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Inativação fotodinâmica de microrganismos com ftalocianinas multicarregadas

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Photodynamic inactivation of microrganisms with multicharged phthalocyanines

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo de Biotecnologia Industrial e Ambiental, realizada sob a orientação científica do Doutor João Paulo Costa Tomé, Investigador Principal do Departamento de Química da Universidade de Aveiro e sob a co-orientação Doutora Maria Ângela Sousa Dias Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

o júri

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palavras-chave

Inativação fotodinâmica de microrganismos (PDI), ftalocianinas, bactérias, *Escherichia coli*, bioluminescência, biofilmes

resumo

O objetivo deste trabalho foi sintetizar e caracterizar novas ftalocianinas (Pc) para serem testadas na inativação fotodinâmica de microrganismos. Nesse sentido tentou-se obter uma nova família de ftalocianinas de zinco(II) tetra e octa-substituídas nas posições periféricas. Os compostos foram caracterizados através de espectrometria de massa e espetroscopia de RMN. Foi avaliada a eficiência de inativação de seis zinco(II)Pc tetra- octa- e hexadeca- substituídas com grupos DMAP numa estirpe de *Escherichia coli* recombinante bioluminescente na sua forma planctónica. Os ensaios foram realizados a uma concentração de 20 μM de fotossensibilizador e luzes vermelha e branca com uma potência de 150 mW cm^{-2}, como fontes de irradiação. Foram ainda realizados ensaios de inativação fotodinâmica de biofilmes da mesma estirpe bacteriana usando a luz vermelha e os PS que apresentaram melhores resultados nos ensaios com a forma planctónica. Foram também realizados testes de geração de $^1O_2$, solubilidade, rendimento quântico de fluorescência, fotoestabilidade, estabilidade e uptake com as ZnPcDMAP.

As Pc **4** e **5** apresentaram maior eficiência na inativação de *E. coli* na sua forma livre, causando reduções de 4 log na bioluminescência da bactéria. Contudo, mostraram reduzida eficiência na inativação fotodinâmica de biofilmes, causando reduções de apenas 2 log na bioluminescência. Em conclusão, Pc **4** e **5** são fotossensibilizadores promissores para a inativação fotodinâmica de *E. coli* na forma livre. No entanto, ainda é preciso encontrar as condições ideais para inativação mais eficiente de biofilmes.

keywords Photodynamic inactivation (PDI), microorganisms, phthalocyanine, bacteria, *Escherichia coli*, bioluminescence, biofilms

abstract The main goal of this work was to synthesize and characterize new phthalocyanines (Pc) for the photodynamic inactivation of microorganisms. For this, the synthesis of a new family of zinc(II)Pc tetra- and octa- substituted at the peripheral positions was attempt. The compounds were characterized by mass spectrometry and NMR spectroscopy. The inactivation efficiency of six Zinc(II) Pc, tetra- octa- and hexadeca-substituted with DMAP groups was evaluated against a recombinant bioluminescent strain of *Escherichia coli* in its planktonic form. The experiments were carried out at a concentration of 20 μM of photosensitizer, and either red or white light, with a fluency rate of 150 mW cm^{-2} as energy source. Assays of photodynamic inactivation of biofilms of the bioluminescent strain were also conducted with red light and the PS that demonstrated better performance in the photodynamic inactivation of free cells. The generation of $^1O_2$, the solubility, fluorescence quantum yield, photostability and cellular uptake were also assessed. Pc **4** and **5** presented the highest inactivation efficiency in the planktonic form of the bioluminescent *E.coli*, causing reductions of 4 log in their light emission. These molecules were however much less effective against biofilms of the same strain, causing reductions of approximately 2 log in the light emission. In conclusion, Pc **4** and **5** are promising photosensitizers for the photodynamic inactivation of planktonic *E.coli*, but it still necessary to find ideal conditions for the efficient inactivation of biofilms.

Index

Acronyms and Abbreviations

λ	Wavelength
d	Doublet
dd	Double doublet
J	Coupling constant
m	Multiplet
s	Singlet
δ	Chemical shift
$^1O_2$	Singlet oxygen
Φ*f*	Fluorescence quantum yield
Abs	Absorbance
CFU	Colony forming units
DMAE	Dimethylaminoethanol
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPBF	1,3-diphenylisobenzofuran
EPS	Extracellular polysaccharide matrix
IUPAC	International Union of Pure and Applied Chemistry
LED	Light emitting diode
MALDI	Matrix assisted laser desorption/ ionization
MS	Mass spectrometry
NMR	Nuclear magnetic resonance

NMR ^{1}H	Proton Nuclear magnetic resonance
NMR ^{13}C	Carbon thirteen nuclear magnetic resonance of
PBS	Phosphate buffered saline
Pc	Phthalocyanine
PDI	Photodynamic inactivation
PDT	Photodynamic therapy
PS	Photosensitizer
QS	Quorum sensing
RLU	Relative light units
rpm	Revolution per minute
r.t	Room temperature
ROS	Oxygen reactive species
SDS	Sodium dodecyl sulphate
TSA	Trypic soy agar
TSB	Trypic soy broth
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UV-vis	Ultraviolet-visible

Chapter 1-Introduction

1.1. The photodynamic effect

Light has been used to treat diseases since ancient civilizations like the Egyptian, Chinese and Indian. Individuals afflicted by particular conditions, like vitiligo, would ingest plants and expose themselves to solar light to mitigate the symptoms of the disease. In ancient Greece this treatment was designated as heliotheraphy.[1] In 1901, after demonstrating that solar light could be used to treat *lupus vulgaris*, Niels Finsen introduced for the first time the term phototherapy.[2] Later, in 1904, Herman von Tappeiner and Jodlbauer verified that oxygen had to be present to destroy protozoa paramecium with light in presence of acridine orange, using the term photodynamic effect to describe the process.[3]

1.1.1. Mechanisms

The photophysical processes underlying the photodynamic inactivation occur when the photosensitizer absorbs light in an appropriated wavelength and the electrons are transferred from its singlet ground state (S_0) to its first excited singlet-state (S_1). This singlet state, S_1, has a short-lifetime and because of this the PS tends to return to the ground state by relaxation emitting the light absorbed as fluorescence or by internal conversion. Also, the relaxation to the S_0 state can occur by another pathway called intersystem crossing. This phenomenon takes place when the first excited state electrons reach to S_0 state, the electrons of the PS are transferred to an intermediate state called first excited triplet state (T_1) and then can decay to the S_0 by phosphorescence. The energy transferred from T_1 to biological substrates or molecular oxygen will generate singlet oxygen or the other reactive oxygen species (ROS), leading to cell inactivation.[4] For a photosensitizer to be considered a good singlet oxygen generator it must be able to go through this pathway with high efficiency.[5] The photo-physical and chemical mechanism is illustrated in Figure 1.

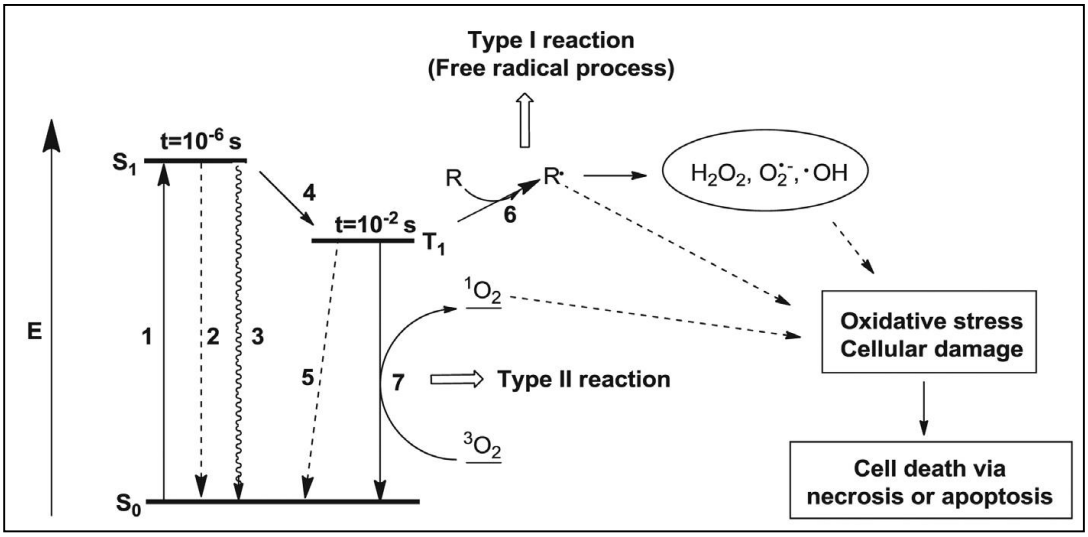
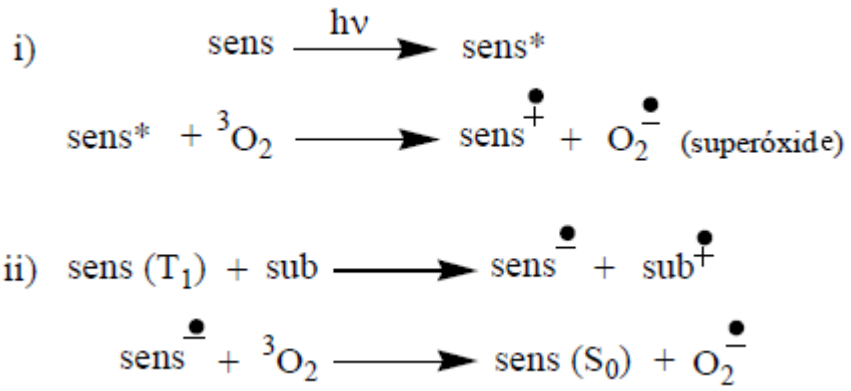
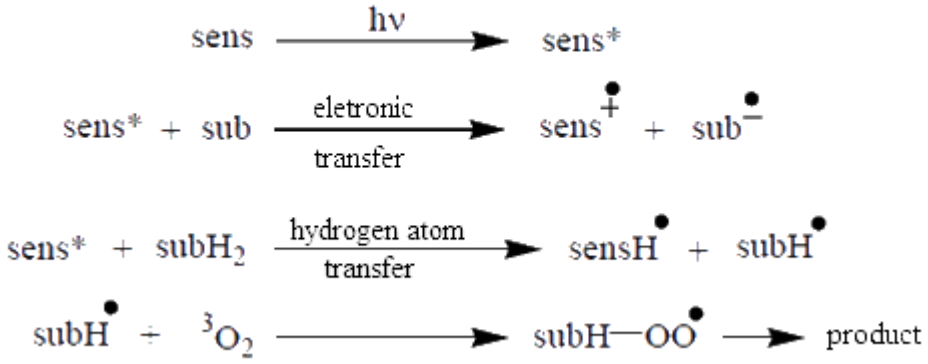


Figure 1 - Jablonski diagram describing the mechanisms involved in the production of singlet oxygen: where E is energy, 1) absorption, 2) fluorescence, 3) internal conversion, 4) intersystem crossing, 5) phosphorescence, 6) formation of free radicals (R).[4]

The PDI process can occur mainly by two types of photochemical pathways, designated as type I and type II pathways, wherein all of them result in oxidation of biomolecules present in the cells leading to cell death. In the type I pathway, the photosensitizers in its excited state generate radical species by transferring electrons to a substrate or by capturing hydrogen atoms from it. This type of mechanism can occur when electrons are transferred from the excited photosensitizer to triplet oxygen forming superoxide (Scheme 1). The later one can suffer dismutation to hydrogen peroxide that is a toxic substance causing cell damage. In the type II pathway the electron transference occurs between the T_1 photosensitizer and the triplet ground state of molecular oxygen, leading to the production of singlet oxygen (Scheme 2). The most common mechanism in the photodynamic inactivation of cells is the type II mechanism. [5,6]



Scheme 1- Schematic representation of type I mechanism[5]



Scheme 2- Schematic representation of type II mechanism[5]

1.1.2. Light Sources

The sun was the first light source used in photodynamic therapy, and although it may be the most suitable light source for environmental applications, it was not ideal for treatments with selective wavelengths and it was necessary to focus the light in the region to be treated.[7] New sources had to be developed for clinical photodynamic therapy (PDT). Initially, PDT was performed with xenon arc lamps and slides projectors with red filters to eliminate short wavelengths. Although the conventional lamps were inexpensive, simple and safe to use, they were not the best alternative because of the thermal effect, the low light intensity and difficulty to control light dose. Light-emitting diodes (LED) were also used because they could generate

high-energy light of desired wavelength and could be assembled in a range of geometries and sizes. Other commonly used light sources are lasers, such as argon dye, potassium–titanium–phosphate (KTP) dye, metal vapour lasers and diode lasers.[8,9] Comparing solar light with artificial lamps, depending on the power supply/ lamp, the second ones can provide higher fluency rates.[10,11] However, it should be taken into account that when high fluency are used the PS cannot absorb all photons, and for the photodynamic inactivation occur the absorption spectrum of the PS must have a good adjustment with the emission spectra of the light source.[12,13]

The range of wavelengths that is normally used in photodynamic inactivation of microrganisms is between 300-800 nm and in PDT is between 650-800 nm.[14] Normally, at the longer wavelengths deeper penetration of the radiation and better efficiency of inactivation are achieved. Thus, the development of PS that absorb at longer wavelengths, such as phthalocyanines, naphthalocyanine and bacteriochlorins, in order to get greater efficiency of photodynamic inactivation, is a major challenge for the scientists.[15]

Many PS are prone to photo-destruction by photobleaching, when upon illumination, the free radical reacts with the PS leading to reduction of its efficiency for further photosensitization effect, and loss of absorbance.[15]

1.1.3. Oxygen and reactive species

A highly reactive oxygen species, singlet oxygen ($^1O_2$), was found to play an important role in biological systems. For example, it was associated to antibacterial or antimicrobial agent and with cell death in the PDT therapeutic processes.[16]

In the photodynamic inactivation process, singlet oxygen is produced by electronic energy transfer from the excited state of a sensitizer to molecular oxygen.[16] It can be generated by wide range of heterocyclic aromatic compounds and it has a short life time, of about 200 ns.[17,18] When $^1O_2$ is produced inside or very close to the cells, it will interact with different intracellular molecules, depending on the site of generation. This will influence the responses of the cell, *i.e.* depending on site of production, different responses are triggered by the cells.

It has been suggested that in bacteria and eukaryotic cells, cytoplasmic membrane is the main site of lethal damage via photosensitization and singlet oxygen. However, other intracellular molecules can also be vital targets for this toxic agent.[19] When penetration of $^1O_2$ is limited by the outer membrane, such as in case of Gram-negative bacteria, secondary products are generated by the reaction of $^1O_2$ with the membrane, which plays a role as additional toxic species contributing to further destabilize the membrane and ultimately enhance the penetration of singlet oxygen.[20]

The photodynamic inactivation of cells is widely influenced by the photochemical yield of the $^1O_2$ and this depends on the intrinsic characteristic of the PS. PS that present tendency to aggregate with increasing of concentrations, results in lower singlet oxygen quantum yields.[6] To monitor the capacity of PS to generate singlet oxygen, it can be used direct or indirect methods. The determination of $^1O_2$ luminescence by near-infra-red (NIR) emission method has been used for direct quantification of $^1O_2$ life-time and quantum yield. The production of $^1O_2$ can be qualitatively measured by the indirect methods of *p*-nitrosodimethylalanine and 1,3-diphenylisobenzofuran (DPBF). In the DPBF method the production of $^1O_2$ is proportional to the loss of absorbance of DPBF, because the reaction of $^1O_2$ with the coloured acceptor DPBF leads to formation of the uncoloured compound *o*-dibenzoylbenzene.[21]

1.1.4. Photosensitizers

Photosensitizers are natural or synthetic organic compounds that when excited by a specific wavelength have the ability to absorb light and transform it into energy, to induce reaction in other molecules, that ultimately will lead to damage on biological targets.[22]

In 1960, Lipson and Schartz discovered a photosensitizer that was named as hematoporphyrin (HPD). Few years later, this compound was partially purified, made available under the commercial name of Photofrin® and used to treat several types of cancer.[22] This is considered the first generation of photosensitizers and since then, new generations of PS, second and third generations, were developed with different characteristics and for different applications. The second generation PS include, texaphyrins, phthalocyanines, chlorin e6; benzoporphyrin derivative (BPD) and bacterioclorophyll. The third generation PSs correspond to conjugates of

second generation PS coupled with antibodies.[23] The first generation of PS are characterized by weak absorption at red wavelength region, while the second generation of PS have very strong absorption at those wavelengths. In third generation the antibodies are coupled to the PS in order to transport the photosensitizer to target cells.[23]

The efficiency of the PS depend on its properties and on the intended application.[24] In the case of photodynamic inactivation of microorganisms, the PS should ideally present the following characteristics:

- Active against several groups of microorganisms;
- Low toxicity in the absence of light and cytotoxic only when excited with light of defined wavelength;
- High extinction coefficients, particularly at long wavelength, for deep tissue penetration of light;
- High triplet and singlet oxygen quantum yield;
- Preferential retention by target cells.[25]

For the photodynamic inactivation of microorganisms, both in the planktonic and sessile forms, compounds such as chlorines, bacteriochlorins, phenothiazines, porphyrins and phthalocyanines have been tested. [26–28]

Some examples of PS used in the photodynamic inactivation of bacteria in planktonic and biofilm forms are represented in Table 1.

Table 1 - Example of photosensitizers used in photodynamic inactivation of bacteria in planktonic and biofilm forms (extracted from the review by Almeida et al., 2011).[29]

Photosensitizer	Planktonic cells	Biofilms
Hematoporphyrin	*Bacillus subtilis, Streptococcus fecalis, Pseudomonas aureginosa,staphilococcus aureus Micrococcus luteus, Escherichia coli, Proteus vulgaris, Klebsiella pneumonia, Enterobacter clocae, Serratia marcescens, Bacillus cereus*	*Phorphyromonas gengivalis,Fusobacterium nucleatum,Streptococcusnsanguinis, Actinomyce actinomycetemcomitans*
Chlorin derivatives	*Porphyromonas gingivalis, Actinomyces actinomycetemcomitans, Actinomyces viscosis, Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Fusobacterium nucleaum, Streptoccocci*	*Porphyromonas gingivaliss, Actinomyces viscosis, Actinomycesnaeslundi*
Porphyrin	*Escherichia coli, Staphylococus aureus, Vibrio auguillarum, Entereococus seriolicida, Ainobacter baumannii, Diendococcus radiodurans, Yersinia enterocolitica, Mycobacterium smegmatis, Fecal coliformes, Pseudomonas aureginosa, Staphylococcus epidermais, Entereococcus fecatis, Bacillus subtilis, Prevotella intermedia, Propionibacterium acnes, Porphyromonas gingivalis*	*Provotella intermedia, Propinobacteruim acnes, Streptococcus mutans, Porphyromonas gingivalis, Actinomyces odontolyticus*
ALA	*Haemophilus parainfluenzae, Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli, Enterococcus hirae, Propinobacterium acnes, Bacillus cereus, Staphylococcus strains, Salmonella enteric, Proteobacterium acnes*	*Pseudomonas aeruginosa*

1.1.4.1. Phthalocyanines (Pc)

Phthalocyanines are blue coloured photosensitizers with a planar aromatic macrocycle structure and with high chemical and thermal stability, which make them widely used in industries.[30,31] The name phthalocyanine comes from the Greek words naphtha (rock oil) and cyanine (dark blue).

Phthalocyanines were first discovered in 1907 by Braun and Tcherniak during experiments with *o*-cyanobenzamide.[32] In their studies, they found that when this compound was heated, a blue coloured solid was obtained. This product was a metal-free compound and therefore it was identified as a metal-free phthalocyanine.[32] In 1927, Diesbach and co-workers described for the first time a synthetic route to obtain phthalocyanines from 1,2-dibromobenzene. They found that 1,2-dibromobenzene when treated with copper(I)cyanide in boiling quinoline for eight hour results in a blue product. This was the first time that a copper phthalocyanine (CuPc) was synthesized; however, they were not able to describe the structure of the compound. In 1928, a new metal-phthalocyanine was discovered by a Scottish dye company from the reaction of phthalic anhydride with ammonia, in a glass-lined reactor. This new compound was identified as an iron (III) phthalocyanine (FePc). Although the synthetic routes for Pc had been described, the structure was only unrevealed in 1933 by Linstead, with the analysis of metal phthalocyanines and products from degradations, and later confirmed by Robertson by X-ray crystallography.[32]

The structure of the phthalocyanines is similar to that of porphyrins and therefore, Pc are also known as tetra-azaporphyrins. The molecular structure of a Pc consists in four isoindole units linked by aza nitrogen atoms, whereas porphyrins are composed by four pyrrole units linked by methylene carbon bridges. The isoindole units of the Pc present 18 π-electrons delocalized over an arrangement of carbon and nitrogen atoms (Figure 2).[1,30]

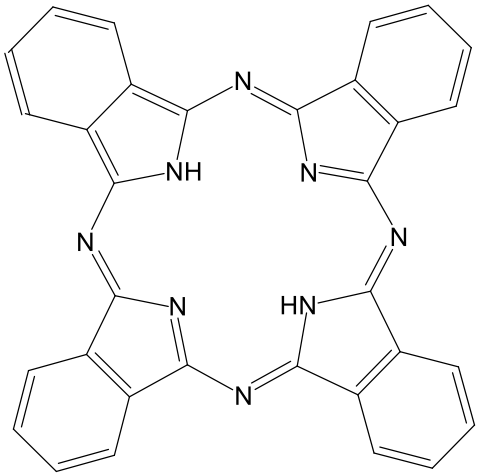


Figure 2- Molecular structure of free-base phthalocyanine (Pc)

The phthalocyanine derivatives are molecules with interesting optical properties both in near visible and near infrared (NIR) region, intense bright colour, high thermal and chemical stability and strong delocalized structure. Because of these features, the Pc has received a great attention in different areas, such as in textile industry, photography, electronic industry and photodynamic therapy.[32,33]

The phthalocyanines show two strong and well-resolved bands in their absorption spectra the Q band and the B band (Soret band) (Figure 3). The most intense band is the Q band that lies within the visible region at the wavelengths between 650-700 nm and less intense one is the B band that lies at the ultraviolet region at 340 nm. The capacity to absorb in the red region confers the characteristic colours of these compounds. However, the intensity of the colour can be slightly varied by introduction of metal in the central core of the Pc.[32]

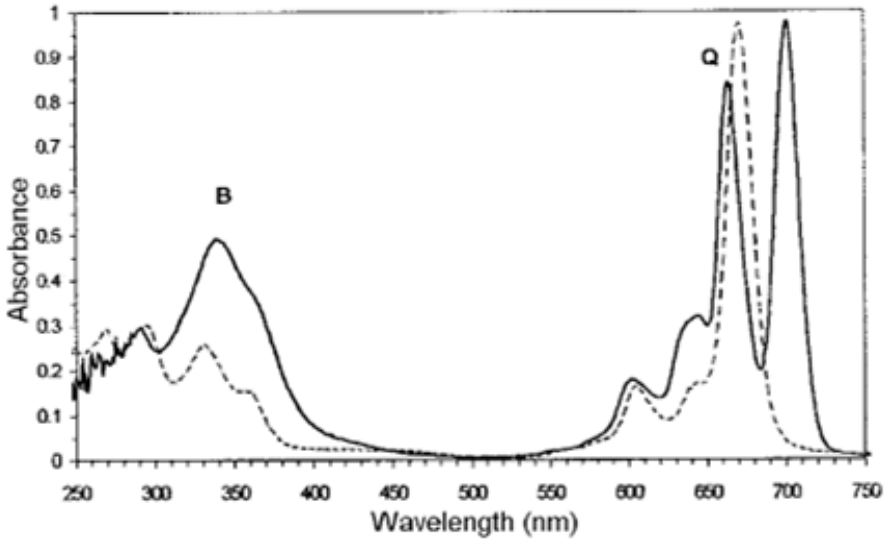


Figure 3 - Absorption spectra of a metal-free-phthalocyanine (solid line) and a metal-phthalocyanine (dotted line).[34]

In the absorption spectra of the metal-free Pc two Q-bands peaks are observed whereas the metal Pc shows a single Q-band peak in the red region. This happens because of the differences of symmetry in these two types of Pc.[35] In the metal-free phthalocyanines, two of the isoindole nitrogen are carry hydrogen atoms and the other two are involved in iminic type functions. Thus, in later, the Q-band is split in two components whereas in the metal Pc, the incorporation of a metal ion inside the central cavity affects the system in a way that a thermodynamically stable delocalized dianion with higher symmetry is obtained. However, this is only observed for symmetrical substituted Pc. If the periphery is asymmetrically substituted, a split Q-band will appear. In addition, the position of Q band and B bands can also be affected by the nature of the metal introduced in macrocycle. The variation arises due to decrease of co-extinction coefficient of both bands upon metal introduction.[35]

On the PDI field, the phthalocyanines are regarded with great interest because of their ability to absorb in the red region, 600-700 nm, and because they are very efficient producers of singlet oxygen.[30] The capacity to absorb light with long wavelength is a very important attribute in PS for clinical application, because it allows a deeper penetration treatment on target tissue or cells.[33] However, Pc molecules have the tendency to form dimmers and aggregates, what leads

to lower photodynamic inactivation efficiency. Aggregation can cause insolubility in many solvents and reduces the lifetime of the PS excited state, causing low quantum yields of excited states and of singlet oxygen generation. Aggregation phenomena can be detected from the absorption/emission spectra of the molecules.[36]

Phthalocyanines are compounds with high chemical flexibility and thus, the preparation of analogous Pc with better physical, electronic and optical properties is possible.

The two hydrogen atoms in the central core of the Pc can be replaced by 70 types of different metals, leading to the formation of metal-phthalocyanines with additional features and optimized physical responses.[35] In addition to changes in the core of the macrocycle, it is also possible to make substitutions on the peripheral positions. The substitutions that are introduced in peripheral position are known as β- and α-substitutions.

According to IUPAC nomenclature, the Pc macrocycle is numbered, wherein the position 1, 4, 8, 11, 15, 18, 22 and 25 correspond to the α-position and the 2, 3, 9, 16, 17, 23 and 24 to the β-positions (Figure 4).[37] Accordingly, various types of functional groups and molecules can be refereed as substituents at α or β positions.

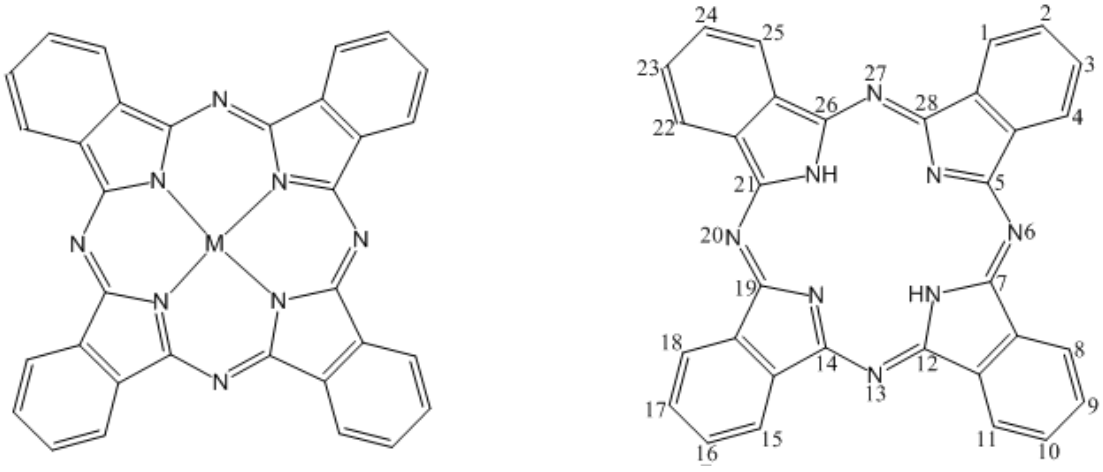


Figure 4- Molecular structure of a metalo-phthalocyanine (MPc) and a metal-free-phthalocyanine

The introduction of substituents on the molecule may considerably change its properties. For instance, in order to improve the solubility, as well as the optical and redox properties, alkyl or bulky groups can be introduced in the peripheral positions of the macrocycle.[31]

The peripheral substitutions can affect the Q-band. The substitutions in those positions can be carried out with electron-puller or electron-donating groups and depending on which group is introduced, different variations on its Q-band peaks can be observed. It is known that the β-substitutions with electron-pullers tend to cause a shift of the Q-band to the red region of the visible spectrum while the substitution with electron-donating groups do not affect strongly the Pc Q-band absorption maxima. The α-substitutions are known to cause stronger effects in the absorption spectra when compared with β- substitutions. However, the effect caused by the substitutions in the absorption spectra depends on each particular substituent and on the number of substituents.[35]

1.1.4.2. Synthesis of phthalocyanines

The synthesis of unsubstituted/substituted metal-free phthalocyanines (H_2Pc) and metal-phthalocyanines (MPc) can be carried out through different methodologies and different precursors. The most common precursors used to obtain Pc are the phthalyl derivatives, phthalonitriles or 1,3-diiminoisoindoline and normally Pc is accomplished through the cyclotetramerization of one of these precursors.[38,39]

For the synthesis of H_2Pc the most common precursor used is the phthalonitrile, which by its cyclotetramerization will give rise to a new Pc. For the cyclotetramerization to occur, initially it is necessary the formation of a diiminoisoindoline through the reaction of the phthalonitrile with one salt, after this the diiminoisoindoline will condense in appropriate conditions and H_2Pc will be formed.[37]

The synthesis of MPc can be done using precursors as phthalonitriles; diiminoisoindoline; phthalic anhydride or phthalamide, being the phthalonitriles the most common. In the case of

MPc synthesis the cyclotetramerization is carried out through the reaction of the precursor with metal salt and a nitrogen source such as urea (Figure 5). Another alternative to obtain MPc is through the reaction of H_2Pc or LiPc with an appropriate metal salt. For the complete metallation of the phthalocyanine an aromatic solvent such as 1-chloronaphthalene or quinine has to be added to the reaction.[37]

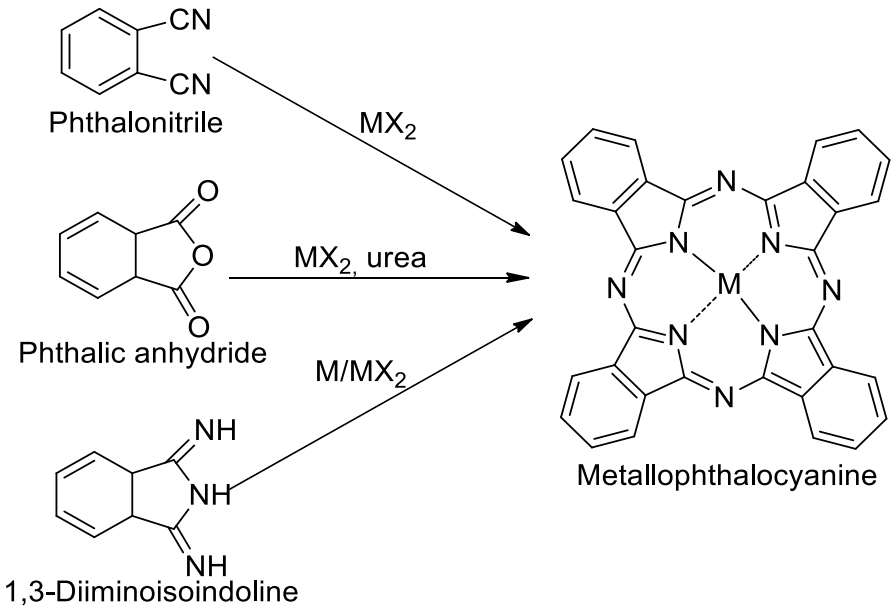


Figure 5 - Synthetic routs for metal-phthalocyanine synthesis

An alternative to obtain substituted phthalocyanines is the previous introduction of substituents groups in the precursor followed by their cyclotetramerization.[23]

The tetra-substituted phthalocyanines prepared from four identical precursor lead to a mixture of four isomers with different symmetries, D_{2h}, C_{4h}, C_{2v} and C_s (Figure 6). This mixture contains 12.5% of D_{2h} isomer, 12.5% of C_{4h} isomer, 25% of C_{2v} isomer and 50% of C_s isomer. In addition there is two types of tetra-substituted Pc, β and α substituted Pc. The β substituted can be obtained from 4 substituted phthalonitriles whereas the α substituted is obtained from 3-substituted phthalonitriles.[39] The octa-substituted phthalocyanines can be synthesized by a cyclotetramerization reaction, where 3,6 and 4,5-disubstituted phthalonitriles will produce octa-substituted Pc.[37]

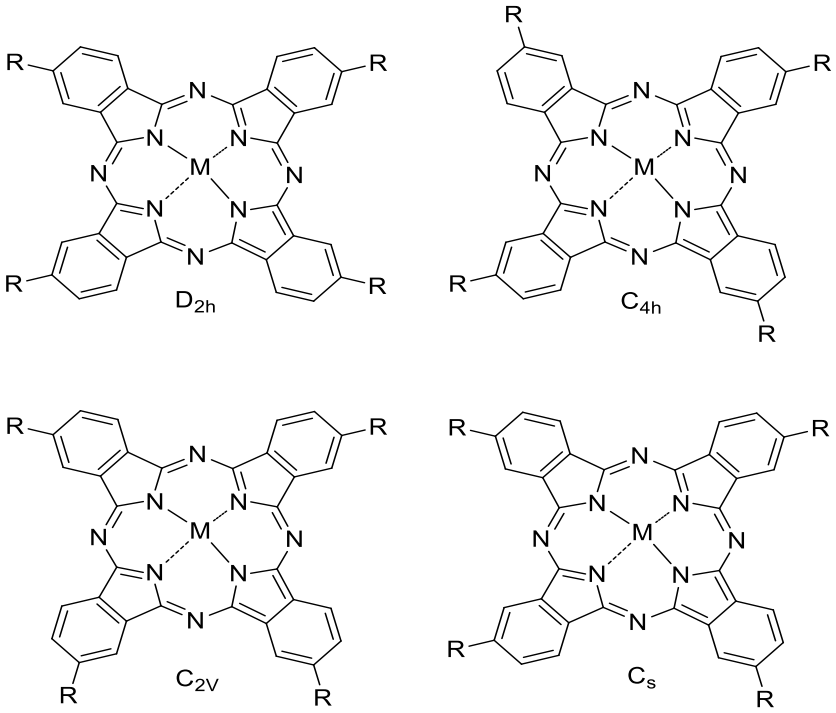


Figure 6 - The possible regiosomers of tetra-substituted phthalocyanine derivatives

The synthesis of substituted Pc can be also done by a nucleophilic substitution of an existing phthalocyanine. One example is the nucleophilic substitution of fluorine atoms in the hexadecafluorinated zinc phthalocyanine ($ZnPcF_{16}$), ZnPc analogue, by other atoms or molecular groups. These reactions are considered a rapid way to obtain phthalocyanine. In the case of $ZnPcF_{16}$ the fluorine is an excellent leaving group what facilitates the replacement. In addition, this molecule presents different reactivity of the fluorine groups in α and β positions and because of this it is easier to obtain octa-substituted Pc since that the β fluorine atoms are more reactive than the α fluorine. For the octa-substituted Pc the remaining fluorine atoms constitute a major advantage since that they can protect the chromophore from oxidation. Beside this the fluorine atoms confers to the molecule thermal stability.[40,41]

1.2. Photodynamic inactivation of microorganisms

The concept of PDT dates back to when the research group of Oscar Raab from Tappeiner used eosin, together with light, to treat skin cancer. Although initially used in the treatment of cancer, Oscar Raab also observed the toxic effects of acridine hydrochloride on *Paramecia caudatum.* After these developments, studies were pursued by other investigators. The inactivation of P*roteus vulgaris* was demonstrated and oxygen was identified as an essential requirement for the antimicrobial activity of fluorescent dyes.[26] In the context of anti-microbial approaches, the concept, photodynamic inactivation (PDI) was established as the inactivation or limitation of growth of microorganisms throught photodynamic effectt.[26]

The PDI of microorganism requires the interaction between three elements: a photosensitizer (PS), light and molecular oxygen. Individually, they are non-toxic agents but when combined they will cause lethal damages.[42]

The photosensitizer, localized in the target cells, when activated by low doses of visible light of appropriate wavelength, will induce photochemical activation of molecular oxygen into its triplet state, singlet oxygen or other oxygen reactive species (ROS). Singlet oxygen is extremely toxic to cells because there is no efficient cellular mechanism of defence or detoxification against this oxygen specie. The targets of the cytotoxic effect are biological molecules such as: proteins, nucleic acids and lipids, and the damage is exerted by oxidation reactions.[19,43–45]

In order to get an efficient PDI or a complete inactivation of the target microorganism some requirements must be fulfilled during the photosensitization process:

- Efficient adsorption (uptake) of the PS by the microorganism
- Lack of induction of resistance responses upon multiple treatments
- Low toxicity in comparison with other anti-microbial agents.

The PDI approach can be used to inactivate undesired microorganisms in different contexts and it has been gaining interest for environmental applications such as the disinfection of water or wastewater.[24,46] It is also envisaged for clinical applications, namely to treat infections caused by microorganisms that are becoming less susceptible to current antibiotics. In this case, the method is known as photodynamic antimicrobial chemotherapy (PACT).[24] In the clinical field,

PDT is used not only to treat infection but still, and actually since longer, to treat tumours instead of the traditional methods like surgery, radiotherapy and chemotherapy.[47]

1.2.1. Photodynamic inactivation of bacteria

PDI has proved to be an efficient way to inactivate different groups of microorganisms such as virus, bacteria and fungi. However, there are considerable variations in the susceptibility of different microorganisms. For instance, Gram-positive and Gram-negative bacteria are not equally susceptible to the photodynamic inactivation process. It is known that neutral and anionic PS are very efficient against Gram-positive bacteria, but not against Gram-negative bacteria. The latter are more resistant to such PS. This difference is due to structural differences of the cell wall of these microorganisms.[14] The high susceptibility of Gram-positive bacteria can be explained by the fact of having a membrane that is surrounded only by a layer of peptidoglycan allowing the PS to cross the cell wall and reach the cell membrane.[46] The Gram-negative cell wall has a more complex constitution, with a lipid bilayer outer membrane, periplasm and peptidoglycan layer. The outer membrane represents an additional barrier for the PS and generally, neutral or anionic PS that can efficiently bound to Gram-positive cell wall and mediate PDI, do not efficiently bind to the Gram-negative cell wall.[48,49] For the occurrence of photodynamic inactivation of Gram-negative bacteria, neutral or anionic PS must be combined with other biological molecules, such as nona-peptide polimyxine B or ethylenediamine tetra-acetic acid (EDTA), which will act as outer membrane disorganizing substances. These molecules allow the inactivation because they will increase the permeability of Gram-negative outer membrane so that the PS can penetrate to locations where the oxidative species (ROS) will cause a fatal damage to the cell.[48] Nevertheless, it is also now possible to perform the direct inactivation of Gram-negative bacteria using cationic photosensitizers, such as Zn-phthalocyanines, meso substituted cationic porphyrins and poly-L-lysine substituted chlorine e6, etc.[50–52] These cationic photosensitizers bind to the Gram-negative bacteria surface and their polycationic nature allow the penetration through the outer membrane by disturbing the lipopolysaccharide layer.[52] These PS can bind more easily to the outer membrane due to enhancement of electrostatic interactions between the positive charge of the PS and negative

charges in the bacteria membrane.[53,54] Therefore, primary damage of the cell wall occurs and then the penetration of PS is achieved.[27]

Other factors can also influence the difference in PDI susceptibility of different bacteria groups, such as differences in antioxidant enzymes, DNA repair mechanisms or number of microbial cell.[52,55]

1.2.2. Photodynamic inactivation of biofilms

1.2.2.1. Biofilms structure and physiology

Many microorganisms can occur in nature aggregated or physically attached to surfaces or environmental interfaces in the form of biofilms and are more frequently found in this form in nature.[56] To form a biofilm, bacteria transit from a planktonic stage to a sessile stage. Once in this stage the biofilm is formed and it will be protected by an extracellular polysaccharide matrix produced by the microorganisms.[57] Biofilms can be described briefly as a community of microorganisms that form an assembly with a complex structure capable of attaching to surfaces and interfaces.[58]

Biofilms represent an advantageous lifestyle because surfaces tend to concentrate nutrients and by attaching, bacteria are better provided with substrates, become less affected by changes in environmental conditions, and display enhanced resistance to many antimicrobial agents and to host defenses.[59–62] Once organized in a biofilm, the microorganisms will be express characteristics that may be different from those exhibited by the planktonic forms due to changes in general physiology, metabolism, and gene expression.[63]

Biofilms can be found on various microhabitats: natural environments and inert surfaces, living tissues (e.g. plant roots, intestinal tracts), medical devices (e.g. pacemakers, catheters, dental plaque), industrial equipment (pipelines), and they can occur in solid-liquid, solid-air and in liquid interfaces.[57,64,65]

To form a biofilm, the microorganisms will only select the environment that provides them better conditions to grow according to chemical and physical signals detected from the

environment. When the right conditions are not met, the biofilm will disassociate into individual cell that will seek a new favorable habitat.[59,66] The transition from the swimming form to the sessile form depend on the microorganism species, environmental factors and on particular genetic determinants. The processes involve five distinct fundamental steps: pre-conditioning of the adhesion surface; attachment of microorganisms; microcolony formation; macrocolony development and dispersal (Figure 7).[67]

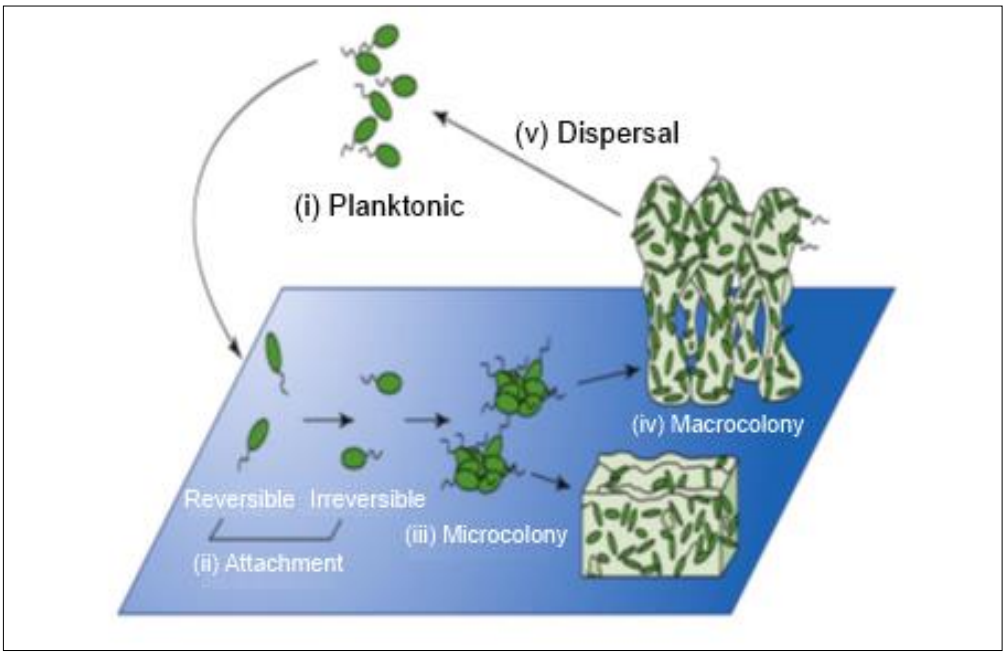


Figure 7 – Process of biofilm formation[67]

Normally, the attachment is divided into two stages, one reversible and other irreversible, given that the first attachment is weak and it can be easily reverted by fluid shear forces.[60] In the first stage, a planktonic organism is transported nearby the surface to initiate adherence and for this some physicochemical variables are needed. Once close enough to the surface, the microorganism will bind to it, in response to a net of attractive or repulsive forces. These forces include electrostatic and hydrophobic interactions, steric hindrance, Van Der Walls forces and hydrodynamic forces. The longevity of this first adhesion will depend on the sum of these variables and also on the presence of organic substrates near to the surface, since microorganisms tend to aggregate in nutrient-rich environments. In the second stage, which is

considered irreversible, molecular reactions between bacterial surface structures and substratum surfaces are enhanced. This implies a tighter adhesion of bacteria to a surface by the bridging function of bacterial surface polymeric structures. The microorganisms will bind to the surface by the production of exo-polysaccharides and or specific attachment structures such as pili or fimbriae. After this stage, a much stronger physical or chemical agent is necessary to detach the cells. When the microorganism is irreversibly attached, the biofilm maturation begins. During the maturation, its density and complexity increase, the organisms start to replicate and producing extracellular components that will interact with the organic and inorganic molecules of the environment to create the extracellular polymeric matrix (EPS).[59,68] The micro-colony formation will occur with the clonal growth of the adherent cells and the micro-colonies will grow forming the macro-colonies. Finally, in the last dispersal step, the macrocolonies can release cells from the biofilm that will return to the planktonic form to eventually initiate the colonization of a different microhabitat.[67]

Biofilms are known to be structurally heterogeneous, consisting of a complex matrix, which is the major part (around 97% of the biofilm), composed by water and extracellular substances like polysaccharides and proteins, and cell clutters formed by microorganisms of the same species or a mixture of different microorganisms.[69,70] It is well known that each of the components plays an important role in the biofilm formation and maintenance. For example water is required for processes of diffusion into and within the biofilms, cell clusters will produce the EPS matrix and signalling molecules that are important for cell-cell communication (quorum sensing) and EPS will play a role in the architecture and maintenance of the biofilms structure.

The extracellular polymeric substances include polysaccharides (mainly hexoses and pentoses) which are actually major components of the biofilm matrix.[71] The matrix also contains proteins, nucleic acids (DNA and RNA), lipids, heteropolymers and humic substances.[72]

The EPS contribute for the formation and maintenance of biofilm structure, and the morphology of the colonies is more affected by the capacity of the cells to produce EPS.[73]

The biofilm EPS play a role in the determination of the strength, elasticity and adsorption capacity of the biofilms. It confers physical/chemical protection, facilitates metabolic

interactions between cells, works as a supply of substrates for growth and acts as a protective mechanism against desiccation and toxic substances, such as biocides and antibiotics.[72,74]

The transition from the planktonic to the sessile form involves a phenotypic change determined by a shift in gene expression which in term, is triggered by cell to cell communication mechanisms.

Quorum sensing (QS) is a density-dependent form of cell-cell chemical communication trough signal molecules.[75] Microorganism can use QS to coordinate for example the formation of biofilms, swarming and the production of polysaccharides.[76]

The cell-cell communication underlies the triggering and reinforcement of common group responses during biofilm formation. This, communication is regulated by signalling molecules that are released by some cells, and detected and perceived by other cells and also by producer cell.[67,75] At certain concentrations they can trigger the expression of multiple genes that regulate important biological functions such as transfer of plasmids, mobility, aggregation, luminescence, biosynthesis of antibiotics, expression of virulence factors, symbiosis, and development of the biofilm.[77]

1.2.2.2. Biofilm resistance

Microorganisms organized in biofilms are more resistant to antimicrobials than the planktonic form. This resistance is provided by different defense mechanisms that have been developed by biofilms. The EPS matrix constitutes one of the principal barriers, by preventing the penetration of antimicrobials into the biofilms.[78] Several studies have demonstrated the difficulty of many antibiotics to diffuse into biofilms, suggesting that the antibiotics bind to the EPS, being thereby retained in the extracellular matrix, which prevents of reaching the cells within the biofilm. Not only the inhibition of diffusion is involved in the antibiotic resistance, but the production of enzymes that catalyze antibiotics activity, or the matrix charge can also play a role, through the inhibition of drug activity or by retarding the penetration providing more time to develop resistance.[78,79] The grow rate can influence the susceptibility of biofilms to antimicrobials. It is known that the slow growth of cells in mature biofilms increases the resistance due to increased cell density.[78] The activation of stress responses by biofilms in order to fight external agents is

very common. These stress responses result in the physiological changes or in the production of enzymes or toxins to protect from anti-microbial agents.[78,80] QS will mediate the activation of these stress responses, by the production of interacting signals ,which allows the microorganisms to sense when there is a limitation of cells in the environment and expresses the genes to inhibit the effect of the antibiotics.[79,80] The existence of persistent cells, that can be found either in planktonic or biofilm communities is one of the reasons of antimicrobial resistance.[81] However it is more frequent to find persistent cells in biofilms than planktonic communities, which may be other explanation for the lower susceptibly of biofilms are antibiotics.[82]

1.2.2.3. Negative impact of biofilms

Biofilms are found in different areas of industry, environment and health. Their presence may have beneficial or detrimental impacts, depending on the characteristics and functions of the organisms and of the attaching surface, but very often biofilms development is associated with chronic infection, biodeterioration and biocorrosion with significant human and economic losses.

Biofouling refers to the undesired accumulation of microbial and extracellular material of microorganisms on surfaces that causes deterioration of materials and affects the efficiency of several processes which those surfaces are involved.[83] This phenomenon can occur in several situations like the dental plaque, the colonization of medical devices, the deterioration of metal surfaces such as in ship hulls and buildings, in pipelines and industrial structures.[56] In industries, biofouling is a major concern due to the metallic corrosion affects. Water cooling systems, increases the resistance to heat energy transfer and increases fluid frictional resistance when film thickness surpasses the monolayer.[84] In membrane systems, the attachment of microorganisms can lead to the decrease the membrane flux and increase filtration pressure and thus demand for the frequent cleaning and replacement of the equipments.[76] In buildings, biofouling in walls can influence the heat uptake and so the energy demands inside the building. In ships it leads to reduction in speed, increase the fuel consumption or even enhance the corrosion of the hulls.[56] All these problems will conduce to loss of energy, increasing of costs

and loss of quality in the final products. Beside all the environmental issues caused by biofilms, they are also involved in several human infections, being responsible for over 60% of infections caused by bacteria. Biofilms are responsible for several human infections, among which periodontitis, cystic fibrosis pneumonia, chronic urinary tract infection, tonsillitis, rhinosinusitis, otitis media, wound infections, infectious kidney stones, bacterial prostatitis, etc. Beside this, they can also associated to medical devices causing serious infection.[79,85]

1.2.2.4. Inactivation of biofilms

Because of these impacts, environmentally sustainable and cost-effective approaches for biofilm control or inactivation are urgently needed. To minimize the losses caused by the attachment of microorganisms to the surfaces and overcome their intristic and acquired resistance, some alternatives have been attempted, such as the modification of nature of the solid surface, optimization of operation conditions, physical and chemical cleaning, use of biocides, synthetic dispersant or enzymes.[76,84] Although traditional chemical methods still the most commonly used, new alternatives are being introduced. One example is photodynamic inactivation (PDI) that proved to be an economic, environment-friendly approach to biofouling problems.[24]

PDI is widely investigated as a therapeutic alternative for biofilms infections, since it is a multitarget process and there is a lack of antimicrobial resistance. PS used in PDI present high reactivity and this allow interactions either with cellular and non-cellular biomolecules, which for the inactivation of biofilms is an important advantage since their extracellular matrix are equally susceptible to the photosensitizing effect.[86] It is expected for biofilms to be more resistant to PDI than planktonic cells and in fact they are. However it was demonstrated that the increasing of the light dose, time exposure or PS concentration will lead to higher PDI efficiency in biofilms.[87–89]

Many PS have been reported as efficient drugs for the photodynamic inactivation of biofilms, namely methylene blue and toluidine blue O, considered effective PS to treat oral and medical infection caused by biofilms.[90,91] Beside these two dyes, macrocyclic photosensitizers like phthalocyanines, porphyrins and chlorines are used in PDI of attached microorganisms Reductions of 4 and 3 log on the concentration of surviving *Pseudomonas aeruginosa* and

Streptococus epidermis wre observed with Tetra-Py^+-Me and Tri-Py^+-C_{14}-Py^+-Me but this PS have the inconvenient of presenting dark toxicity, reductions of 6.5, 6.3 and 2.8 log of *Candida albicans*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively.[6,88] Zinc-phthalocyanines have been also reported as efficient PS against biofilms. A study of photodynamic inactivation with Zn(II) Pc reported the destruction of dental-plaque biofilms.[92] The fungus *Candida albicans* can be completely inactivated with Si(IV) phthalocyanine derivative under soft condition.[93]

1.2.3. Bioluminescence as reporter for photodynamic inactivation assays

Bioluminescence is considered a fast and effective method to monitor the photodynamic inactivation of microorganisms. It can be used in direct, continuous and non-destructive approaches to follow the inactivation processes in real time.[94]

The bioluminescence method consists in a process in which organisms produce light by reactions that involve consumption of energy and that are tightly related to the metabolic activity of the cell. These reactions are catalysed by luciferases.[95,96]

Different bacteria are able to produce light and they can be found in terrestrial, freshwater and marine environments. All bioluminescent bacteria are classified as Gram-negative and normally they are included in one of the genders: *Vibrio*, *Photobacterium* and *Photorhabdus* (*formerlly Xenorhabdus*), that are naturally light producers.[94] The luciferase gene is now well characterized and cloned, which allows bacteria that are not natural light-emitting organisms to emit light. Normally, the transformed bacteria are Gram negative, such as *Escherichia coli.*

In bacteria, the light emitting reaction consists in the oxidation of fatty aldehyde and riboflavin phosphate $FHNH_2$ with emission of light.[97]

The most common methods used to follow infections require several steps, for example to follow animal infections it is necessary to sacrifice the animal, remove the infected tissue, homogenise, make serial dilutions, plate and count the colonies.[49] By using bioluminescence, a directed correlation between viable cells and the emission of light can be established, since only active cells can be detected. Therefore, a decrease in bacteria bioluminescence reflects a decrease in the number of active cells or a decrease in the average level of activity of the

population.[98] In the beginning of the year 2000, bioluminescent bacteria started to be used and it proved to be more effective, simpler, faster and sensitive than conventional plating methods to make the screening of viable bacteria after PDI exposure. Since then, this approach has been widely used to do the screening of effective PS used to inhibit the growth of the bacteria and its bioluminescence.[94] In addition to these advantages, another advantage is that different than other chemicals, the PS does not interfere with the bioluminescence of the microorganism.[94]

Bioluminescence method was also considered an efficient method to monitor the photodynamic inactivation of biofilms infections.[99,100] The possibility to use this method seems to overcome one of the major problems pointed for the traditional techniques that is the extraction of bacteria from the surface.[101] Light emission of biofilms tends to be lower than in planktonic cells, due to nutrient starvation and oxygen availability which reduce power of bioluminescence.[101,102] However, this does not affect the number of viable cells, thus it important to establish a non-luminescent end point in the PDI process.

1.3. Objectives

The aim of this work was to produce new water-soluble zinc-phthalocyanine for the control of bacteria, either in the planktonic or in the biofilm forms. For that, a zinc-phthalocyanine with peripheral substitution with cysteamine hydrochloride was synthesized and characterized by spectroscopic methods. Further photophysic studies of this compound and other phthalocyanine derivatives, that were previously synthesized and characterized, were conducted in order to assess their potential as photosensitizers for inactivation of microorganisms. After the initial screening with a rapid bioluminescence test based on planktonic cells of a recombinant strain of *Escherichia coli*, selected phthalocyanines were tested against biofilm prepared with the same strain.

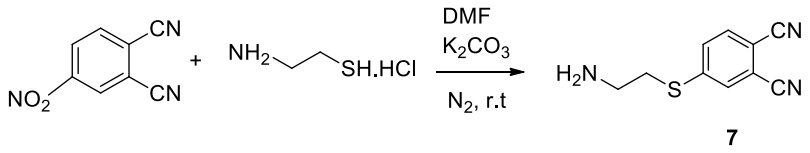
Chapter 2 – Synthesis and characterization of new phthalocyanines

2.1. Synthesis optimization

2.1.1. Synthesis of thiocysteamine phthalonitriles

For the synthesis of new cationic zinc-phthalocyanines different synthetic methodologies were followed. In an initial phase, we tried to obtain new phthalocyanine derivatives containing cysteamine units at the peripheral positions. To obtain these molecules, the synthetic route was divided into three steps: (i) synthesis of mono and di-substituted cysteamine phthalonitrile derivatives; (ii) cyclic tetramerization of phthalonitriles to corresponding Zn(II)phthalocyanines; (iii) protection and deprotection of Zn(II)phthalocyanines.

4-thiocysteaminesubstituted phthalonitrile, **7**, (Scheme 3) was obtained by nucleophilic substitution reaction. 4-nitrophtalonitre (5.75 mmol) reacted with cysteamine hydrochloric acid (1.43 mmol) in presence of potassium carbonate (K_2CO_3) (1.54 mmol). The reaction mixture was kept at 50 ºC and under stirring. After 24 hours, the reaction was stopped, distilled water were added. The formed precipitate was filtered and washed with dichloromethane and water to remove unreacted starting materials if any. It was not possible to proceed for further purifications because the obtained powder was not soluble in any of the regular solvents. The powder was dried and submitted to NMR and mass spectrometry, but the results were inconclusive.



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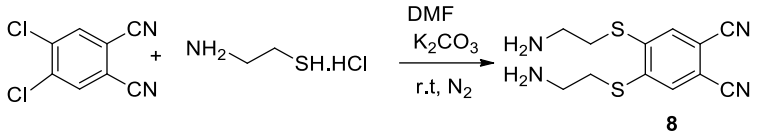
Scheme 3

To obtain the di-substituted phthalonitrile with cysteamine, compound **8**, (Scheme 4) 0.51 mmol of 4,5-dichlorophtalonitre was reacted with 1.22 mmol of cysteamine hydrochloric acid in presence of 1.09 mmol of K_2CO_3. The reaction mixture was kept at room temperature and under stirring. After 2 hours, the reaction was stopped and distilled water was added. The formed

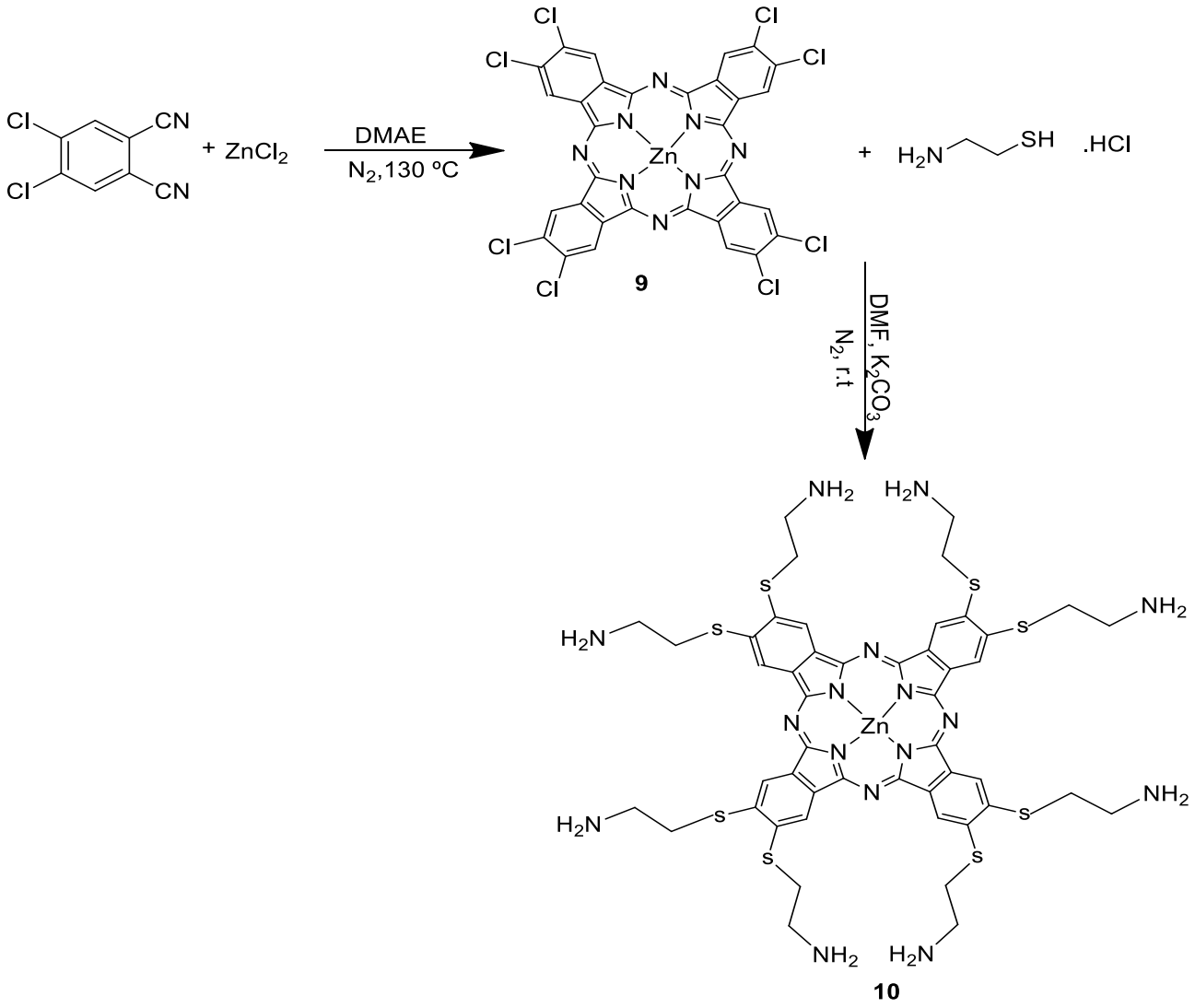
precipitate was filtered and washed with water and dried. The TLC showed the presence of small amounts of starting material along with two other products with close separation between them. Therefore, in order to separate the two products, it was necessary to prepare TLC plates using dichloromethane (DCM) with 15% of methanol as eluent. The product was separated from the silica by filtration and later dried. Further, the compound was characterized by ^{1}H NMR, FT-IR, and mass spectroscopic techniques, but the results were still inconclusive.



Scheme 4

2.1.2. Synthesis of (octakis[4,5-bis[thiocysteamine]phthalocyaninato])zinc(II)

Since the synthesis/characterization of the thiocysteaminephthalocyanine using the corresponding phthalonitriles revealed to be difficult, new approaches for the synthesis were attempted. In the new synthetic rout, the objective was to obtain the phthalocyanine as starting material. For that, a reaction with 1.01 mmol (200 mg) of 4,5-dichlorophthalonitrile and 0.5 mmol (681 mg) anhydrous zinc chloride under nitrogen atmosphere and reflux conditions was conducted. After 24 hours the reaction was stopped and petroleum ether was added to dissolve the crude material. The product was precipitated and re-precipitated in methanol.



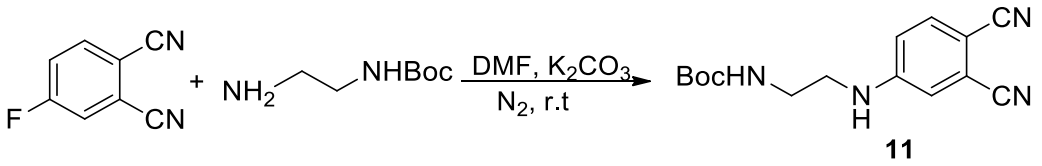
Scheme 5

After having the phthalocyanine **9**, the synthesis of the thiocysteamine substituted, Pc **10**, by reacting the compound **10** with cysteamine hydrochloride was attempted in order to get peripheral substitutions (Scheme 5). For this, 50 mg ZnPc **10** reacted with 70 mg cysteamine hydrochloride acid using DMF as solvent and K_2CO_3 as base. The reaction was kept at room temperature and nitrogen reflux for 24 hours. The reaction could not be controlled by TLC since the starting material did not move in the TLC plate.

2.1.3. Synthesis of aminoethylcarbamate phthalonitriles

Following all the unsuccessful attempts to obtain phthalonitriles or a phthalocyanine substituted with thiocysteamine groups, a different substituent containing amine groups connected by an alkyl spacer was used. The objective was to introduce a protected amine group in the phthalonitrile, and then, to perform the cyclotetramerization of the phthalonitrile, the hydrolysis and finally the cationization of the phthalocyanine.

The phthalonitrile **11 (**Scheme 6**)** was synthesized by reacting the 4-fluorophthalonitrile (0.6 mmol) with tert-butyl(2-aminoethyl)carbamate (1.03 mmol) using DMF as solvent and K_2CO_3 as base. The reaction was stirred under nitrogen atmosphere and at room temperature. The progress of the reaction was monitored by TLC. After 2 hours, the TLC revealed the appearance of two spots, one of which was identified as starting material and the other indicating the formation of the product. We further extended the reaction for 24 hours, but reaction was not was not complete. Because of this, we decided to keep the reaction at 50 ºC for more 2 hours. However, no change was observed in the progression of the reaction, so we stopped the reaction. Distilled water was added to the reaction mixture and extracted with dichloromethane. The organic layer was collected and the solvent was evaporated under reduced pressure. Tetrahydrofuran was added and evaporated in order to remove some remaining DMF. The product was purified by column chromatography using silica gel as stationary phase, dichloromethane/hexane (9:1) and then dichloromethane/methanol (98:2) as eluents (yield 55%). The product fraction was characterized by ^{1}H NMR and mass spectrometry.



Scheme 6

^{1}H NMR spectrum of compound **11** (Figure 8) showed one doublet at δ 7.67 ppm and one multiplet at δ 6.90-6.94 ppm, corresponding to the protons of benzene ring at 5, 6 and 3 positions; one triplet and one doublet at δ 7.13 and δ 7.29 ppm, referring to the aliphatic protons;

two double doublets at δ 3.07 and δ 3.20 ppm related to the protons of alkyl chain and one singlet at δ 1.36 ppm, due to the resonances of the CH_3 protons. ^{13}C spectrum show 10 signals, in which 3 corresponded to the carbons in benzene ring, 1 corresponded to cyanine groups and the others are referent to the methyl, ethyl, carbonyl groups (Figure 9).

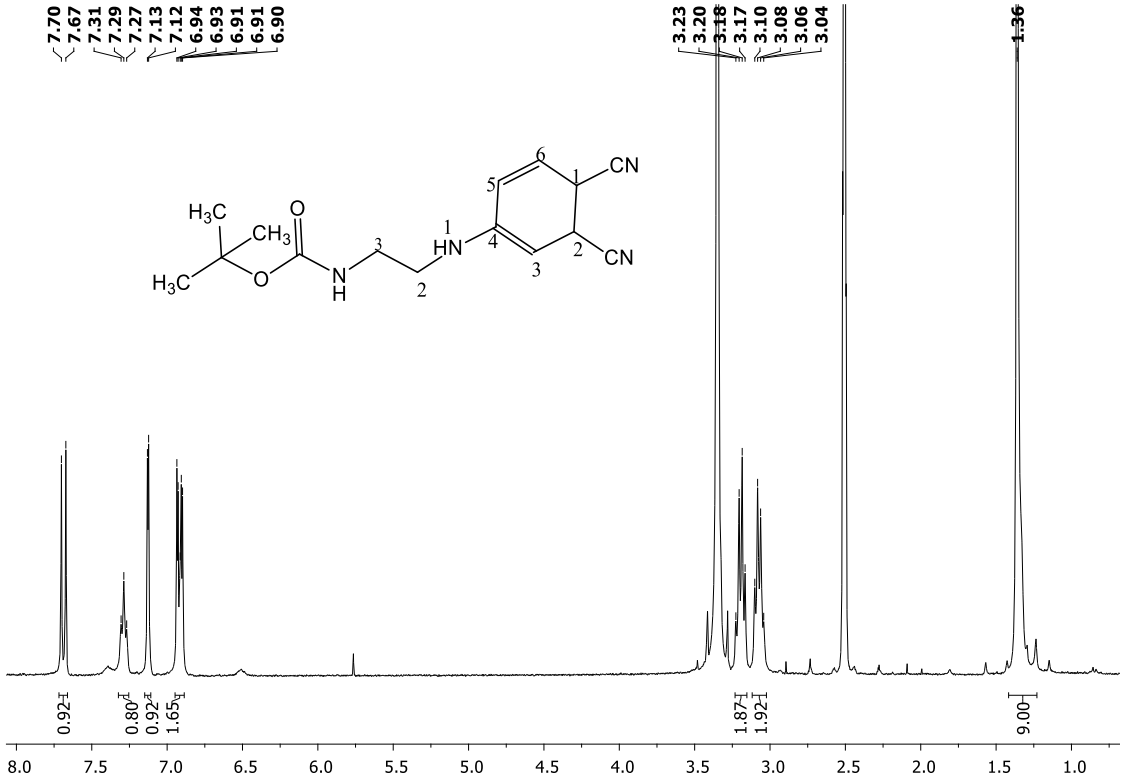


Figure 8- ^{1}H NMR (300 MHz) spectra of the phthalonitrile **11** in DMSO-d_e

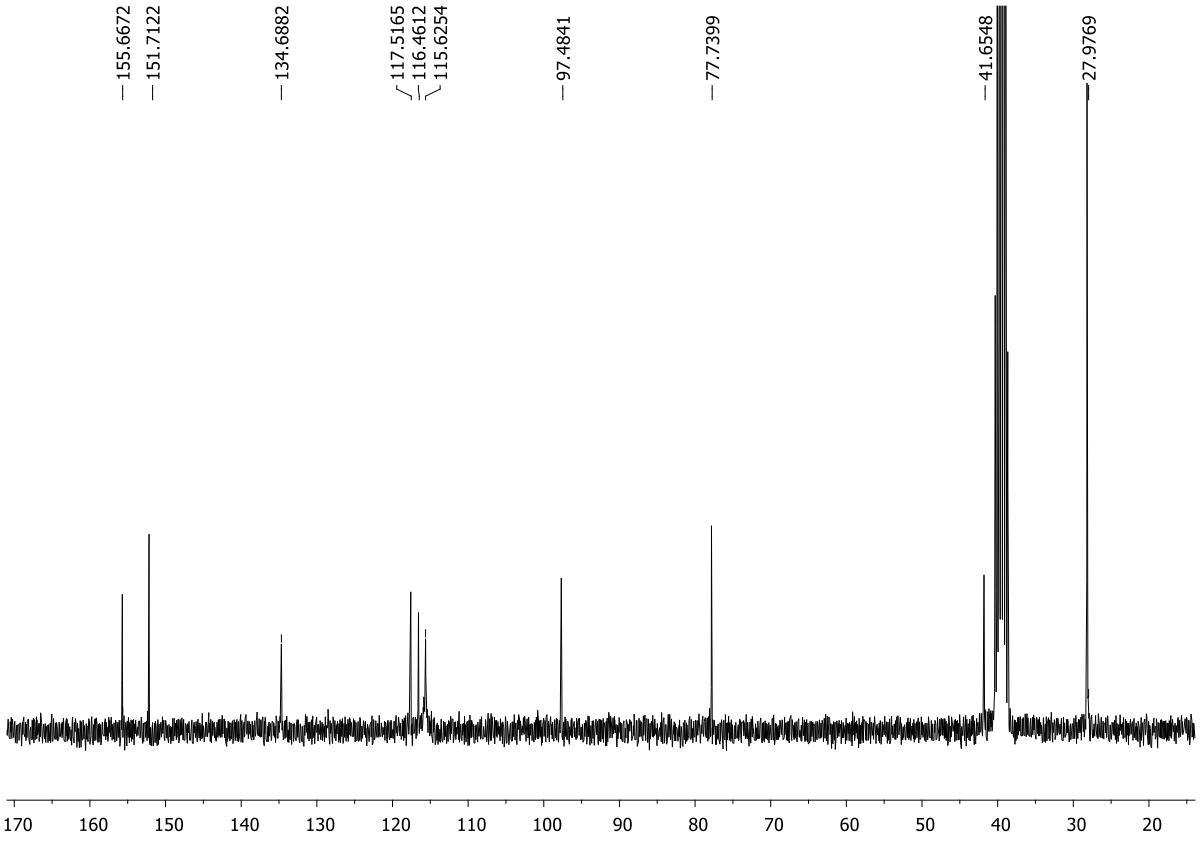
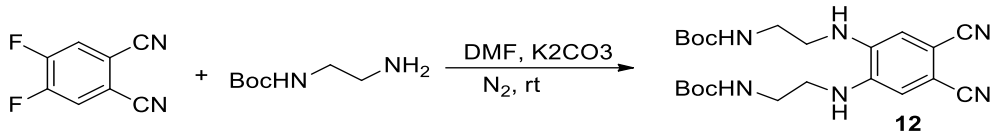


Figure 9- ^{13}C NMR (125.77 MHz) spectra of the phthalonitrile **11** in DMSO-d_6

Phthalonitrile **12** (Scheme 7) was obtained from reaction of 4,5-flourphthalonitrile (0.6 mmol) and tert-butyl(2-aminoethyl)carbamate (1.4 mmol) in presence of DMF and K_2CO_3. The reaction mixture was kept overnight stirring, under nitrogen atmosphere and at room temperature. After this period, the TLC revealed that the reaction was complete. The reaction mixture was extracted using distilled water and dichloromethane. The organic layer was evaporated under vacuum. Toluene was added and evaporated in order to remove some remaining DMF. This product was precipitated with hexane and dichloromethane and dried under vacuum. ^1H NMR confirmed the proposed structure of di-substituted phthalonitrile **12**, yielding 63%.



Scheme 7

^{1}H NMR spectrum (Figure 10) shows two doublets at δ 7.86 and δ 7.40 ppm, corresponding to the protons of benzene ring at positions 3 and 6; one doublet at δ 7.15 and one triplet at δ 6.91 referring to the resonances of the aliphatic protons; two double doublet at δ 3.11 and δ 3.26 ppm, corresponding to the protons 3 and 2, respectively; one mutiplet at δ 1.37 ppm, referring to the resonances of the methyl protons. Due to the symmetry of the phthalonitrile, the ^{13}C NMR spectrum showed 8 signals, each one corresponding to 2 carbons from a total of 16 carbons (Figure 11).

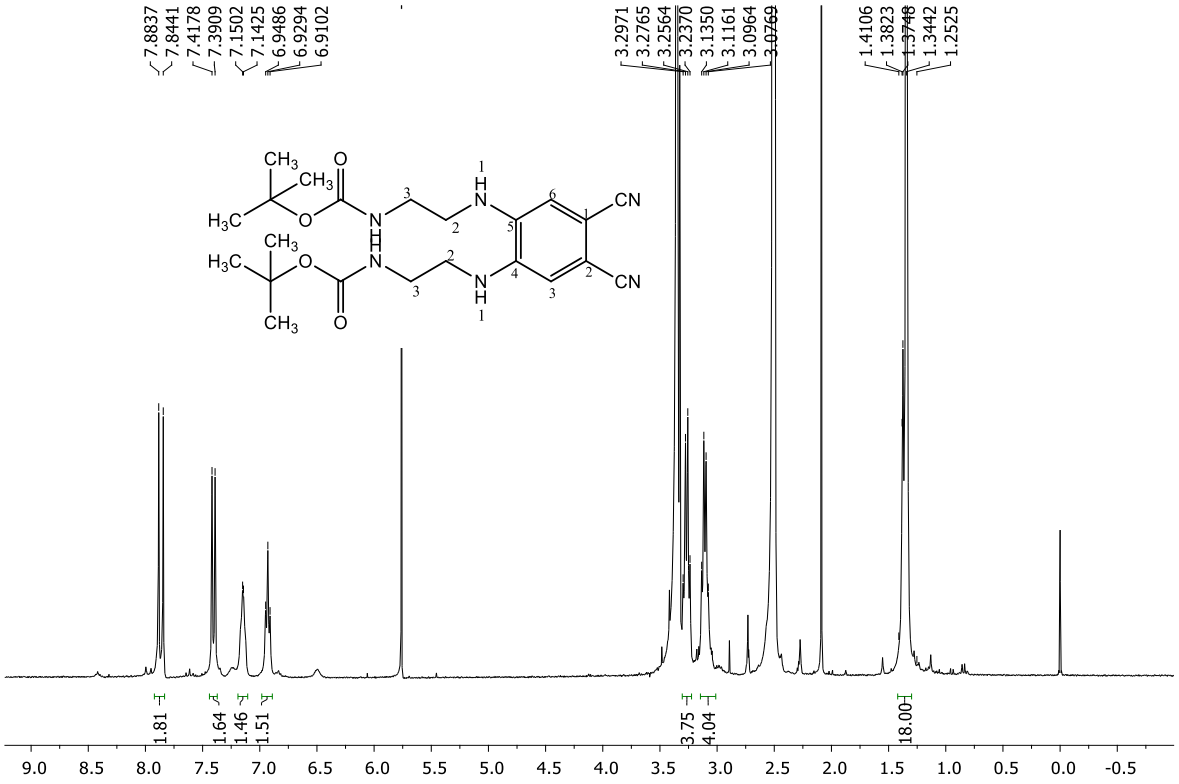


Figure 10-^{1}H NMR (300 MHz) spectra of phthalonitrile **12** in DMSO-d_6

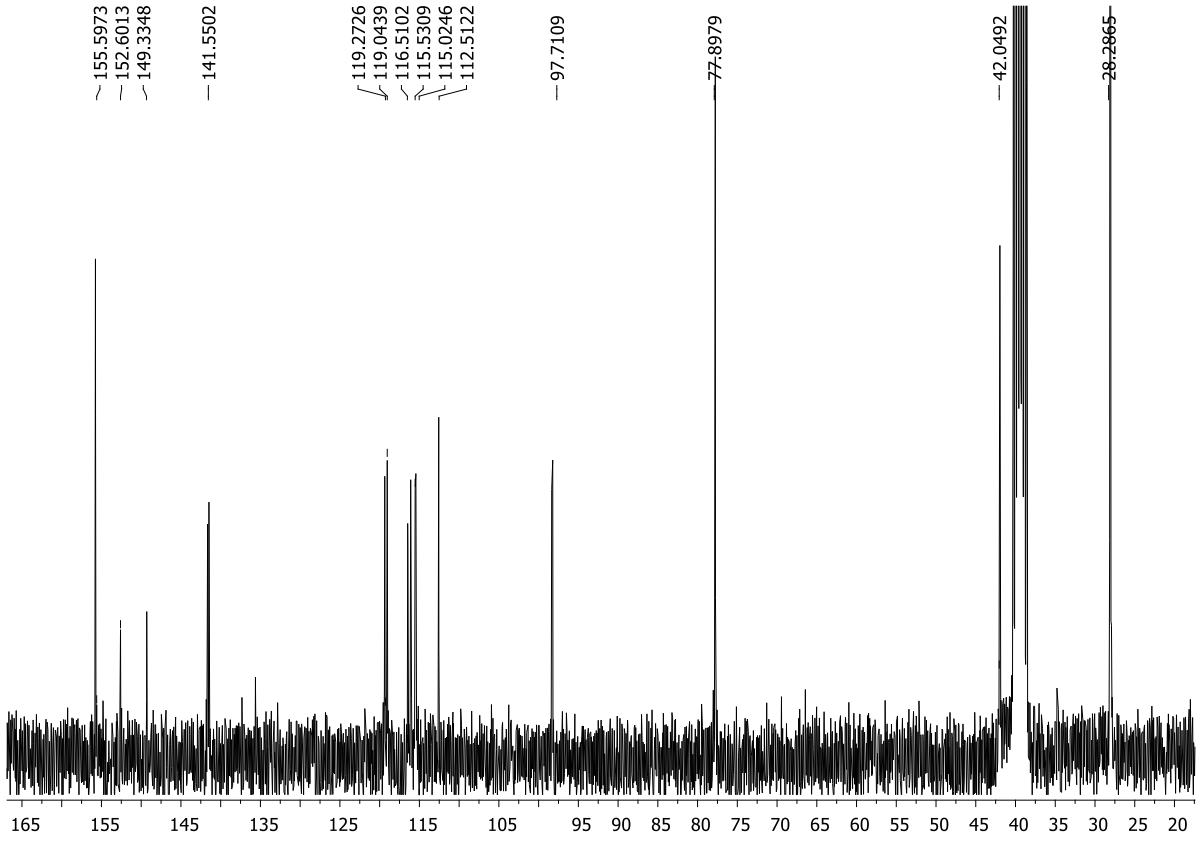
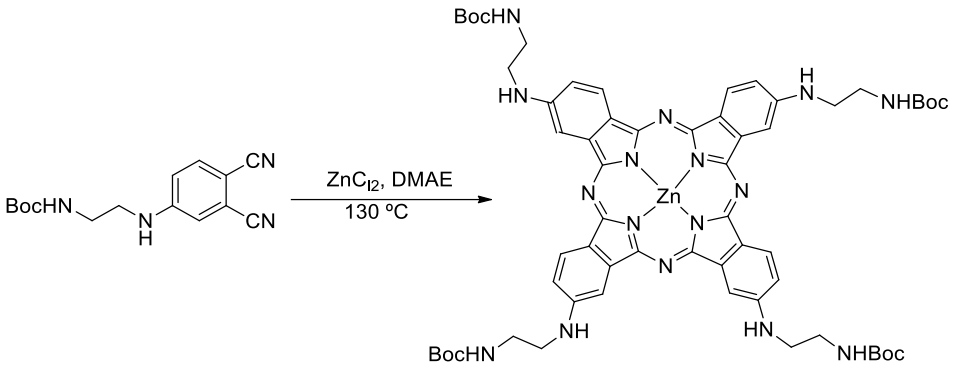


Figure 11- ^{13}C NMR (125.77 MHz) spectra of the phthalonitrile **12** in DMSO-d_6.

2.1.4. Synthesis of [tert-butyl(amino)carbamate]phthalocyaninato)zinc(II)

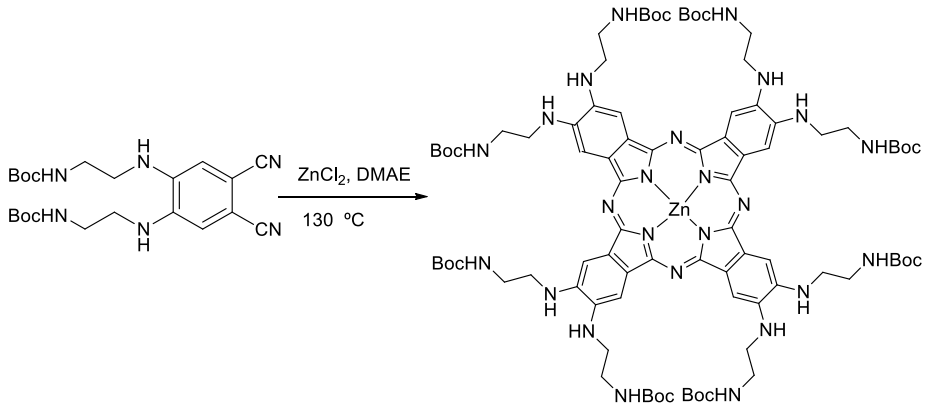
Pc **13** (Scheme 8) was synthesized by cyclotetramerization of phthalonitrile **11** (0.51 mmol) with anhydrous zinc chloride (0.30 mmol) in presence of DMAE (1 mL). The reaction was kept overnight stirring at 130 ºC. After this time the TLC revealed that the reaction was complete, the crude product was precipitated with hexane and ethyl acetate and filtered under vacuum. The filtrated was purified by column chromatography with silica gel as stationary phase and dichloromethane: methanol (8:2) as eluent.



13

Scheme 8

Pc **14** (Scheme 8) was obtained from the cyclotetramerization of phthalonitrile **12** (0.32 mmol) with anhydrous zinc chloride (0.26 mmol) in the presence of DMAE (1 mL). The reaction was kept overnight with stirring and under 130 ºC. After this time the TLC revealed that the reaction was complete. The crude product was precipitated with hexane and ethyl acetate and filtered under vacuum. The filtrated was purified by column chromatography with silica gel as stationary phase and dichloromethane: acetone as eluent. The phthalocyanine was obtained in 6% yield.



14

Scheme 9

The structure of Pc **14** was confirmed by the ^{1}H NMR. ^{1}H NMR spectrum showed three multiplets, one corresponding to the resonances of the α protons of the macrocycle at δ 8.96-8.99; δ 8.88-8.91; δ 8.55-8.76 other at δ 7.24-7.29; δ 6.41-6.96 corresponding to the aliphatic

protons and the other at δ 1.31-1.56 referring to the methyl protons of Boc group. The signal of protons in the alkyl groups was overlapped by the solvent signal. (Figure 12).

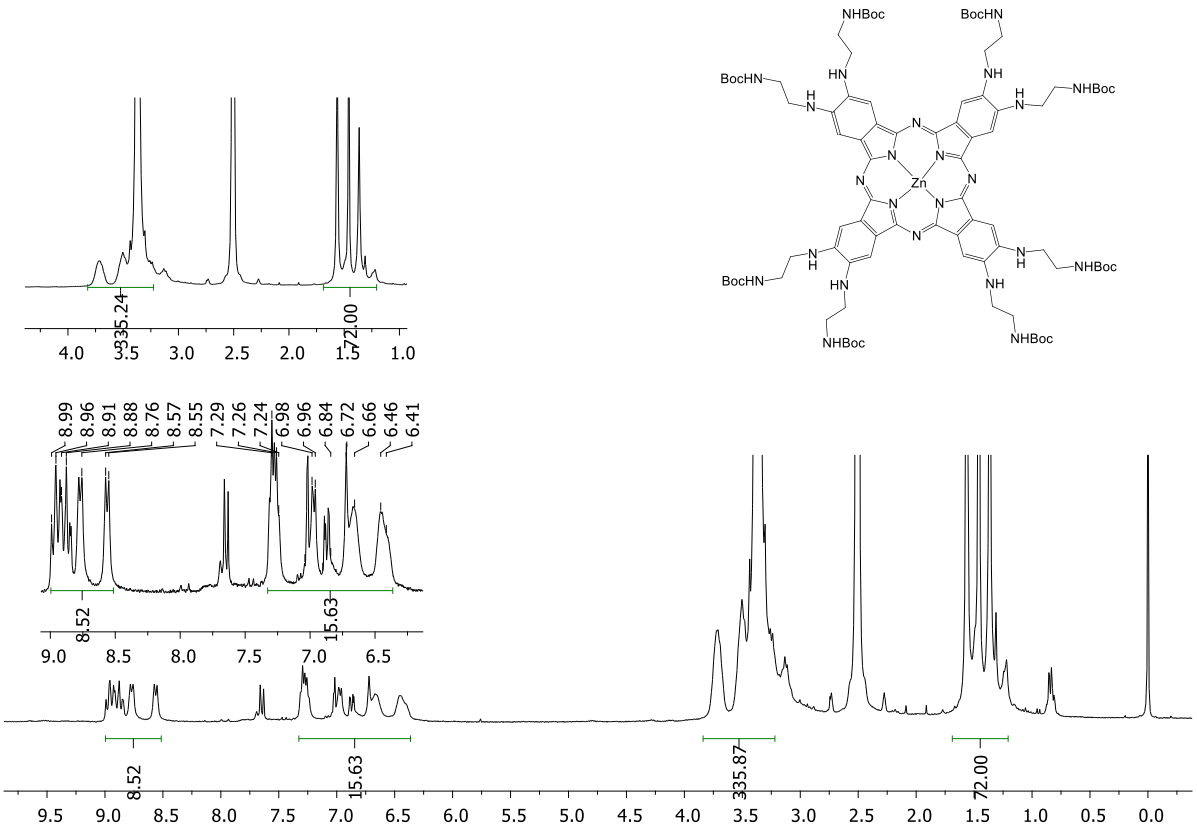
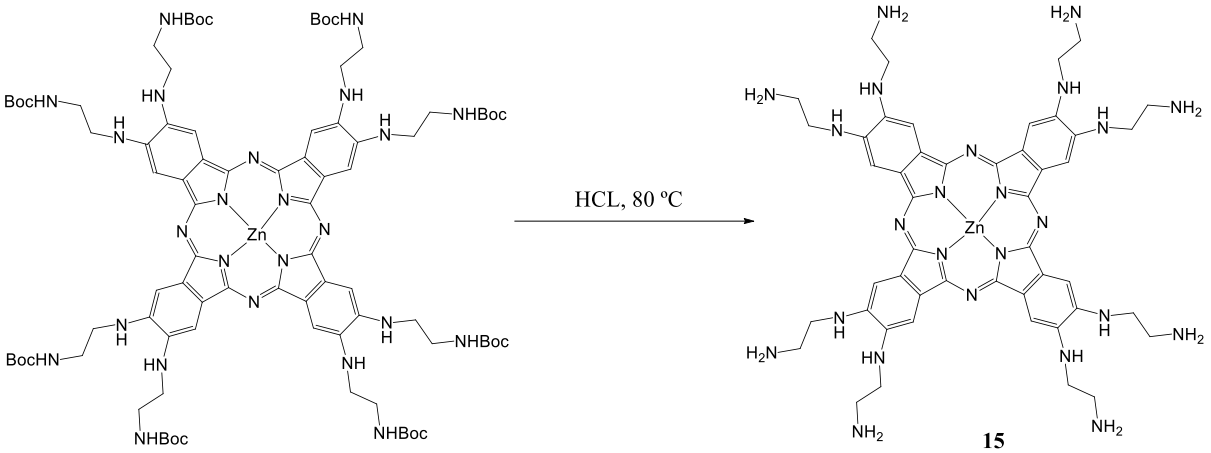


Figure 12-^{1}H NMR (300 MHz) spectra of the hthalocianine 1**4** in DMSO-d_6

After performing the synthesis of the phthalocyanine **14**, the following step was the hydrolyze of the Boc groups. For this, a reaction mixture of compound **14**, 1.35 mmol, and 1 mL of hydrochloride acid was kept at 80 ºC and stirring during 5 hours. The TLC revealed that the starting material was completely reacted and a new product was formed. The hydrochloride acid was removed by filtration and the filtrated was washed with acetone (Scheme 10).



scheme 10

2.2. Experimental procedures.

Tert-butyl(3-amino)ethyl)carbamatephthalonitrile (11): 4-fluorophthalonitrile (0.84 mmol), tert-butyl (2-aminoethyl)carbamate (1.3 mmol) and dry K_2CO_3 (1.08 mmol) was added in 3 mL of DMF. The mixture was kept with stirring under nitrogen atmosphere and at room temperature for 24 hours. After this time, distilled water was added to the reaction mixture and extracted with dichloromethane. The organic layer was evaporated under reduced pressure. The product was purified by column chromatography with silica as stationary phase and CH_2Cl_2-MeOH (9.8:0.2). The product was dried under vacuum. ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 7.7 (d, *J* =9, 1H), 7.3(t, *J* = 12, 1H, NH), 7.1 (d, *J*= 3, 1H, NH), 6.90-6.94 (m, 2H), 3.1 (dd, *J*=12 and 24 , 2H, CH_2), 3.2 (dd, *J*= 12 and 24, 2H, CH_2), 1.4 (s, 9H, CH_3). ^{13}C NMR (125.77 MHz, DMSO, ppm): δ 27.9, 41.7, 77.7, 97.5, 115.6, 116.5, 117.5, 134.7, 151.7, 155.7. ESI_MS calculated for $C_{15}H_{18}N_4O_2$: 286.33 found: 327.1 $[M+H]^+$.

Ditert-butyl(bis(ethane-2,1-diyl))dicarbamate phthalonitrile (12): 4,5-diflourophthalonitrile (0.61mmol), tert-butyl(2-aminoethyl)carbamate (1.33 mmol), K_2CO_3 (0.88 mmol) was dissolved in 3 mL of DMF. The reaction mixture was left with uniform stirring under nitrogen atmosphere and at room temperature, overnight. The completion of the reaction was monitored by TLC. Distilled water was added to the reaction mixture and extracted with dichloromethane. Residual DMF was removed with the addition of toluene. The compound was further purified by column chromatography over silica gel with dichloromethane: methanol (9.8:0.2). ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 7,9 (d, *J* =24, 2H), 7.4 (d, *J* =18, 2H), 7.2 (s, 2H, NH), 6.93 (t, *J* = 12, 2H, NH), 3.3 (m, 4H, CH_2), 3.1 (dd, *J*= 12 and 24, 4H, CH_2), 1.4 (m, 18H, CH_3), ^{13}C NMR (125.77 MHz, DMSO, ppm): δ 28.3, 42.0, 77.9, 97.7, 112.5, 115.0, 115.5, 116.5, 119.0, 119.3, 141.6, 149.3, 152.6, 155.6

2,3,9,10,16,17,23,24-octakis(ditert-butyl(bis(ethane-2,1-diyl))dicarbamate) phthalocyaninato)zinc(II) (**14**):

A reaction mixture of 0.32 mmol of phthalonitrile **11** and 0.26 mmol of anhydrous zinc chloride was kept under stirring and at 130 ºC in 3 mL of DMAE overnight. The reaction was stopped and the product was washed with hexane and precipitated with hexane and ethyl acetate. The product was purified by column chromatography over silica gel and dichloromethane: acetone

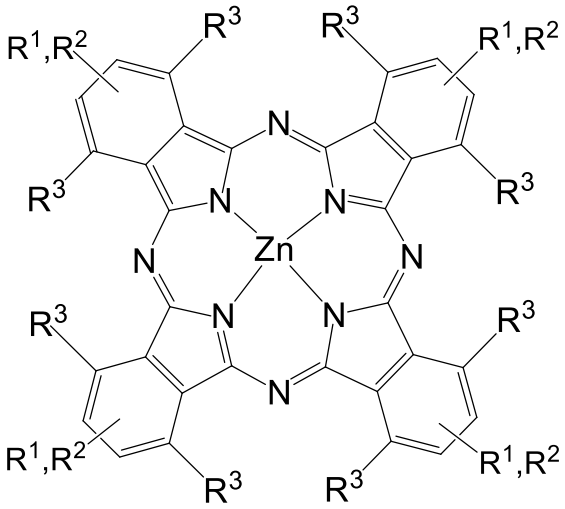
as eluent. ^{1}H NMR (300 MHz, DMSO, ppm): δ 8.96-8.96; 8.88-8.91; 8.88; 8.55-8.76 (m, 8H, β-H) δ 7.24-7.29; 6.41-6.98 (m, 15H, NH) δ 1.31-1.56 (m, 72H, CH_3) ^{13}C NMR (125.77 MHz, DMSO, ppm)

Chapter 3 – Photochemical, Photophysical and Photobiologycal studies

3.1. Experimental procedure of photophysical and photochemical studies

3.1.1. Photosensitizers

The photodynamic studies were carried out using six new cationic phthalocyanine derivatives (Figure 13). A stock solution for each compound was prepared at a concentration of 500 µM in DMSO. All phthalocyanines included in this study, namely those produced in previous work and for which the synthesis is not described, were synthesized by the Organic Chemistry group of University of Aveiro (QOPNA)



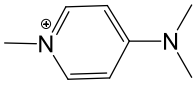
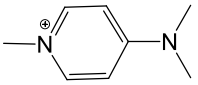
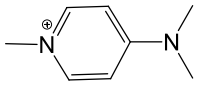
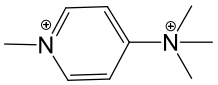
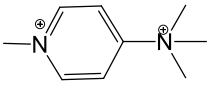
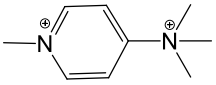
Pc	R^1	R^2	R^3
1		H	H
2		$R^2=R^1$	H
3		$R^2=R^1$	F
4		H	H
5		$R^2=R^1$	H
6		$R^2=R^1$	F

Figure 13 – Structure of photosensitizers used on the photodynamic studies

3.1.2. Fluorescence quantum yield

The fluorescence quantum yields of the six phthalocyanines in DMF were measured in 1 x 1 cm quartz optical cells under room temperature on a spectrofluorimeter Fluromax 3 (Horiba Jovin Yvon). The fluorescence quantum yield of each phthalocyanine were calculated by the comparison of the area of the emission bands by comparison of the area below the corrected emission spectrum of the ZnPc, used as fluorescence standard at λ_{exc}=630 nm with Φf= 0.2 in DMF. In all cases, the absorbance of samples was kept at 0.2 at 630 nm, excitation wavelength.[103] The fluorescence quantum yields were calculated according to the equation below (Equation1),

$$\Phi_f^{sample} = \Phi_f^{standard} \frac{AUC^{sample}\,(1 - 10^{\,Abs_{standard}})}{AUC^{standard}(1 - 10^{\,Abs_{sample}})}$$

Equation 1

in which the AUC is the integrated area under the fluorescence curves of the phthalocyanines and Abs the absorbance of sample and standard at excitation wavelength.

3.1.3. Solubility

The solubility of cationic phthalocyanines derivatives 1-6 in PBS and DMSO was assessed by UV-visible spectroscopy. Concentration, between 2.0 and 20 µM, obtained by addition of aliquots of each phthalocyanine. UV-visible spectra were determined 5 min after the addition. The intensity of the Q-band versus phthalocyanine concentration was plotted in a graphic for linear regression to verify the compliance with Beer-Lambert law.

3.1.4. Singlet oxygen generation

The ability of the Pc to generate singlet oxygen was assessed by the qualitative method of 1,3-diphenylisobensofuran (DPBF).

A stock solution of DPBF at 10.0 mM in DMF/Water (9:1) and stock solution of each phthalocyanine at 500 µM were used. The reaction mixture of 50 µM of DPBF and 0.5 µM of

each phthalocyanine in DMF/water (9:1) were irradiated with LED at a Fluency rate of 9.0 mW cm^{-2}, in glass cuvettes under magnetic stirring, at room temperature. The absorption decay of the DPBF at 414nm was measured at 3 minutes intervals during a total irradiation time of 15 min. The percentage of DPBF absorption decay, proportional to $^1O_2$ production, was assessed by the difference between the initial absorbance and the absorbance after the irradiation time.[104]

3.1.5. Photostability and stability

To study the photostability of the photosensitizers, each Pc was diluted in 2 mL of PBS to a concentration of 20 μM, and irradiated at same conditions used in the biological studies (150 mW cm^{-2}). The Pc were irradiated during 30 minutes under magnetic stirring at room temperature. The UV-visible spectra were collected at 5 minutes intervals during the irradiation in order to assess the the photostability of Pc. The photostability was assessed by monitoring the intensity of the Q band at the different time intervals. At same time, stability studies were carried out by irradiating the PS, protected from light by aluminium foil and under same conditions. The photostability and stability assays of each Pc were carried out at the same time and the percentage of decay was calculated according to I_t/I_0 (%), where I_t is the intensity of the band at a given time and I_0 is the intensity of the band before irradiation.

3.2. Photodynamic inactivation assays

3.2.1. Bacterial biological model

For the photodynamic inactivation assays a recombinant bioluminescent strain of *E. coli* was used, it was obtained from a previous work.[105] Before the assays a fresh liquid culture of the bacteria was prepared in medium containing the antibiotics, ampicillin (100 mg mL^{-1}) and chloramphenicol (25 mg mL^{-1}). To prepare the culture, the antibiotics were aseptically added to 30 mL of a Tryptic Soy Broth medium (TSB; Liofilchem), and one aliquot of 240 μL of culture

was inoculated. The culture was grown at 25 ºC for 20 hours.This culture was used for the asssays of photodynamic inactivation of planktonic cells.

3.2.2. Preparation of biofilms

For the preparation of the *E. coli* biofilms to be used in photodynamic inactivation, a fresh liquid culture of the recombinant *E.coli* was grown overnight in TSB medium with the antibiotics, ampicillin (100 mg mL^{-1}) and chloramphenicol (25 mg mL^{-1}), at 150 rpm and 26 ºC. An aliquot (1.0 mL) of this culture was aseptically transferred to 30 mL of a medium containing casamino acid (1.0%), yeast extract (0.12%) ampicillin (100 mg mL^{-1}) and chloramphenicol (25 mg mL^{-1}). Aliquots of 1.5 mL were distributed by eppendorf microtubes and incubated at 26 ºC. After 24 hours of incubation the medium was discarded and fresh medium was added to the biofilms which were further incubated for 4 days at 26 ºC.

3.2.3. Relation between bioluminescence and colony forming units

To establish the correlation between light emission, expressed in relative light units (RLU) and the concentration colony forming units (CFU), a culture of bioluminescent *E.coli* was grown overnight, and serially diluted (10^{-1} to 10^{-7}) in phosphate buffered saline (PBS) buffered. The luminescence was measured in each dilution and an aliquot of each dilution was sireally diluted and pour-plated in triplicate in Tryptic Soy Agar (TSA). The plates were incubated at 37 ºC during 24 hours and colonies were counted in the most suitable dilutions for the calculation of the concentration of colony forming units (CFU mL^{-1}) in the cell suspension.

3.2.4. Experimental set up for the photodynamic inactivation of planktonic cells

Bacterial cultures grown overnight were 10-fold diluted in PBS to a final concentration corresponding to 10^7 CFU $mL^{-1}$and kept under stirring at room temperature for 10 min. This bacterial suspension was equally distributed in 12-well plates and appropriate quantities of the three stock solutions of phthalocyanine derivatives (500 µmol.L^{-1}) were added to achieve a final concentration of 20 µM in a total volume of 4.5 mL of cell suspension. The samples were protect from light with aluminium foil and incubated for 15 min under stirring, at room temperature, to promote PS binding to the cells. Light and dark controls were included in the experiment. Light

control was irradiated without the phthalocyanine and the dark control, containing 20 µM was protected from light with aluminium foil during the course of the experiment. Three independent assays were carried out for each compound.

3.2.5. Experimental set up for the photodynamic inactivation of biofilms

The liquid medium in the tubes containing the biofilms was discarded and replaced by an equal volume of PBS, and the biofilms were allowed to stabilize for 10 minutes. After this time, the Pc solution was added and the suspensions were incubated for 15 minutes, in the dark to promote the PS adsorption. The biofilms immobilized in the microtubes were irradiated with red light. Light and dark controls were included in the experiments. Light and the dark controls, were included in the experiment. Three independent assays were carried out for each compound. In these experiments two different conditions were used, irradiation with 20 µM and 40 µM of Pc, during 30 and 60 minutes, respectively.

Irradiation conditions

For the irradiation of the samples with red light (620-750) or white light (400-800), light was delivered by an illumination system (LC-122-LumaCare, London) containing a Halogen/quartz 250 W lamp coupled to different interchanges optic fibber probes (620-750 nm and 400-800 nm).The light was delivered at a fluency rate of 150 mW cm^{-2}, measured with a potentiometer Coherent FieldMaxII-Top combined with a Coherent PowerSens PS19Q energy sensor. The samples were irradiated during 30 min under stirring and at room temperature for planktonic bacteria and 30 and 60 min without stirring for biofilm cultures.

Bioluminescence measurements

The inactivation kinetics was followed by measuring the bioluminescence of the bacteria during the irradiation time. Aliquots of the samples and controls were collected at times 0, 5, 10, 15, 20, 25 and 30 minutes for experiments with planktonic bacteria. Bioluminescence was read in triplicate in a luminometer (GLOMAX^{TM} 20/20 Luminometer). In the experiments with biofilms, bioluminescence was read in triplicate in the luminometer after 0, 5, 10, 15, 20, 25 in

the first set of experiments with the lowest Pc concentration and after 30 minutes and 0, 10, 20, 30, 40, 50 and 60 minutes for the experiments with the highest Pc concentration.

3.2.6. Adsorption of the photosensitizers to planktonic cells

A bacterial suspension (10^7 CFU mL^{-1}) was incubated in dark for 15 min in presence of 20 μM of the phthalocyanine derivatives (**1-6**), with magnetic stirring under room temperature for the binding of the PS to the cells. After this period, 1.0 mL of this suspension was centrifuged during 15 min at 13 G in order to separate the cells from the PS solution. The supernatant was rejected and the pellet was washed with 1.0 mL PBS and centrifuged again, in order to further remove unbonded PS. The cells were digested with 1.0 mL of a solution containing 2% of dodecyl sodium sulphate (SDS) and 0.1 M of NaOH and the mixture was kept in the dark at 4 ºC at least 24h until the clearance of the solution. The concentration of the PS in the digested extracts was determined by fluorescence. The samples were excited at 610 nm and emission spectra were collected in the 620-850 nm range. Fluorescence corresponding to the PS bound to the cells was calculated from the intensity of fluorescence by interpolation with a calibration plot build with known PS concentrations in the digestion solution.

3.3. Results

3.3.1. Fluorescence quantum yield

All the compounds emit fluorescence and the derivatives Pc**4** and Pc**5** have higher fluorescence quantum yield, followed by Pc**2,** Pc**1,** Pc**3** and Pc**6**. (Table 2)

Table 2 - Fluorescence quantum yields of compounds **1-6** in DMF using ZnPc at λext= 630 nm and Φf=0.2 nm**.**

Compound	Φf
Pc1	**0.01**
Pc2	**0.02**
Pc3	**0.005**
Pc4	**0.37**
Pc5	**0.39**
Pc6	**0.001**

3.3.2. Solubility

The solubility of the six phthalocyanine derivatives (**Pc 1-6**) in DMSO and PBS at different concentrations was monitored by UV-visible spectroscopy. The results show two intense bands, corresponding to the soret and Q-band of the Pcs (Figure 14 and 15). The graphs obtained by plotting the Q band intensity of each Pc versus concentration show, that all the six compounds presented a linear regression in DMSO (Figures 14), which indicates that in this solvent they follow the Beer-Lambert law. The studies conducted in PBS show that all the Pcs also present linear regression, at the tested concentrations (Figure 15).

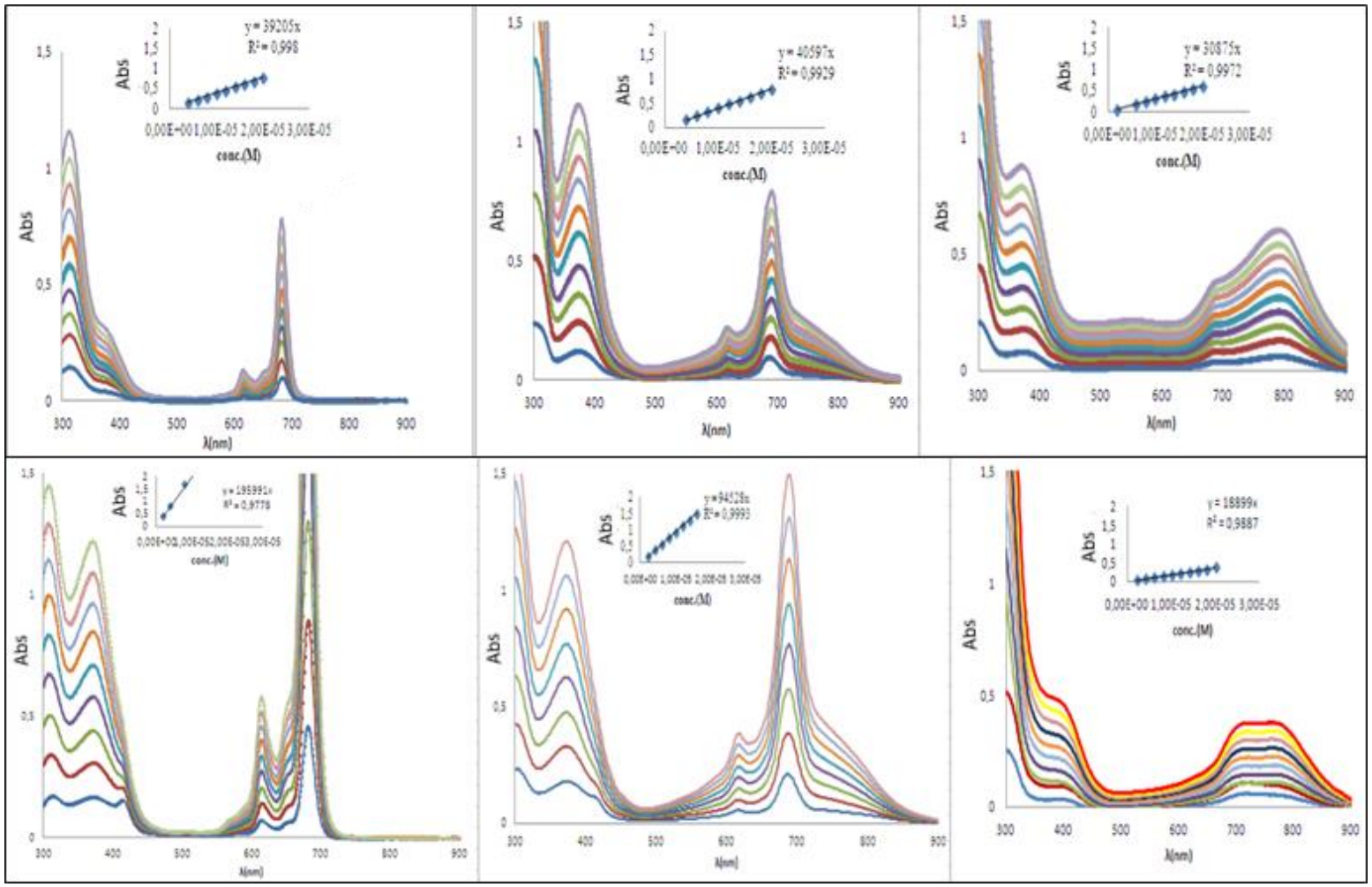


Figure 14 - UV-visible spectra of Pcs **1-6** at different concentrations in DMSO. The linear regression graphics represent the Q-band absorbance of each Pc versus the concentrations in M.

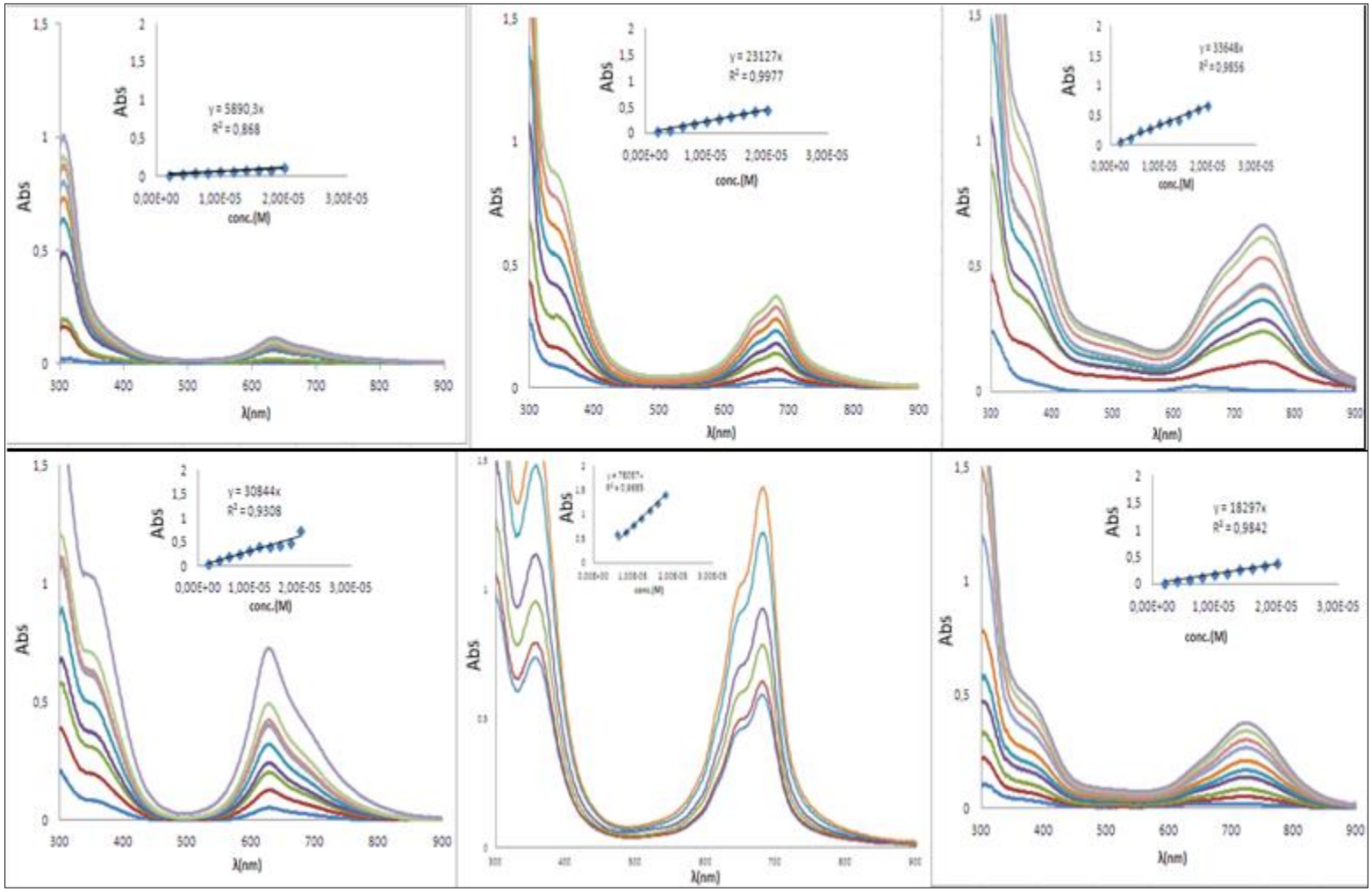


Figure 15 - UV-visible spectra of Pcs **1-6** at different concentrations in PBS. The linear regression graphics represent the Q-band absorbance of each versus the concentrations in M.

3.7.2. Singlet oxygen generation

The results obtained with the indirect method of 1,3-diphenylisobenzofuran show that all the phthalocyanine derivatives were able to generate singlet oxygen ($^1O_2$) causing the decay of absorbance of the DPBF. The cationic phthalocyanines presented better performance than the conjugated Pc, being the **Pc4** and **Pc5** the best $^1O_2$ generators with an absorbance decay of DPBF higher than 90% after 15 min of irradiation (Figure 16).

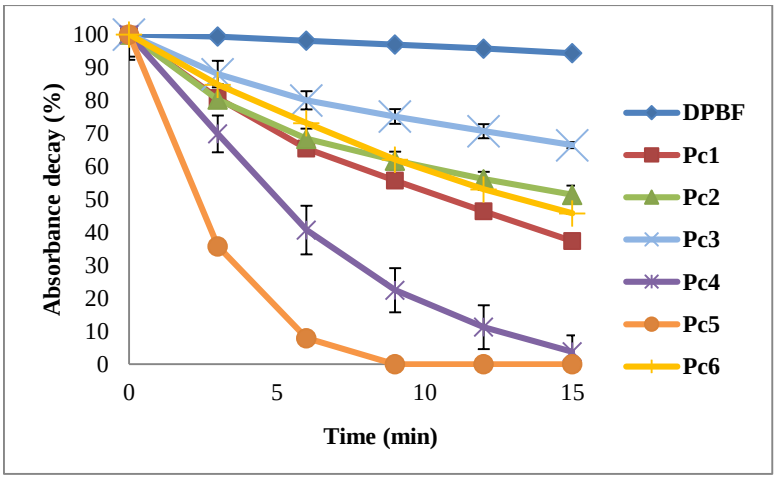


Figure 16 - Singlet Oxygen generation of Pcs **1-6** assessed by the indirect method of 1,3-diphenylisobenzofuran DPBF (50 µM) in DMF/H2O (9:1) upon irradiation with white light with LED, at flounce rate of 9.0 mW cm^2 with or without Pc (05µM).Values correspond to average of two independent experiments. Error bars represent standard deviation of the mean.

3.7.3. Photostability and stability

The photostability studies with red light revealed that, with the exception of the Pc **3, 5** and **6** all the others compounds, **1, 2** and **4** were photostable. Upon white light, the compounds showed to be photostable at the conditions that they were irradiated, however, the compound **5** revealed signs of photodegradation such as with red light. All the Pc were stable in absence of light. (Table 3)

Table 3 - Photostability and stability study of the Pcs **1-6** accessed by UV-visible spectroscopy after 30 minutes of irradiation under red and white light.

Compound	Phtotostability (%)		Stability (%)	
	Red light	**White light**	**Red light**	**White light**
Pc1	91.0	94.3	96.9	97.0
Pc2	85.7	93.1	100	100
Pc3	57.1	85.1	87.5	89.5
Pc4	93.4	88.5	100	93.9
Pc5	66.9	65.4	100	100
Pc6	69.5	95.5	98.48	98.3

3.7.4. Relation between bioluminescence and colony forming units

The relation between the bioluminescence and the concentration of viable cells of *E.coli*, expressed in CFU mL^{-1} is represented in figure 17. The results show a significant linear correlation (R^2=0.9414) between the light emission units and the colony counts.

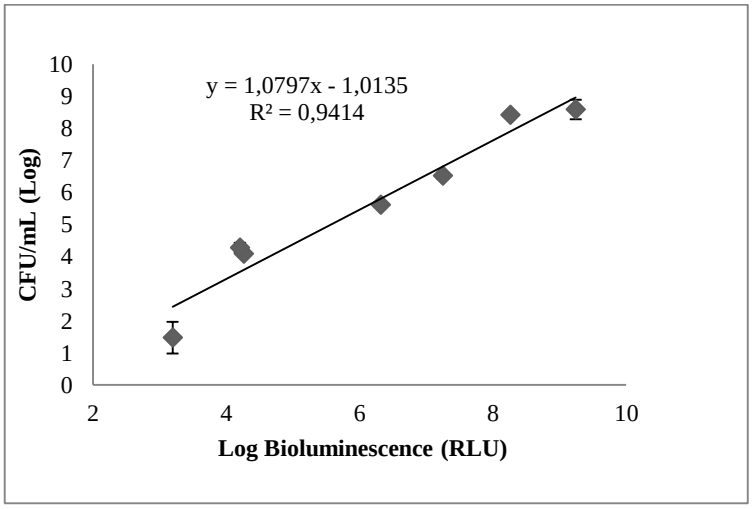


Figure 17 - Linear correlation between bioluminescence signal and colony counts of an overnight culture of bioluminescent *E. coli* serially diluted in PBS, with bioluminescence expressed in relative light units and colony counts expressed in CFU mL^{-1}

3.7.5. Photodynamic inactivation of planktonic cells of bioluminescent *E.coli*

The profiles of photodynamic inactivation of the bioluminescent *E.coli* strain using **Pc 1-6**, with red and white light are presented in Figures 18 and 19, respectively.

The results obtained with both lights show that the PS did not present dark toxicity against bacterial strain in presence of 20.0 µM Pc, since the bioluminescence remained constant in dark controls. Also, direct cytotoxic effect triggered by light in absence of the PS (light controls) were not detected, since there was not a significant variation in light emissions variation during the experiments. Under red light, Pc **4** and **5** were the most efficient PS, causing reductions of approximately 4.0 log in light emission. Inactivation with Pc **1** and **3** was very small and the light emission was not different from the controls. With Pc **6**, 2.0 log reduction in light emission was obtained. Upon irradiation with white light Pc **6** and **4** were more efficient in the photodynamic inactivation of the bacteria, causing a reduction of approximately 2.0 log followed by Pc **5** with a reductions of 1.5 log. Pc **1** and **3** did not significantly affect light emission. The comparison of both lights shows that with red light, a higher efficiency was achieved, particularty with Pc **4** and **5**. However, under white light, inactivation efficiency decreased for all Pc, with exception of Pc **6**, for which results were similar.

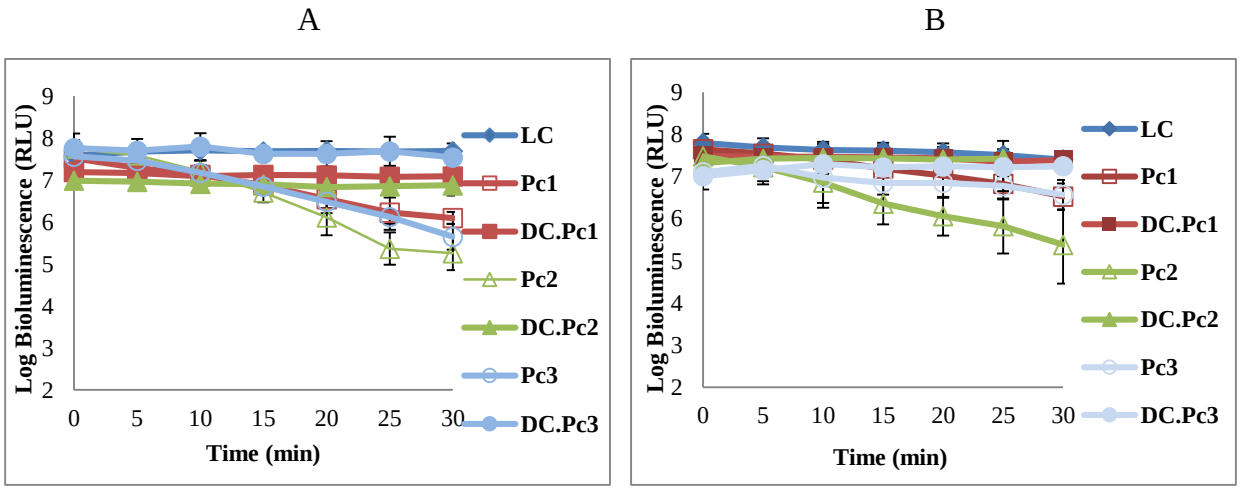


Figure 18- Photodynamic inactivation of bioluminescent *E. coli* with Pc**1-3** under red (A) and white (B) light at a fluency rate of 150 mW cm^{-2} in presence of 20 µM of each phthalocyanine. The error bars represent the standard deviation of three independent assays.

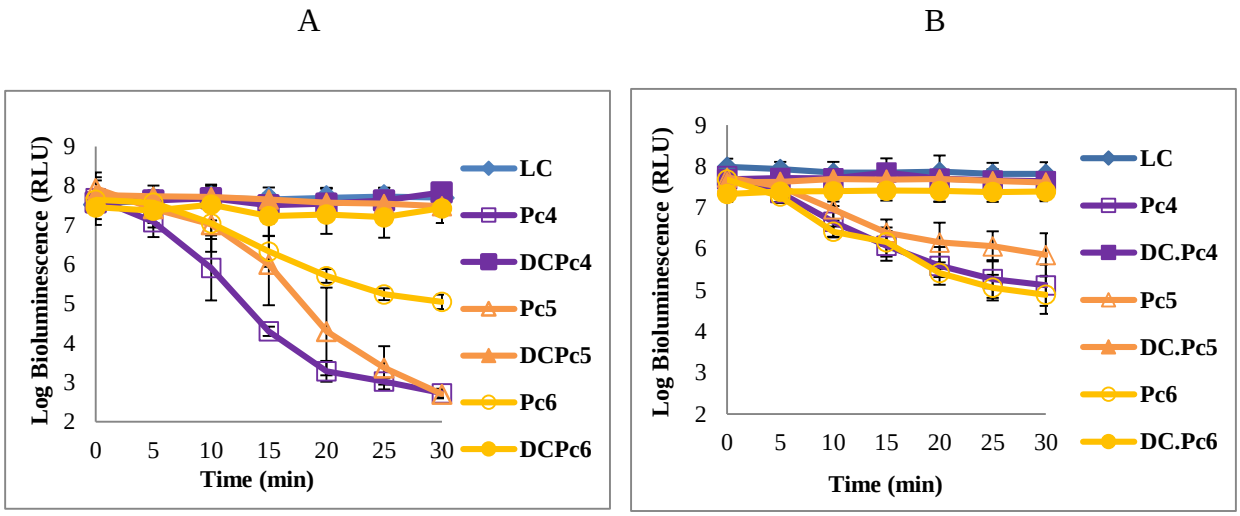


Figure 19- Photodynamic inactivation of bioluminescent *E. coli* with Pc **4-6** under red (A) and white (B) light at a fluence rate of 150 mW cm^{-2} presence of 20 µM of each phthalocyanine. The error bars represent standard deviation of three independent assays.

3.7.6. Photodynamic inactivation of biofilms of bioluminescent *E.coli*

The kinetics of photodynamic inactivation of biofilms by using the Pc **4** and **5** at a concentration of 20 µM and a fluency rate of 150 mW cm^{-2} under red light is represented in Figure 20-A . The bioluminescence of the biofilms was not affected neither during the irradiation in absence of the PS nor in dark with 20 µM of PS. Pc **4** was more efficient than Pc **5**, causing a total reduction in the light emission of 2.3 log during 30 minutes of irradiation. The photodynamic inactivation with Pc **5**, in the same conditions was less efficient with a decrease of only 1.8 log in light emission. During the first 15 minutes the redutions caused by the two Pc were similar, but after 20 minutes of irradiation in presence Pc **4** the inactivation proceeded at slightly higher rate than with Pc **5**. In the experiments conducted with higher PS concantration (40 µM, Figure 20-B), Pc **4** caused a reduction of 2.4 log in the biofilm light emission, and Pc **5** caused a lower redution (1.5 log). At this concentration there was still not dark toxicity related to any of the tested Pc.

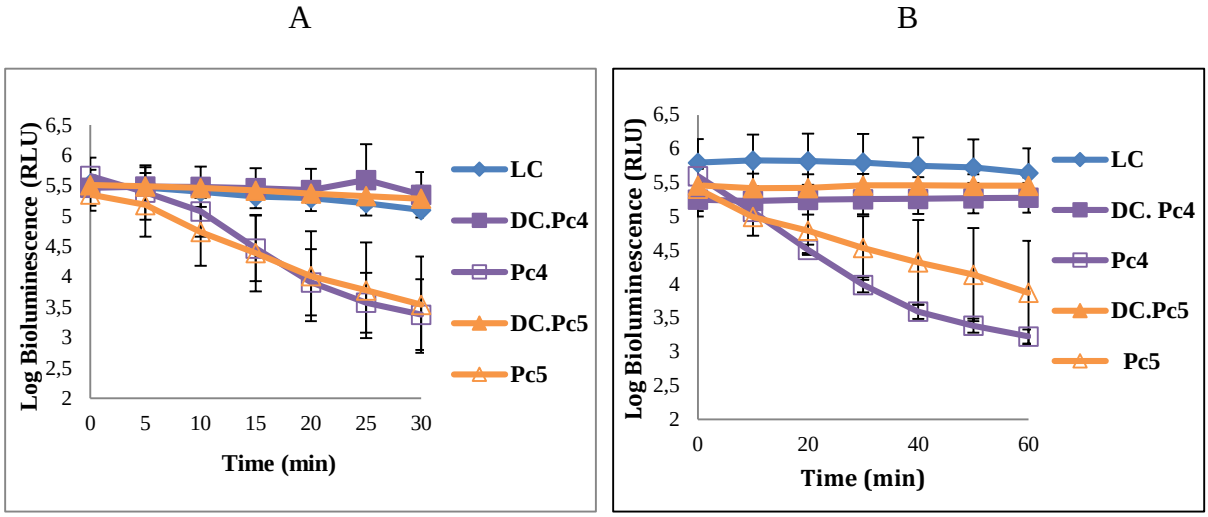


Figure 20- Photodynamic inactivation of bioluminescent *E. coli* biofilms with Pc **4** and **5** under red light at a fluency rate of 150 mw cm^{-2} presence of 20 µM (A) and 40 µM (B) of each phthalocyanine. The error bars represent the standard deviation of three independent assays.

3.7.7. Adsorption of the photosensitizers to planktonic cells

The results of the adsorption of each phthalocyanine derivative to *E. coli* cells after incubation in dark for 15 minutes are represented in Figure 21. Pc demonstrated highest affinity to the bacteria cells, with average uptake value of $1.11x10^{9}$ molecules (PS) CFU^{-1}. Pc **3**, **4** and **5** showed the lowest uptakes values ranging from $3.64x10^{6}$ to $7.26x10^{6}$ molecules (PS) CFU^{-1}.

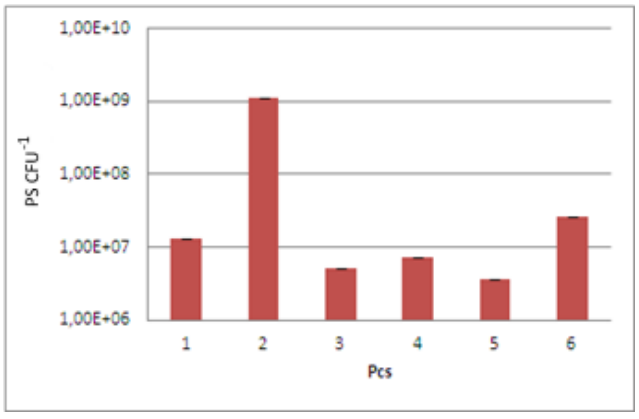


Figure 21 - Uptake of the phthalocyanines **1-6** in the bioluminescent in presence of 20 µmol L^{-1} *E.coli* after 15min of incubation in dark. Error bars represent the standard deviation.

Chapter 4- Discussion and Conclusion

4.1. Discussion

Considering the natural and the acquired resistance of microorganisms to the antimicrobial agents, particularly critical in the case of biofilms, there is an increasing interest on PDI as an alternative anti-microbial approach. In this work we aimed the design of new cationic phthalocyanines and the exploration of their potential as photosensitizers for the photodynamic inactivation of microorganisms, either in planktonic or in biofilm forms.

Phthalocyanines bearing amino and alkyl groups have been widely investigated due to the interesting electronic properties conferred by these groups. In this work we tried to obtain different alkylamino Pc by following different synthesis strategies. However, in our first attempt of introducing thiocysteamine groups by nucleophilic substitutions, either in phthalonitriles or in the octa-chlorophtalocyaninatozinc(II), we could not obtain the desired compounds. The compounds were not soluble in most regular solvents, most probably due the presence of the NH_2 groups. In a new approach we could overcome the solubility problem by using a protected amino substituent, obtaining compounds with higher solubility. The synthesis method used to obtain phthalonitriles was very efficient resulting in a high yield of production for both phthalonitriles. From these phthalonitriles we were able to develop the conjugated Pc, although with low yield due to impurities in the compounds.

The results of the biological assays conducted with the different phthalocyanines demonstrate that the molecules differ in the photosensitization efficiency of the bioluminescent *E.coli*. Only two of the six studied Pc, **4** and **5,** revealed potential for application in the PDI, at least under red light, since they caused significant inactivation of the *E.coli* survival (4 log reductions). Under white light, the reductions were generally smaller, being Pc **4** and **6** the most efficient in in the later conditions.

The better performance of Pc **4** and **5** against a gram-negative model bacterium can be related to the physico-chemical properties of the molecules, namely in terms of solubility in aqueous medium and capacity to generate $^1O_2$. These two Pcs showed to be the most efficient $^1O_2$ generators, causing a decrease of over 90% in DPBF absorbance. The capacity to produce $^1O_2$ must be interpreted in the light of structure of the molecule and of other photophysical features. Pc **3** and **6** present broad Q-bands in DMSO, but in PBS the Q-bands are broader for all six Pc.

The variation of the Q-bands suggests that almost all Pc have monomeric behaviour in DMSO (**1, 2, 4** and **5**) and that all the Pc have non-monomeric behaviour in PBS. This tendency to form non-monomeric structures can affect the capacity of the Pc **1, 2, 3** and **6** to generate $^1O_2$, since these structures tend to dissipate the energy of the excited states, thus compromising the PDI efficiency.[106] Although, Pc **4** and **5** also have tendency to form non-monomeric structures in PBS, comparatively to the other Pc their Q-band is much intense, making them good singlet oxygen producers. The higher tendency of Pc **3** and **6** to form non-monomeric structures either in DMSO or in PBS, is due to the fluorine atoms in the macrocycles, leading to the formation of planar molecules with tendency to stack.

The photosensitization potential of the different Pc may also be related with the number of charges the molecules. The results indicate that an increasing in the number of charges lead to an increase in the PDI efficiency, since Pc **1**, **2**, **3**, tetra and octa-cationic Pcs, were less efficient than their conjugates, **4**, **5**, **6**, octa- and hexadeca-cationic Pc. A relation between the number of charges and the photosensitization efficiency was reported with pyrrolidine-fused chlorins and isobacteriochlorines.[107] However in another study comparing the inactivation efficiency of three cationic thio-pyridinium phthalocyanines showed that a higher number of positive charges had no effect on the PS efficiency.[104]

The affinity of the PS by the cells can also play an important role in the PDI process, since for the photodynamic inactivation to occur, the PS must be close enough to the cellular target so that singlet oxygen may act within a very short time interval. However in this study we could not demonstrate a consistent relation between the amount of PS adsorbed and the efficiency of photodynamic inactivation. The most efficient PSs in terms of PDI did not correspond to the molecules with highest affinity to bacterial cells. Pc **2** showed a high uptake value but this can be an artefact due to its low solubility in the PBS medium and possibility of aggregation and precipitation on the call surface. Other studies, however, show that the antimicrobial PDI is not dependent on surface-bond PS but on permeation of the cell membrane by unbounded PS molecules.[108]

The comparison of the inactivation efficiency with white and red light reveals Pc **4** and **5** as particularly active under red light. The performance of Pc **1, 2, 3** and **6** did not change significantly the light wavelength range. The better results of Pc **5** with red light are likely due

to the higher overlapping of Q-band with the emission spectrum of the light source (Figure 22). The adjustment of the wavelength of the delivered light with the absorption spectrum of the PS is a major determinant of the outcome of the photodynamic effect. The same PS may considerably change in photodynamic inactivation efficiency depending on the light source, even if all other photodynamic parameters, namely PS concentration and fluency rate remain constant.[12,104] The Q-band of Pc **4** shows higher overlapping with white light, however, inactivation was more efficient with red light. This is probably associated to the amount of photons adsorbed by the PS under red light, i.e., although the overlapping area is smaller the absorption rate of photons emitted by red light is enough to cause a higher photodynamic inactivation of the bacteria.

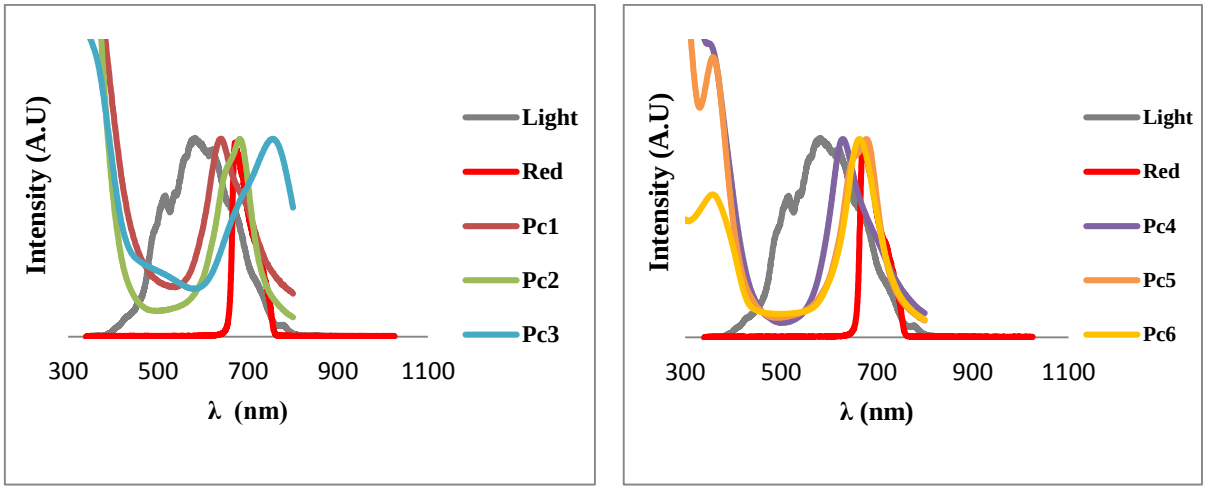


Figure 22 - Normalized UV-Vis spectra of Pc **1-6** in PBS and white and red light source emission.

One of the objectives of this study was to compare the inactivation efficiency of the photosensitizer against planktonic cells and biofilms. After doing the PDI studies with bioluminescent *E.coli* in the planktonic for and the selection of the most promising PS, the inactivation profile against bioluminescent *E.coli* in biofilm form was tested in similar irradiation conditions. Experiments with 20 µM of Pc achieved modest inactivation of approximately 2 log. In a second set of experiments, the PS concentration was doublet to 40 µM and the irradiation time was extended to 1 hour in the attempt of improving the inactivation efficiency. However, these modifications were not successful. This can be due to PS aggregation

observed when the PS concentration is high, causing a decrease in it molar absorbance at the wavelength absorption maximum.[109]

Planktonic cells are more susceptible to photosensitization with Pc **4** and **5** than the biofilms. Beside the stronger reductions observed in the planktonic cell, we also notice that the 2 log reduction achieved in the biofilms experiments was achieved after 30 minutes of irradiation, while the same reduction was reached after 10 and 15 minutes of irradiation (Pc **4** and **5** respectively) in the planktonic cells. Our results are consistent with others that observed higher reductions in the viability of the planktonic *Staphylococcus aureus* and *Pseudomonas aruginosa* than in the biofilm cells.[87,88] Biofilms are imbedded in an extracellular polymeric matrix and the chemical composition of the matrix depending on the charge or hydrophobicity, may decrease or increase the binding of the PS to the cells.[110] In the case of *E.coli* biofilms, the extracellular matrix acted more like a barrier to the penetration of the PS than as trap for the PS making biofilm cells less susceptible to photosensitization. Biofilms present higher cell density comparing to planktonic cells. This may also explain the different results obtained in this study, since it is known that by increasing the cell number the amount of PS that bound to the cells will decrease.[6] The interpretation of the results of the photodynamic inactivation of a bioluminescent biofilm must also take into consideration the conditions in which is produced in order to ensure that the reduction observed in the biofilms bioluminescence reflects a reduction in the number of viable cells. In future studies, the inactivation must be assessed by a non-luminescent endpoint, such as plating and colony counting colonies.

4.2. Conclusion

In conclusion, new amino-phthalonitriles and their corresponding multi-amino Pc were synthesized, although not all could be fully characterized. The need of alternative treatments against bacterial resistance has led to find new approaches such as photodynamic inactivation of microorganisms. This strategy appears as an interesting alternative for the treatment of bacterial infections. For this purpose, new photosensitizers have been designed with improved physico-chemical properties that increase the efficiency and the possibility to inactivate a wide

range of microorganisms, and microbial biofilms. Pc **4** and **5** were efficient singlet oxygen producers and were soluble in organic and aqueous mediums while Pc **1**, **2, 3** and **6** produced less singlet oxygen generators and were soluble in aqueous medium. This study demonstrates that the nature and number of substituents can strongly affect the singlet oxygen generation and solubility of the PS, and that the presence of fluorine groups in the macrocycle can diminish the photosensitizing effect of the PS.

Biofilms still represent a challenge to photodynamic inactivation. However PDI experiment with the planktonic cells shows that two of the tested Pc may be promising photosensitizers for Gram-negative bacteria. The efficiency of the photodynamic inactivation process is determined by a combination of attributes like singlet oxygen, solubility in aqueous medium, wavelength absorption and also in the adjustment of the light to the absorption spectrum of the Pc. Pc **4** and **5** may be considered promising photosensitizers to treat infections caused by Gram-negative *E.coli*, but for complete inactivation of biofilms infection repeated treatments may be required.

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