and the ability to transfer these genes into prokaryotic and eukaryotic organisms have greatly extended the capacity and potential uses of bacterial bioluminescence (Meighen 1993) therefore allowing the detection of antimicrobial activity in real time (Vesterlund, Paltta et al. 2004). Amongst the applications of these recombinant bacteria are clinical research (Contag, Jenkins et al. 2000; Rocchetta, Boylan et al. 2001; Jawhara and Mordon 2004; Demidova, Gad et al. 2005; Doyle, Nawotka et al. 2006), environmental research (Burlage, Sayler et al. 1990; Ptitsyn, Horneck et al. 1997; Verschaeve 1999; Johnson 2005; Grande, Pietro et al. 2007) and biotechnology (Maoz, Mayr et al. 2002; Kadurugamuwa, Sin et al. 2003). In the clinical studies, photodynamic therapy is applied to laboratory animal models inoculated with genetically engineered bioluminescent bacteria in order to assess *in vivo* and in real time the progress of infectious disease by optical detection and monitoring of bioluminescence, through sensitive imaging cameras (Contag, Contag et al. 1995 ; Francis, Joh et al. 2000; Francis, Yu et al. 2001; Hamblin, O'Donnell et al. 2002; Demidova, Gad et al. 2005; Doyle, Nawotka

et al. 2006). Light output from these bioluminescent bacteria is a highly sensitive reporter of metabolic activity (Marincs 2000) and can therefore be used to monitor the real time effects of antimicrobials on bacterial metabolism (Salisbury, Pfoestl et al. 1999; Rocchetta, Boylan et al. 2001). Furthermore, in experimental systems in which a strong correlation between bioluminescence and viable counts can be demonstrated, measurement of bioluminescence offers a rapid, alternative method for monitoring bacterial viability (Marincs 2000; Rocchetta, Boylan et al. 2001). Light output is noncumulative, reflecting actual metabolic rate, and can be measured directly, continuously and nondestructively in high-throughput screening or continuous-culture models (Beard, Salisbury et al. 2002). Thus, the transformation of pathogenic bacteria into indicator bioluminescent strains allows using a rapid, sensitive and cost-effective methodology (Simon, Fremaux et al. 2001; Beard, Salisbury et al. 2002; Hamblin, O'Donnell et al. 2002; Vesterlund, Paltta et al. 2004; Jawhara and Mordon 2006) to evaluate the efficiency of PI, in order to become possible, in the future, the field implementation of photodynamic antimicrobial therapy.

As far as we know, the application of bioluminescent bacteria in photodynamic antimicrobial therapy with the purpose to monitor antibacterial activity of cationic porphyrins to further application to wastewater disinfection has not yet been reported. In this study, we aim to assess the antibacterial effect of three cationic *meso*-substituted porphyrin compounds on the metabolic activity of recombinant bioluminescent *Escherichia coli* (*E. coli*) under artificial and solar irradiation.

MATERIALS AND METHODS

PHOTOSENSITIZERS

Porphyrins 5,10,15,20-tetrakis(1-methylpiridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^+-Me), 5-(pentafluorophenyl)-10,15,20-tris(1-methylpiridinium-4-yl)porphyrin tri-iodide (Tri-Py^+-Me-PF) and 5-phenyl-10,15,20-tris(1-methylpiridinium-4-yl)porphyrin tri-iodide (Tri-Py^+-Me-CO_2Me) (Fig. 4.1) used in this work were prepared in two steps. First, the neutral porphyrins were obtained from 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, Chertkov et al. 2002; Tome, Neves et al. 2004). After being separated by column chromatography (silica), the pyridyl groups of each porphyrin 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. All porhyrins were kindly provided by the Organic Chemistry group from Chemistry Department of University of Aveiro.

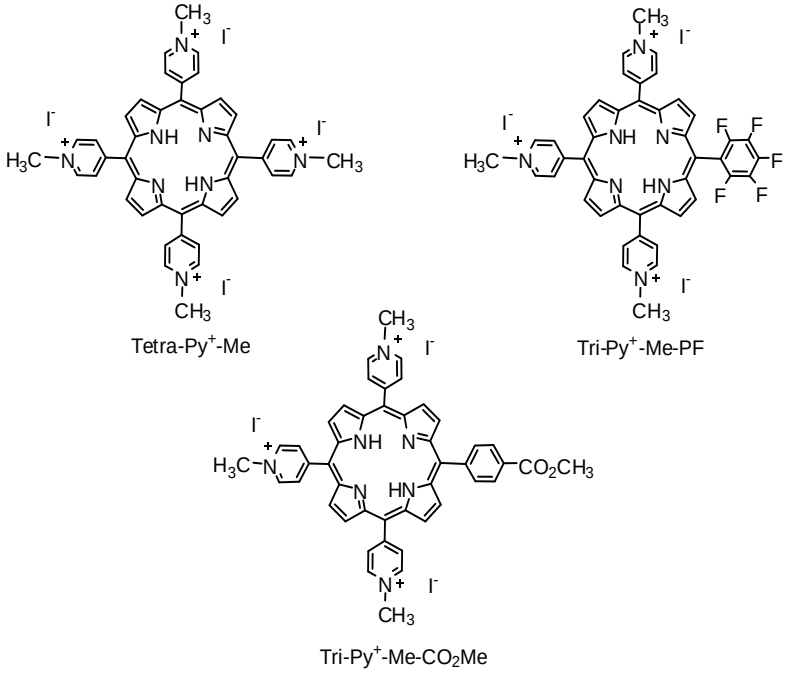

Figure 4.1 Structure of the three porphyrin derivatives used for photoinactivation of bioluminescent *E. coli.*

BACTERIAL STRAIN, PLASMID CONSTRUCTS AND E. COLI TRANSFORMATION

In this study, we have used two plasmids that were cloned into *E. coli*. These plasmids contain the *lux* operon from the bioluminescent marine bacterium *Vibrio fischeri (V. fischeri),* required to produce light. The plasmid pHK724 contains a ColE1 replicon, an ampicillin resistance marker and *luxR* gene whose gene product is a transcription regulatory protein. The plasmid pHK555 contains a P15A replicon, a chloramphenicol

resistance marker and a functional *luxICDABE* operon. The *luxR* gene of pHK555 is inactive because of the insertion of phage DNA. When pHK724 is transformed into *E. coli* containing pHK555, the resultant colonies grow on selective media and are bioluminescent (Kaplan and Greenberg 1987; Slock, VanRiet et al. 1990).

In our laboratory, chemically competent cells of *E. coli* with plasmid pHK555 were prepared and were further transformed with plasmid pHK724, as described earlier (Sambrook, Fritsch et al. 1989), resulting in a bioluminescent strain.

BACTERIAL GROWTH CONDITIONS

Bioluminescent *E. coli* were grown on Luria Bertani agar (LB, Merck) supplemented with 50 mg mL^{-1} of ampicillin (Amp) and with 34 mg mL^{-1} of chloramphenicol (Cm). A stock was stored at -80°C in 10% glycerol. Before each PI assay, one colony of bioluminescent bacteria was aseptically inoculated into 30 mL of triptic soy broth (TSB, Merck) supplemented with both antibiotics (150µL Amp/100 mL TSB and 60 µL Cm/100 mL TSB) and were grown for one day, at room temperature, at 100 rpm stirring. Then an aliquot of this culture was subcultured in 30 mL of fresh TSB with both antibiotics and grew overnight also at room temperature, to reach stationary growth phase ($OD_{600} \approx 1.3$).

PHOTOSENSITIZATION PROCEDURE

EXPERIMENTAL SETUP

Bacterial cultures grown overnight were diluted tenfold in phosphate buffered saline, pH 7.4, to a final concentration of 10^6 colony forming units (CFU) mL^{-1}. This bacterial suspension was equally distributed in 50 mL or 100 mL sterilized and acid-washed glass beakers. Then, appropriate quantities of the three porphyrins under study were added to achieve final concentrations of 0.5 µM, 1.0 µM and 5.0 µM (total volume was 10 mL per beaker). The samples were protected from light exposure with aluminium foil and incubated during 10 minutes under 100 rpm stirring, at 25-30°C, to promote the porphyrin binding to *E. coli* cells. Light and dark controls were carried out during the experiments. The light control microcosm was not added with porphyrin and was exposed to light. The dark control was added with the higher concentration of PS (5.0 µM) and was protected from irradiation with aluminium foil.

IRRADIATION CONDITIONS

Following the pre-irradiation incubation period, the samples were exposed to two different light sources, during 270 minutes, under 100 rpm stirring.

In laboratorial experiments with artificial light, it was used white light (PAR radiation, 13 OSRAM 21 lamps of 18 W each, 380-700 nm) with a fluence rate of 40 Wm^{-2}. Samples were placed on a tray with clamps and the bottom of the tray was covered with water in order to maintain a constant temperature in the samples (20-25°C) due to light heating, once this bioluminescent indicator strain contains the *V. fischeri lux* genes, which are from marine origin and emit light preferentially at temperatures above 30°C (Hill, Rees et al. 1993).

The experiments with solar irradiation were done outside of the laboratory. Samples were exposed to solar PAR light on sunny summer days, in the Littoral Centre of Portugal, where the averaged PAR light fluence was 620 W m^{-2} (measured with a radiometer LI-COR Model LI-250). It was used only the PAR radiation of the solar spectrum in order to avoid ultraviolet inactivation of the bacteria during the exposure time with the porphyrins. Samples were also placed on a tray with clamps and the bottom was covered with water. Water temperature was monitored and maintained above 30°C. To filter the ultraviolet radiation, samples were covered with a glass plate of 0.5 cm thick.

BIOLUMINESCENCE MONITORING

In both irradiation experiments, aliquots of treated and control samples were collected at time 0 and after 15, 30, 60, 90, 180 and 270 minutes of light exposure and the bioluminescence was measured in a luminometer (TD-20/20 Luminometer**,** Turner Designs, Inc., USA).

MONITORING OD_{600}, BIOLUMINESCENCE AND CFU OF A GROWING CULTURE

To assess the correlation between CFU number and bioluminescent signal of our indicator strain, one assay was done during bacterial growth without porphyrin or light exposure. In 30 mL of fresh TSB added with appropriate concentration of antibiotics, an aliquot of bioluminescent *E. coli* was aseptically transferred and was grown at 20-25°C,

under 100 rpm. At time 0 and at 30 minute intervals, an aliquot of the bacterial culture was aseptically transferred to a cuvette and red on the luminometer. To obtain the CFU number, aliquots of 500 µL of undiluted and serially diluted bacterial culture were pour plated in TSA medium. After 24 hours of incubation at 37°C, the number of colonies was counted on the most convenient series of dilution.

STATISTICAL ANALYSIS

All experiments for all three porphyrins were done in duplicate. Statistical analysis was performed by using SPSS (SPSS 15.0 for Windows, SPSS Inc., USA). Normal distributions were assessed by Kolmogorov-Smirnov test. The significance of both porphyrin derivatives and irradiation time on bacterial inactivation was assessed by two-way univariate analysis of variance (ANOVA) model with the Bonferroni *post-hoc* test. A value of $p < 0.05$ was considered significant.

RESULTS

MONITORING BIOLUMINESCENCE AND CFU OF A GROWING CULTURE

A positive linear correlation ($R^2 = 0,931$) was observed between bioluminescence and viable counts (Fig. 4.2).

ARTIFICIAL WHITE LIGHT EXPERIMENTS

Comparing the experiments done in the artificial light conditions (40 W m^{-2}), bioluminescence values show the difference in the PI patterns of the three porphyrins. For the lower concentrations used (0.5 and 1.0 µM), the tricationic porphyrins (Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me) were most effective than tetracationic (Tetra-Py^+-Me) ($p <$ 0.005, ANOVA) (Fig. 4.3A, 4.4A and 4.5A). They caused more than a 4 log decrease in bioluminescence (reaching the limits of detectable), after 270 minutes of irradiation. Tetracationic porphyrin (Tetra-Py^+-Me), however, was able to cause a 4.43 log decrease in bioluminescence only with the higher concentration (5.0 µM) after 270 minutes, while after that period it was observed only a 0.5 and a 0.65 log decrease in bioluminescence with lower concentrations (0.5 µM and 1.0 µM respectively) (Fig. 4.5A). Therefore, with

5.0 μM, the difference in the PI pattern was not significantly different amongst the three porphyrins ($p > 0.005$, ANOVA).

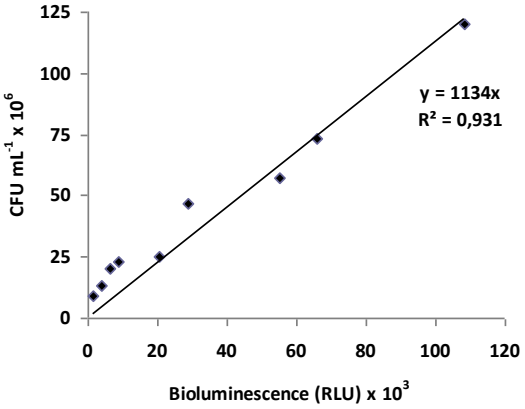

Figure 4.2 Relationship between bioluminescence and viable counts of a growing culture of recombinant *E. coli.* Bioluminescence is expressed in relative light units (RLU) and viable counts in CFU mL^{-1}.

SOLAR LIGHT EXPERIMENTS

The results of the experiments done under solar PAR light conditions (≈620 W m^{-2}) show that the three porphyrins studied were able to cause a decrease in the bioluminescence signal to the limit of detectable after 270 minutes of irradiation with the three concentrations tested (more than 4 log decrease) (Fig. 4.3B, 4.4B and 4.5B). However, for lower concentration values of Tri-Py^+-Me-PF the drop happens earlier (≈ 3 log reduction of bioluminescence after 15 minutes) than for the other porphyrins. These differences are significant ($p < 0.005$, ANOVA) from the observed for Tri-Py^+-Me-CO_2Me (0.46 log decrease with both 0.5 and 1.0 μM after 15 minutes) and for Tetra-Py^+-Me (0.89 log and 2.46 log decrease with 0.5 and 1.0 μM respectively). For the highest concentration, the difference between all porphyrins was significantly different ($p < 0.005$, ANOVA), being the Tri-Py^+-Me-PF the most effective followed by Tetra-Py^+-Me and then by Tri-Py^+-Me-CO_2Me.

CONTROL SAMPLES

In the experiments done under artificial light conditions, light and dark controls

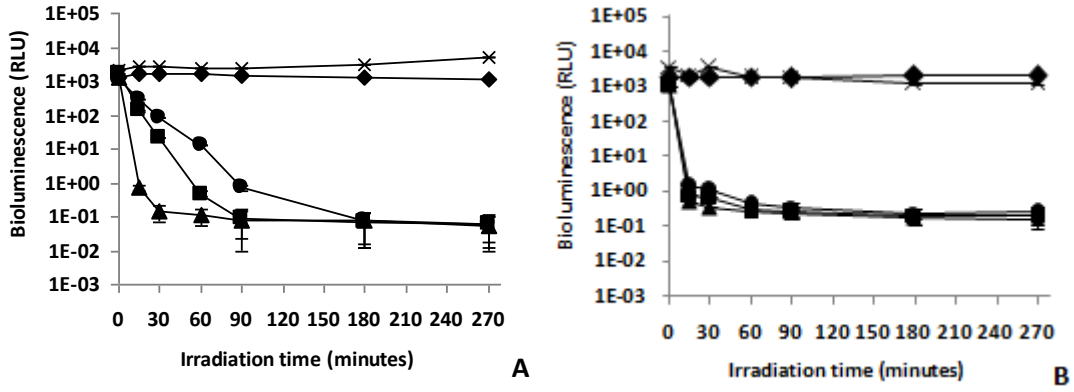

Figure 4.3 Bioluminescent monitoring of *E. coli* treated with porphyrin Tri-Py^{+}-Me-PF, after 270 minutes of 40 W m^{-2} irradiation (**A**) and of 620 W m^{-2} irradiation (**B**). The values are expressed as the means shown of two independent experiments; error bars indicate the standard deviation. (× light control, ◆ dark control, • 0.5 μM, ■ 1.0 μM, ▲ 5.0 μM).

results show that the viability of recombinant bioluminescent *E. coli* is not affected neither by irradiation itself nor by any of the PS tested in the dark with 5.0 μM of porphyrin (≈ 3 log RLU maintained during all irradiation period) (Figs. 4.3A, 4.4A and 4.5A).

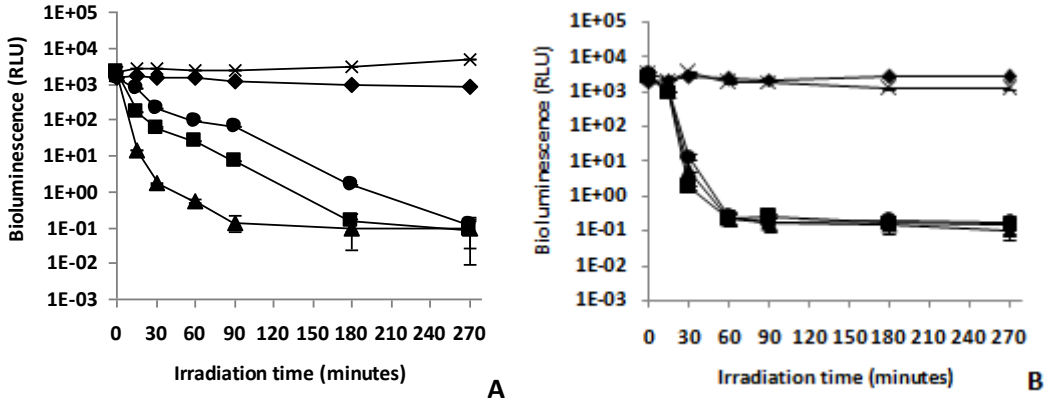

Figure 4.4 Bioluminescent monitoring of *E. coli* treated with porphyrin Tri-Py^{+}-Me-CO_2Me, after 270 minutes of 40 W m^{-2} irradiation (**A**) and of 620 W m^{-2} irradiation (**B**). The values are expressed as the means shown of two independent experiments; error bars indicate the standard deviation. (× light control, ◆ dark control, • 0.5 μM, ■ 1.0 μM, ▲ 5.0 μM).

The same was observed for dark control during solar light experiments. This indicates that the cell viability reduction obtained after irradiation of the treated samples is due to the photosensitization effect of the porphyrin.

DISCUSSION

Photodynamic antimicrobial therapy has been considered to be a promising alternative to treat skin lesions and dental infections (Komerik, Nakanishi et al. 2003; Maisch, Szeimies et al. 2004; Jori, Fabris et al. 2006). The possibility to use this approach in the wastewater treatment, in order to avoid and reduce the environmental pollution and promote the reuse of water for crop irrigation, for example, has led to the study of the efficiency of different PS under different light fluencies on the inactivation of

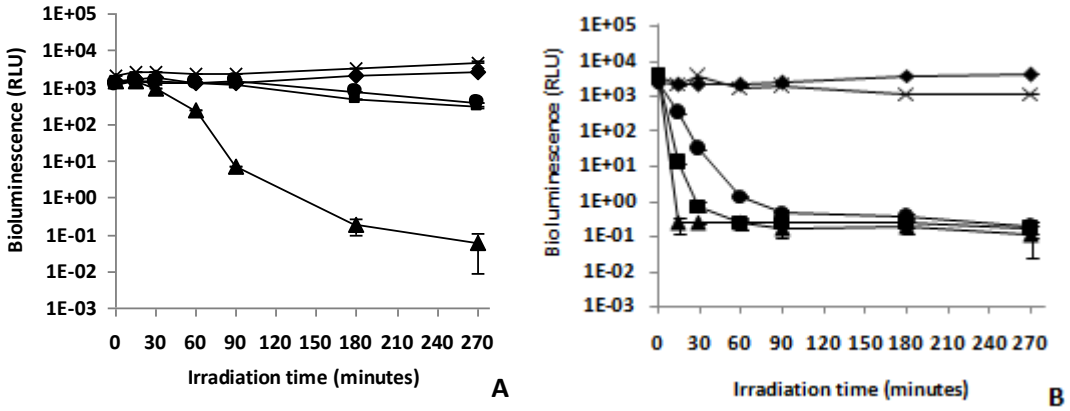

Figure 4.5 Bioluminescent monitoring of *E. coli* treated with porphyrin Tetra-Py^{+}-Me, after 270 minutes of 40 W m^{-2} irradiation (**A**) and of 620 W m^{-2} irradiation (**B**). The values are expressed as the means shown of two independent experiments; error bars indicate the standard deviation. (✕ light control, ◆ dark control, ● 0.5 μM, ■ 1.0 μM, ▲ 5.0 μM).

different microorganisms (Alouini and Jemli 2001; Jemli, Alouini et al. 2002; Bonnett, Krysteva et al. 2006; Carvalho, Gomes et al. 2007). The conventional methods used to test microbial PI *in vitro* entail laborious techniques of plating, overnight incubation and time consuming counting of CFU. To this end, simpler, faster, cheaper and sensitive methods, as bioluminescence methods, are desirable for PI studies.

In the present study, we have demonstrated that it is possible to rapidly photoinactivate bioluminescent *E. coli* with cationic *meso*-substituted porphyrin derivatives as PS. The bioluminescent indicator strain was obtained by transformation of an ATCC® *E. coli* strain with the *lux* operon (*luxCDABE*) from the marine bacterium *V. fischeri* that emits light continuously without the addition of exogenous substrates (Rocchetta, Boylan et al. 2001). The use of stable bioluminescent bacteria allows the

progress of the PI process to be followed with real time results. The bioluminescence was followed as a function of time to show that bacteria were stable light producers and the light production correlated with the growth of the bacteria (Fig. 4.3).

Experiments done with artificial light, with a fluence of 40 W m^{-2}, show the efficiency of the three porphyrins against bioluminescent *E. coli*, causing a reduction on light signal to the limits of the detectable by the luminometer. The differences between porphyrins are more noticeable at lower concentration values, since PI by tricationic porphyrins is significantly different from that by tetracationic porphyrin. At 5.0 µM, the differences are not significant and all porphyrins show equal effectiveness.

With solar irradiation (≈ 620 W m^{-2}), PI is more effective with Tri-Py^{+}-Me-PF than with Tetra-Py^{+}-Me and Tri-Py^{+}-Me-CO_2Me for all concentration values, besides the PI occurs much faster with this porphyrin (more than a 3 log decrease after only 30 minutes of irradiation). At 5.0 µM, Tri-Py^{+}-Me-PF was more effective than Tetra-Py^{+}-Me and this was more effective than Tri-Py^{+}-Me-CO_2Me.

The results of the experiments with white light are in agreement with previous results obtained for these porphyrins, in a study of charge effect of different cationic porphyrins which used conventional methods (data not shown).

Maintaining all experimental conditions, it can be observed that when a higher light fluence is used (solar PAR radiation nearly 15 fold higher than artificial PAR irradiation) the PI occurs faster for all concentrations of the three PS (near the limits of detectable after 90 minutes of irradiation).

In this study, it was shown that the PI of bioluminescent *E. coli* is achieved with low light fluence (40 W m^{-2}) and in a more efficient and faster way with PAR solar radiation (≈ 620 W m^{-2}). The results obtained, at least for artificial light are in consonance with PI patterns previously obtained for these PS where the conventional method of CFU count was done (data not shown). The results of the present study were obtained immediately once bioluminescence was read after irradiation exposure. For this reason, it can be concluded that the bacterial bioluminescence method is sensitive, real time, simple and cost-effective, in a way it does not need expensive equipment or materials, so it can be used as a rapid method for monitoring PI experiments.

The inactivation of Gram (-) bacteria to the limits of detectable 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 reutilization 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 bacteria from wastewater.

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CHAPTER 5 DISCUSSION

Photodynamic antimicrobial therapy has been considered to be a promising alternative to treat skin lesions and dental infections (Komerik, Nakanishi et al. 2003; Maisch, Szeimies et al. 2004; Jori, Fabris et al. 2006). The possibility to use this approach in wastewater treatment, in order to avoid and reduce the environmental pollution and promote the reuse of water, has led to the study of the efficiency of different PS under different light fluencies on the inactivation of different microorganisms (Alouini and Jemli 2001; Jemli, Alouini et al. 2002; Bonnett, Krysteva et al. 2006; Carvalho, Gomes et al. 2007). Furthermore, photodynamic antimicrobial therapy represents a potential advantage in environmental management, as it allows for the inactivation of high concentrations of microorganisms retained in solid residues during sewage treatments, as in sludges, when activated sludges are used as secondary treatment if this technology will be applied to a thin layer of sludges or if the sludge treatment will be done on a flow system.

According to our results tetra and tricationic porphyrins, when irradiated by the appropriate light, can efficiently inactivate Gram (+) and Gram (-) faecal bacteria, whether they are free or immobilized on solid matrices. The most effective free porphyrin, Tri-Py^{+}-Me-PF, when immobilized on a cationic support, inactivates the bacteria to the limits of detection as observed. The rate and extent of the PI with the seven *meso*-substituted cationic porphyrins studied varies with porphyrin's charge number, the lipophilic character of the *meso*-substituent groups, the charge distribution, light fluence and PS concentration. Photoinactivation with immobilized porphyrins on solid matrices is also dependent on the characteristics of the solid supports. The use of bioluminescent bacteria allows assessing the efficiency of anti-metabolic action of these porphyrins. This method is effective and sensitive besides that is simpler, faster, cheaper and much less laborious than conventional methods and can be used as a screening method for bacterial PI studies *in vitro*.

All the free seven *meso*-substituted cationic porphyrins derivatives tested are effective PS against *E. faecalis* achieving more than 99.999% (≈7 logs) reduction on cell

survival after 270 minutes of irradiation with 5.0 μM. The PI process against *E. coli* is efficient with the three tricationic PS and with the tetracationic PS, achieving more than 99.999% (≈ 7.50 log) reduction after 270 minutes of irradiation with 5.0 μM. The reduction on cell survival, for that time of light exposure and concentration value, is much lower with Di-Py^+-Me-Di-CO_2H *opp* (3.77 log, 99.98%), Di-Py^+-Me-Di-CO_2H *adj* (3.40 log, 99.96%) and Mono-Py^+-Me-Tri-CO_2H (3.28, 99.93%).

The porphyrins with three and four positive charges are the most effective PS against both bacterial strains but Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me are even more efficient than Tetra-Py^+-Me. Some studies showed a high rate of bacterial inactivation with tri- and tetracationic porphyrins compared with di- and monocationic ones (Lazzeri, Rovera et al. 2004; Caminos, Spesia et al. 2005). Merchat et al. (1996) concluded that the number of charges does not affect the activity of the PS against both bacterial Gram types (Merchat 1996). Caminos et al. (2005) showed that the photodynamic activity of a tricationic porphyrin which cationic charges were combined in different patterns with trifluoromethyl groups, in solution or immobilized on agar surfaces, was higher for an *E. coli* strain than that of a tetracationic porphyrin (Caminos, Spesia et al. 2005). Banfi et al. (2006) also concluded that a dicationic porphyrin was more efficient than that of the corresponding tetracationic derivatives against Gram (+) *Staphylococcus aureus* and Gram (-) *E. coli* and *Pseudomonas aeruginosa* (Banfi, Caruso et al. 2006).

With our results, we can say that the number of positive charges affects the PI process, as tetra- and tricationic PS are the most efficient. Nevertheless, the higher inactivation rate observed with Tri-Py^+-Me-PF and Tri-Py^+-Me-CO_2Me than with tetracationic PS suggests that a high number of positive charges can decrease the PI efficiency as already shown in other studies (Jori, personal communication) and/or that the *meso*-substituent groups can also play an important role on bacterial PI process. In fact, it has been shown that positive charges combined with highly lipophilic groups might increase the amphiphilic character of the PS, enhancing its affinity to bacteria (Boyle and Dolphin 1996; Lazzeri, Rovera et al. 2004), thus increasing the photocytotoxic activity (Caminos, Spesia et al. 2005).

The results obtained with Tri-Py^{+}-Me-PF and Tri-Py^{+}-Me-CO_2Me on both bacterial strains were significantly different (p = 0.000, ANOVA) from that obtained with Tri-Py^{+}-Me-CO_2H against *E. coli*. Tri-Py^{+}-Me-PF and Tri-Py^{+}-Me-CO_2Me causes a reduction below the detectable limits (≈ 7 log) after 90 minutes on *E. coli* while Tri-Py^{+}-Me-CO_2H causes a ≈ 5 log reduction after 270 minutes on *E. coli*. The difference observed in the PI pattern with Tri-Py^{+}-Me-CO_2H derivative may be explained by 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 PS, namely its binding preferences, aggregation state and electronic energy levels, an important parameter for the generation of singlet oxygen, for instance. On the other hand, the Tri-Py^{+}-Me-CO_2Me, an ester derivative that has the acid group protected which doesn´t allow ionization, showed a significantly higher inactivation rate for *E. coli* than Tri-Py^{+}-Me-CO_2H. The obtained 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 bacterial inactivation as already observed on similar studies (Boyle and Dolphin 1996; Lazzeri, Rovera et al. 2004; Caminos, Spesia et al. 2005).

The distribution of the charges on the sensitizer is another factor that influences the efficiency of the PI process. In this study, the pattern of inactivation by symmetric and asymmetric dicationic porphyrins was significantly different. Di-Py^{+}-Me-Di-CO_2H *adj* shows much more efficiency than Di-Py^{+}-Me-Di-CO_2H *opp* on *E. coli* with the lower (0.5 µM) and the higher (5.0 µM) concentrations. On *E. faecalis*, Di-Py^{+}-Me-Di-CO_2H *adj* is significantly different from Di-Py^{+}-Me-Di-CO_2H *opp* only with the lower concentration (0.5 µM). According to Kessel et al. (2003) who studied the cell localization and photodynamic efficacy of two dicationic porphyrins varying in charge distribution on Murine L 1210 cells, the dicationic adjacent (10 µM) PS was five-fold more efficient than the dicationic opposite (10 µM) PS (Kessel, Luguya et al. 2003). The two adjacent positive charges in the porphyrin macrocycle should result in a molecular distortion due to electrostatic repulsion. In contrast, the porphyrin with the two opposite positive charges is a much more symmetric molecule. The affinity of these asymmetric cationic molecules for cells

structures was not yet established but it is thought to be a function of hydrophobicity factors, charge distribution or both (Kessel, Luguya et al. 2003).

The PI with the monocationic PS is the most inefficient against *E. coli*, causing a 3.28 log reduction on this strain only after 270 minutes. This result is in agreement with previous results for monocationic sensitizer against Gram (-) bacteria (Merchat 1996; Caminos, Spesia et al. 2005).

Our results show that cationic porphyrins having three and four charges are highly efficient PS against both bacterial strains. The distinct *meso*-substituent groups in the porphyrin structure seem to have different effects on PI with Tri-Py^{+}-Me-PF porphyrin providing the highest log reduction on cell survival.

One of the major concerns about the field implementation of this approach is the contamination of the water output by porphyrins release. One way to overcome this issue is to immobilize the PS, as already showed (Kasermann and Kempf 1998; Artarsky, Dimitrova et al. 2006; Bonnett, Krysteva et al. 2006; Chirvony, Bolotin et al. 2006). The results obtained seem to be promising in the development of photodynamic antimicrobial therapy applied to wastewater treatment. Moreover, the immobilization of porphyrin in magnetic materials allows the removal of the sensitizer after photodynamic action, avoiding the release of the PS to the water output and allows also the PS reuse, due to their stability (Bonnett, Krysteva et al. 2006), which turns this technology a less expensive technique for wastewater disinfection.

The best porphyrin, Tri-Py^{+}-Me-PF, was chosen among the other cationic porphyrins to be immobilized by covalent coupling on nanomagnetic support materials covered with silica (work done by the Organic Chemistry Group from the Chemistry Department of University of Aveiro). Three different magnetic silica nanospheres/porphyrin hybrids were tested: cationic PS coupled to cationic material (Tri-Py^{+}-Me-PF-CS), cationic PS coupled to neutral material (Tri-Py^{+}-Me-PF-NS) and neutral PS coupled to cationic material (Tri-Ph-PF-CS).

Tri-Py^{+}-Me-PF-CS hybrid was the most effective for both Gram types (5 log decrease on cell survival with 20 and 200 μM on *E. faecalis* after 90 minutes and ≈ 5 log decrease on *E. coli* after 180 minutes of irradiation). Tri-Ph-PF-CS was the second most

effective hybrid (5 log reduction on *E. faecalis* cells after 90 minutes with 20 and 200 µM, and 4.82 log for *E. coli* after 180 minutes). The Tri-Py^{+}-Me-PF-NS was also effective against the Gram (+) strain: 5 log reduction after 90 minutes with 20 and 200 µM. However, for *E. coli*, almost none cell viability reduction was observed, even with the highest concentration (≈ 0.25 log after 270 minutes). One can say that, in general, the three hybrids tested are good against *E. faecalis* (more than 3 log reduction on cell viability - bactericidal effect - after 90 minutes of mild fluence rate – 40 W m^{-2}). As already stated, Gram (+) bacteria are easily photoinactivated, not only with cationic PS but also with neutral and anionic ones what is explained by the mode of PS interaction with the outer cell surface (Bertoloni, Rossi et al. 1990; Malik, Ladan et al. 1992).

Tri-Ph-PF-CS (neutral PS coupled to cationic material) was not significantly different from Tri-Py^{+}-Me-PF-CS (cationic PS coupled to cationic material) on *E. coli* PI. Therefore, the presence of charge in the PS is not essential to Gram (-) cell PI, as long as the amino groups of the support material are cationized. Comparing *E. coli* results between hybrid Tri-Py^{+}-Me-PF-CS (cationic PS coupled to cationic material) and Tri-Py^{+}-Me-PF-NS (cationic PS coupled to neutral material), it can be concluded that the presence of positive charge in the support material is needed to achieve PI of this strain. The charge in the material might have two important roles: adhesion to bacterial membrane with consequent disruption and also stabilization among magnetic nanoparticles through cationic charges repulsion, allowing PS availability to photodynamic process.

Tri-Py^{+}-Me-PF-CS and Tri-Ph-PF-CS are very good hybrids to the PI of the tested strains, at significant levels of water contamination (10^5 CFU mL^{-1}) (Bonnett, Krysteva et al. 2006), leading to a bactericidal effect (Knapp and Moody 1992). In other studies, where phthalocyanines were used as PS and were immobilized in silica or chitosan matrices, the results of bacterial PI were considered good to be applied to photodisinfection of microbially polluted waters (Artarsky, Dimitrova et al. 2006) with lower microbial levels in water flow systems (Bonnett, Krysteva et al. 2006). In those studies, a maximum of almost 3 log kill was achieved. In our study, we used a porphyrin derivative coupled to magnetic silica nanoparticles, and for the same initial bacterial level, we achieved a 5 log cell kill.

The magnetic silica nanospheres have the following advantages: the magnetic core of iron oxide allows the removal from the surrounding medium, applying an external magnetic field (Lu, Yin et al. 2002) allowing their recovery and reuse; the silica coating avoids oxidation and consequent degradation of the magnetic core; and is also useful to prevent the aggregation and even the partial exposure of naked magnetite (Liu, Ma et al. 2004). They may also be derivatized with organic groups that promote the covalent coupling to different synthetic porphyrin derivatives as well as they promote easy access of the PS to microorganisms surface, once the nano dimension allows the entrance trough the cell wall (Zhang, Xing et al. 2007). Also, the method of producing the nanomagnetic particles and the porphyrin synthesis allows for a large scale production of these materials, becoming a commercially viable process.

The conventional methods used to test microbial PI *in vitro* entail laborious techniques of plating, overnight incubation and time consuming counting of CFU. Consequently, simpler, faster, cheaper and sensitive methods, as bioluminescence methods, are desirable for PI studies. The use of these stable bioluminescent bacteria allows the progress of the PI process to be followed in real time results with immediate results, in RLU.

A recombinant bioluminescent strain of *E. coli* was constructed using the *lux* operon from marine bacterium *V. fischeri* and the anti-metabolic activity of three cationic non-immobilized *meso*-substituted cationic porphyrins was assessed using two different light sources: artificial white light (40 W m^{-2} of PAR radiation) and solar PAR radiation (≈ 620 W m^{-2}). The bioluminescence was followed as a function of time to show that bacteria were stable light producers and the light production correlated with the growth of the bacteria.

The three porphyrins Tetra-Py^{+}-Me, Tri-Py^{+}-Me-PF and Tri-Py^{+}-Me-CO_2Me tested were the same used in the charge effect study (first part of the thesis) once they proved to be the best PS against the Gram (-) strain *E. coli* and thus a major bioluminescence decrease during PI could be observed.

According to what would be expected, at 40 W m^{-2} of light fluence, the three porphyrins are efficient against bioluminescent *E. coli*, causing a reduction on light signal

to the limits of the detectable by the luminometer (≈ 4 log RLU decrease). At 5.0 µM, the differences are not significant and all porphyrins show equal effectiveness. At lower concentrations of PS, more noticeable differences are detected among porphyrins: PI by tricationic porphyrins is significantly different from that of tetracationic porphyrin. At ≈ 620 W m^{-2} (solar PAR radiation), Tri-Py^{+}-Me-PF is significantly more effective than Tetra-Py^{+}-Me and Tri-Py^{+}-Me-CO_2Me for all concentration values. The PI with this porphyrin occurs much faster (more than a 3 log decrease after only 30 minutes of irradiation). At 5.0 µM, Tri-Py^{+}-Me-PF was more effective than Tetra-Py^{+}-Me and this was more effective than Tri-Py^{+}-Me-CO_2Me. Maintaining all experimental conditions, it can be observed that when a higher light fluence is used (solar PAR radiation nearly 15 fold higher than artificial PAR irradiation), the PI occurs faster for all concentrations of the three PS (near the limits of detectable after 90 minutes of irradiation).

It was shown that the PI of bioluminescent *E. coli* is achieved with low light fluence (40 W m^{-2}) and in a more efficient and faster way with PAR solar radiation (≈ 620 W m^{-2}). The results obtained, at least for artificial light are in consonance with PI patterns previously obtained for these PS where the conventional method of CFU count was done (first part of the thesis). Exception made for the 5.0 µM concentration at which was observed significant difference between the tricationic derivatives and tetracationic one. In the bioluminescence study, there were no significant differences between the three porphyrins for 5.0 M, probably because in this study, an initial value of ≈ 10^6 CFU mL-1 was used, contrasting with 10^7 CFU mL^{-1} in our previous study. As shown by Demidova and Hamblin, cell density is an important variable to take in account in the PI studies because cells compete for binding with the available PS, as well as for reaction with photogenerated cytotoxic species (Demidova and Hamblin 2005).

In the experiments done under artificial light conditions, light and dark controls results show that the viability of recombinant bioluminescent *E. coli* is not affected neither by irradiation itself nor by any of the PS tested in the dark with 5.0 µM of porphyrin (≈ 3 log RLU maintained during all irradiation period). The same was observed for dark control during solar light experiments. This indicates that the cell viability reduction obtained after irradiation of the treated samples is due to the

photosensitization effect of the porphyrin. However, the values of bioluminescence in the light control (bacterial suspension only) were not constant during the solar light experiments, showing a 3 log drop in bioluminescence values after 270 minutes of irradiation. Once the samples were protected from ultraviolet radiation with a 0.5 cm thick glass plate, we believe that the decrease in bioluminescent values in this control was due to temperature increase of the suspension during the experiments. Water temperature in the bottom of the tray where the samples were placed was monitored and maintained above 30°C, approximately at 28°C. However, that temperature value doesn't seem to be adequate for these experiments since in the laboratory experiments with artificial light the water was kept at 20°C and the bioluminescence values of light control maintained constant during all irradiation period. The bioluminescent indicator strain contains the *V. fischeri lux* genes which are from marine origin and emit light preferentially at temperatures above 30°C (Hill, Rees et al. 1993). However, in experiments where such as high light fluence is used, the temperature should be maintained around 20°C.

In conclusion, our results showed that photodynamic technology with immobilized cationic sensitizers efficiently inactivate Gram (+) and Gram (-) faecal bacteria, opening the possibility of using this methodology for wastewater disinfection. The complete PI of both bacteria using low artificial light fluence (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 that white light. In temperate climates, as in Portugal, solar PAR radiation reaches higher fluence rates (about 400 W m^{-2} in winter and 620 W m^{-2} in summer). If this technology is applied to the real context of a flow system and if the sunlight is used as the light source, the time needed for the photodynamic inactivation to occur would be shorter than the 4.5 hours observed in this study. The economical advantages that this approach represents in terms of light source (solar light is freely available), synthesis and production of these immobilized PS, possibility to their removal after PI and further reuse, besides the significant photoinactivation observed, makes photodynamic antimicrobial therapy an interesting option to water disinfection. The bioluminescent method has the great advantage of results being obtained immediately

once bioluminescence is read after irradiation exposure. We consider the bacterial bioluminescence method sensitive, fast, simple and cost-effective, in a way it does not need expensive equipment or materials, so it can be used as a rapid method for monitoring PI experiments.

These results represent a new insight in the development of novel technologies for wastewater treatment and need to be further investigated.

In the future, we aim to carry out experiments with the best hybrids using transformed bioluminescent *E. coli* and *E. faecalis*, in a pilot plant, under different meteorological conditions of light and temperature and further reuse on bacterial inactivation.

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