Universidade de Aveiro Departamento de Biologia
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Patrícia da Silva Santos

Cationic porphyrins in the photoinactivation of viruses in blood

Porfirinas catiónicas na fotoinativação de vírus no sangue

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e Doutora Ana Teresa Peixoto de Campos Gomes, Investigadora do Departamento de Biologia.

O júri

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Palavras-chave Vírus; Transfusão; Sangue; Plasma; Terapia fotodinâmica antimicrobiana; Porfirinas catiónicas; Azul de metileno; Eritrócitos.

Resumo

Nos últimos anos, além da implementação de sistemas de hemovigilância em muitos países, a aplicação de métodos de desinfeção de sangue tem sido utilizada para melhorar a qualidade e segurança das transfusões. No entanto, infeções transmitidas por transfusão de sangue ainda ocorrem. O desenvolvimento de novos métodos para tratar, não apenas o plasma e as plaquetas, mas também o sangue total e os concentrados de eritrócitos, pode ser uma medida importante para diminuir a incidência dessas infeções. Atualmente, técnicas convencionais de desinfeção são usadas apenas para plasma devido aos danos colaterais que estas causam nas frações celulares. A terapia fotodinâmica antimicrobiana (aPDT) representa uma alternativa aos métodos convencionais, mesmo para o sangue total e concentrados de eritrócitos. A aPDT envolve a exposição de um fotosensibilizador (PS) à luz na presença de oxigénio, o que resulta na produção de espécies reativas de oxigénio (ROS) que causam danos irreversíveis nos microrganismos patogénicos. Esta terapia já está aprovada em alguns países, mas é limitada ao uso de três PSs, o azul de metileno (**MB**) para a desinfeção de plasma e a riboflavina e o psoraleno para a desinfeção de plasma e plaquetas.

O objetivo deste estudo foi avaliar o efeito da aPDT usando PSs porfirínicos catiónicos (**Tri-Py(+)-Me**, **Tetra-Py(+)-Me** e **Tetra-S-Py(+)-Me**) na fotoinativação de vírus no plasma e sangue total e os resultados foram comparados com a eficiência de um PS já aprovado para desinfetar plasma, o **MB**. Os possíveis efeitos colaterais da aPDT nos eritrócitos também foram avaliados através de testes de fragilidade osmótica da membrana citoplasmática dos eritrócitos usando concentrações crescentes de NaCl e da contagem de eritrócitos antes e após o tratamento com aPDT. O bacteriófago T4 foi utilizado como modelo de vírus que infetam mamíferos. Para tal, uma suspensão fágica de 10^8 PFU/mL em plasma e sangue total foi exposta à luz branca (150 mW/cm^2) durante 270 minutos com uma concentração de PS de 10 μM.

Os resultados indicaram que os PSs porfirínicos foram mais eficazes que o **MB** na fotoinativação do fago T4 no plasma, especialmente a **Tetra-S-Py(+)-Me**. No entanto, a sua eficiência diminuiu no sangue total, possivelmente devido aos efeitos bloqueadores da aPDT causados pela complexidade da matriz. Nenhum dos PSs testados promoveu stress osmótico e subsequente hemólise nos eritrócitos na condição isotónica. Assim, os derivados porfirínicos, principalmente a **Tetra-S-Py(+)-Me**, podem ser considerados PSs promissores para fotoinativar vírus no plasma, mas são necessárias melhorias para o uso da aPDT em sangue total.

Keywords Viruses; Transfusion; Blood; Plasma; Antimicrobial photodynamic therapy; Cationic porphyrins; Methylene blue; Erythrocytes.

Abstract

In the last years, besides the implementation of haemovigilance systems in many countries, the application of blood disinfection methods has been used in order to improve transfusion quality and safety. However, infections transmitted through blood transfusion still occur. The development of new methods to treat not only plasma and platelets, but also the whole blood and erythrocytes concentrates, can be an important measure to decrease the incidence of blood transfusion infections. Conventional disinfection techniques are currently in use essentially for plasma due to the collateral damage in cellular fractions. Antimicrobial photodynamic therapy (aPDT) represents an alternative to the conventional methods even for the whole blood and erythrocytes concentrates. aPDT involves the exposure of a photosensitizer (PS) to light in the presence of oxygen, which results in the production of reactive oxygen species (ROS) that causes irreversible damage in the pathogenic microorganisms. This therapy is already approved in some countries, but is limited to the use of three PSs, methylene blue (**MB**) for plasma disinfection and riboflavin and psoralen for plasma and platelet disinfection.

The aim of this study was to evaluate the aPDT effect using cationic porphyrinic PSs (**Tri-Py(+)-Me**, **Tetra-Py(+)-Me** and **Tetra-S-Py(+)-Me**) in the photoinactivation of viruses in plasma and whole blood and the results were compared with the efficiency of an already approved PS to disinfect plasma, the **MB**. Possible side effects of aPDT on erythrocytes were also assessed by osmotic fragility tests of erythrocytes cytoplasmic membrane using increasing NaCl concentrations and erythrocytes count before and after aPDT treatment. The T4 bacteriophage was used as a model of mammalian viruses. For this purpose, a phage suspension of 10^8 PFU/mL in plasma and whole blood was exposed to white light (150 mW/cm^2) for 270 minutes with a PS concentration of 10 μM.

The results indicated that porphyrinic PSs were more effective than **MB** in the photoinactivation of T4 phage in plasma, with special emphasis of **Tetra-S-Py(+)-Me**. However, their efficiency decreased in the whole blood, possibly due to the aPDT blocking effects caused by the matrix complexity. None of the PSs tested caused osmotic stress and subsequent haemolysis in the erythrocytes at the isotonic condition. Therefore, porphyrinic derivatives, mainly the **Tetra-S-Py(+)-Me**, can be considered a promising PSs to photoinactivate viruses in plasma, but further improvements are required for aPDT use in whole blood.

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Chapter 1 [General introduction]

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
aPDT	Antimicrobial Photodynamic Therapy
CFU	Colony Forming Units
DMSO	Dimethyl sulfoxide
FDA	Food and Drug Administration
MB	Methylene blue
PBS	Phosphate-Buffered Saline
PFU	Plaque Forming Units
PS	Photosensitizer
ROS	Reactive Oxygen Species
rpm	Rotations per minute
SD method	Solvent-Detergent method
Tetra-Py(+)-Me	5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide
Tetra-S-Py(+)-Me	5,10,15,20-tetrakis[2,3,5,6-tetrafluoro-4-(1-methylpyridinium-4-ylsulfanyl)phenyl]porphyrin tetra-iodide
Tri-Py(+)-Me	5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TTIs	Transfusion Transmissible Infections

THESIS OUTLINE

The main goal of this study was to evaluate the aPDT effect in the photoinactivation of viruses in plasma and whole blood using cationic porphyrinic PSs (**Tri-Py(+)-Me**, **Tetra-Py(+)-Me** and **Tetra-S-Py(+)-Me**) and their efficiency was compared with an approved PS to disinfect plasma, the **MB**. Possible side effects of aPDT on erythrocytes were also evaluated.

The present document is structured in two chapters, which are summarized as follows:

Chapter 1 [General introduction] includes a literature review on blood and blood products disinfection methods focusing on the antimicrobial photodynamic therapy.

Chapter 2 [Viruses inactivation in blood by photodynamic treatment with tetrapyrrolic photosensitizers] consists on the brief introduction to the topic, describes the experimental work, the results obtained and their discussion as well as the main conclusions and future perspectives.

Appendix

[An insight into the synthesis of cationic porphyrin-imidazole derivatives and their photoinactivation efficiency against *Escherichia coli*] is an additional work performed in a collaboration with the Organic Chemistry group of Department of Chemistry (UA) whose main objective was to evaluate the effectiveness of novel porphyrin-imidazole compounds in the photoinactivation of bioluminescent *Escherichia coli* in Phosphate-Buffered Saline (PBS) and their combined effect with potassium iodide. I performed all the biological assessments and the discussion of the biological results of this study. I also participated in the preparation of the manuscript which is in a final phase of submission.

CHAPTER 1

General introduction

1.1 Blood

Blood is an indispensable tissue and has vital functions in human body, such as regulation and protection of organism, transport of substances, among other functions. The blood is composed of figurative elements (red blood cells, leukocytes and platelets) which are suspended in plasma (extracellular matrix) and all the blood cells develop from precursor cells mainly produced in the bone marrow. Plasma is separated from the blood figurative elements by the centrifugation process (Fig. 1). Red blood cells contain haemoglobin and have a function of storage and transport of oxygen to tissues. Leukocytes are crucial in destruction of foreign products that contact with the organism and the platelets mainly act in the mechanism of blood clotting. Plasma includes several proteins, clotting factors and metabolic products in suspension (WHO, 2002).

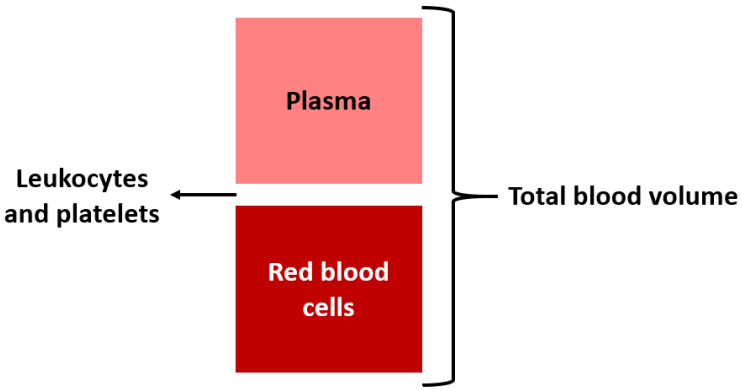

Figure 1 – Blood constitution after centrifugation. Adapted from World Health Organization, [2002].

1.1.1 Blood donations and transfusions

In the blood donation process, whole blood is collected into a sterile and disposable plastic bag that contains an anticoagulant solution (usually with sodium citrate, phosphate, dextrose and adenine, whose functions are represented in the Table 1) (WHO, 2002).

Table 1 – Functions of anticoagulant solution. Retrieved from World Health Organization, [2002].

Solutions		Functions
C	Sodium citrate	Binds with calcium ions in blood in exchange for the sodium salt so the blood does not clot
P	Phosphate	Supports metabolism of the red cells during storage to ensure they release oxygen readily at tissue level
D	Dextrose	Maintains the red cell membrane to increase storage life
A	Adenine	Provides energy source

Whole blood transfusion is used in haemorrhagic cases, because supplies red blood cells, stable coagulation factors and volume (WHO, 2002). However, whole blood properties are only preserved for a limited period and numerous changes occurs after five days of storage. So, although whole blood transfusions are still used, processed blood transfusions are more common currently (Hardwick, 2008).

Thus, whole blood may be processed in different products (red blood cells, platelets and plasma) and can be transfused according to the needs of each patient (Ben-Hur and Goodrich, 2011; Marciel et al., 2017). Consequently, it is possible to maximize the use of one donation, because several patients are beneficiated from a single donation (Hardwick, 2008). The plasma and platelets can also be obtained by apheresis, a process that allows collect these constituents directly from the donor and not by whole blood processing (WHO, 2002).

In accordance with World Health Organization (2016) report, 112,5 million blood donations were collected globally during reporting period and only 11,9 million were apheresis donations while 100,6 million were whole blood donations. However, the level of blood availability for transfusion is not homogeneous in the world because it varies greatly with the region (Fig. 2). Most donations are collected in high and upper middle-income countries in contrast with low and lower middle-income countries. The same pattern occurs with blood processing (Fig. 3), because in developing countries just 50-59% of blood donations are processed while in the developed countries the values are 93-96%.

The different blood components are stored under specific conditions that to ensure its properties (Hardwick, 2008). Red blood cells are used in hypoxia cases caused by bleeding after trauma, surgery or anaemia and can be stored at 2-6 °C for 35 days in preservation solutions (Wainwright, 2002; Hardwick, 2008; Liumbruno et al., 2009; Marciel et al., 2017). The limit of 6 °C is useful to prevent the bacterial contamination in the blood and the limit of 2 °C is crucial to avoid freezing of red blood cells (WHO, 2002). Whole blood has similar storage conditions to red blood cells (WHO, 2002). Platelets are suitable for patients with platelet dysfunction or in the treatment of bleeding disorders originated by thrombocytopenia and can be stored for 5 days at 22 °C to preserve platelet function (WHO, 2002; Shander and Goodnough, 2007; Wainwright et al., 2007; Hardwick, 2008; Marciel et al., 2017). Plasma is indicated to the treatment of coagulopathies or in autoimmune disorders and has a shelf life of 5 years if stored at -18 °C, so it is called fresh frozen plasma (Wainwright, 2000; Wainwright, 2002; Hardwick, 2008; Marciel et al., 2017). This temperature is used to preserve clotting factors (WHO, 2002). In relation to leucocytes, this blood components are not transfused because there is a high risk of containing transfusion transmissible infections (TTIs). Thus, all blood components undergo a leucodepletion phase to eliminate leukocytes (WHO, 2002; Hardwick, 2008).

The quality of transfusion medicine is enhanced by an important haemovigilance system implemented in many countries around the world. Haemovigilance is a tool for investigation and evaluation of the possible side effects after a transfusion, aiming improving transfusion quality and safety. In addition, it also creates measures to prevent some of these events from occurring (Politis et al., 2014). In accordance with Portuguese Institute of Blood and Transplantation, portuguese hemovigilance system is active since 2008 after the publication of the law decree 267/2007-07-24 (Sousa, 2014).

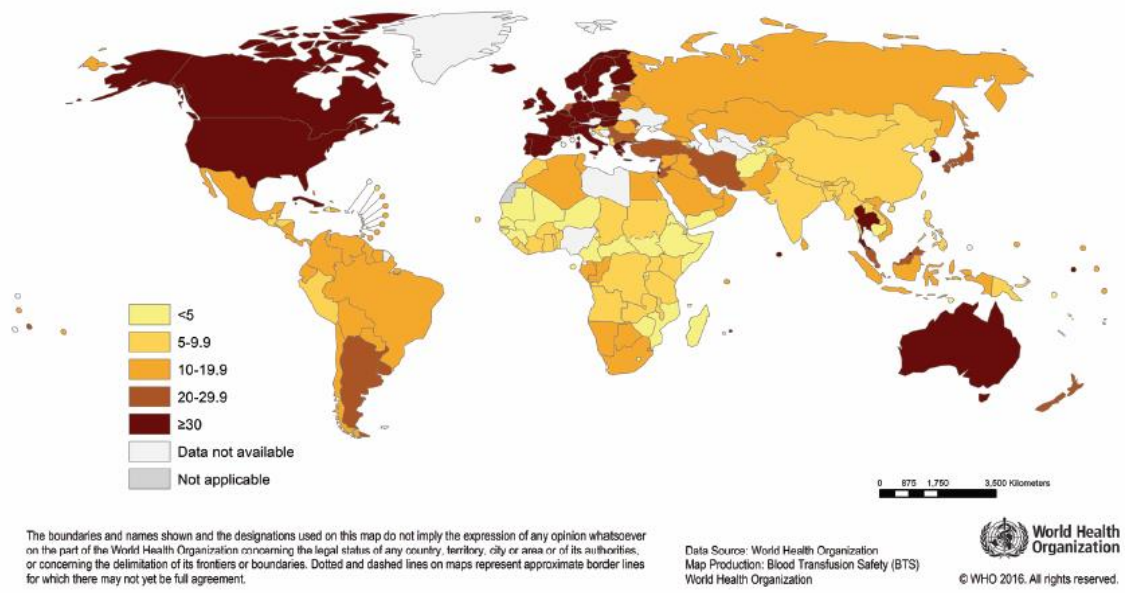

Figure 2 – Global map of whole blood donations per 1000 population. Retrieved from World Health Organization, [2016].

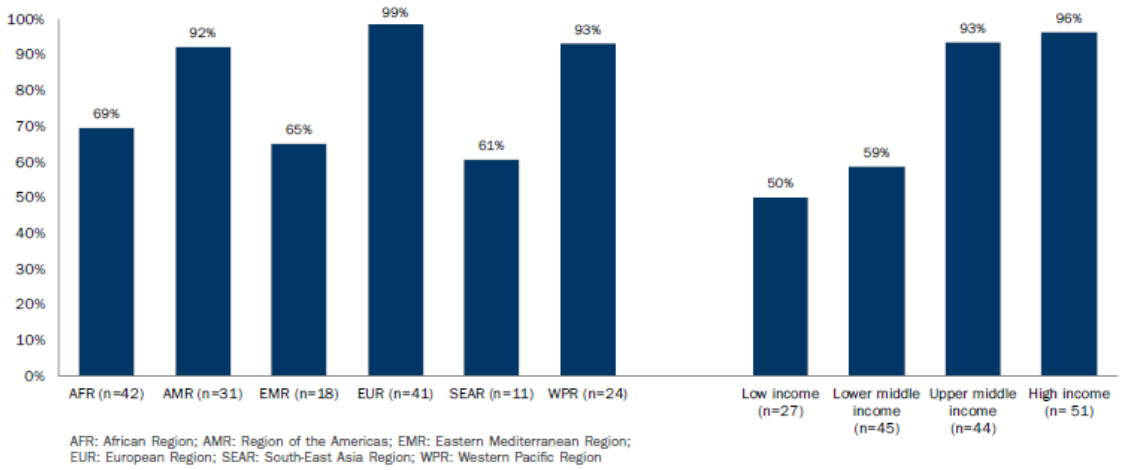

Figure 3 – Percentage of whole blood donations processed into components in different regions. Retrieved from World Health Organization, [2016].

1.1.2 Transfusion transmissible infections (TTIs)

In accordance with World Health Organization (2002), all donated blood units should be tested to TTIs according to the national legislation and the incidence of infections in the donor population. Furthermore, the blood units should not be transfused until all tests required by national standards are negative. In the Europe and North America are realized screening procedures to test the presence of Human Immunodeficiency Virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and syphilis (Salunkhe et al., 2015). Nowadays, some of the procedures used to ensure the safety of transfusions are specific serological and nucleic acid tests, donor screening and transfusion haemovigilance (Di Minno et al., 2016; Marciel et al., 2017). However, despite the safety and efficacy of donated blood products being the main concern of medical community, the emerging infectious diseases remain to be a danger to the blood supply and still occur (Salunkhe et al., 2015; Di Minno et al., 2016; Marciel et al., 2017; Dean et al., 2018). The "immunological window" intensifies this problem, because it refers to the period where pathogenic agents are not detectable but are present (WHO, 2002; Seghatchian and Sousa, 2006; Bihl et al., 2007; Marciel et al., 2017). So, during this period it can be difficult to understand whether the patient is developing infection.

In accordance with Food and Drug Administration (FDA), TTI can be defined as a pathogen that is potentially transmissible through the blood supply and causes severe damage in the organism and may even be fatal (Dean et al., 2018). Many infectious agents can be transmitted through transfusion (Table 2), like bacteria, viruses, fungi or other parasites and may cause diseases mainly in immunosuppressed patient (Di Minno et al., 2016). In the European Union, 111 TTIs were reported between 2010 and 2013, of which 66% bacterial, 32% viral and about 3% parasites (Domanović et al., 2017).

The contamination can be endogenous (donor's blood) or exogenous, because may occur in the blood collection process or in the handling of blood products (Bihl et al., 2007; Salunkhe et al., 2015). For example, the contamination may come from Gram-positive bacteria of skin at the incision site, like *Staphylococcus aureus*, which may proliferate in platelet concentrates due to the storage temperature (WHO, 2002; Brecher and Hay, 2005; Salunkhe et al., 2015).

The microorganism location depends on the fraction of blood, because in red blood cells and platelets the pathogens can be in suspension or associated with cells and in plasma it may only be in suspension (Wainwright, 2002; Marciel et al., 2017). Plasma has a high infection rate, as a result of plasma pooling from diverse donors and 1 in 5,000 platelets can be contaminated due to their storage temperature that is promising for bacterial growth but is required to keep the platelet function (Wainwright, 2002; Eder et al., 2007; Marciel et al., 2017; Dean et al., 2018). Red blood cells have a lower infection rate because the storage temperature is lower (WHO, 2002; Bihl et al., 2007).

Table 2 – Some of the possible blood transfusion transmissible pathogens and their geographic distribution.

MICROORGANISMS	GEOGRAPHIC AREA	REFERENCES
Viruses		
HIV	Widespread in the world	Bihl et al., 2007; Di Minno et al., 2016
Hepatitis A virus	Widespread in the world	Casteel et al., 2004
Hepatitis B virus	Widespread in the world	Bihl et al., 2007; Di Minno et al., 2016
Hepatitis C virus	Widespread in the world	Bihl et al., 2007; Di Minno et al., 2016
West Nile virus	Africa, West Asia, Middle East, Europe and some parts of America	Ludlam et al., 2006; Di Minno et al., 2016; Udvardy, 2018
Cytomegalovirus	Widespread in the world	Di Minno et al., 2016
Bacteria		
Treponema pallidum	Widespread in the world	Di Minno et al., 2016
Staphylococcus aureus	Widespread in the world	Brecher and Hay, 2005; Udvardy, 2018
Escherichia coli	Widespread in the world	Brecher and Hay, 2005; Udvardy, 2018
***Pseudomonas* spp.**	Widespread in the world	Brecher and Hay, 2005; Udvardy, 2018
Protozoa		
Plasmodium falciparum	Endemic in Africa regions	Bihl et al., 2007; Wairimu et al., 2016; Udvardy, 2018
Trypanosoma cruzi	Endemic in some parts of Africa and Central and South America	Bihl et al., 2007; Di Minno et al., 2016; Udvardy, 2018
Babesia microti	Widespread, but predominantly in United States	Di Minno et al., 2016; Udvardy, 2018
Fungi		
***Candida* spp.**	Widespread in the world	Kett et al., 2011; Aubron et al., 2017

The risk of this transmission depends on the blood product and geographical location where occurs the transfusion, because TTIs are more frequent in less developed countries (Table 3) (Salunkhe et al., 2015; WHO, 2016).

Table 3 – Proportion of blood donations with positive results on screening tests to TTIs more common (by World bank income group). Retrieved from World Health Organization, [2016].

Income group	Proportion of blood donations with positive/reactive results (median and interquartile range, %)			
	HIV	HBV	HCV	Syphilis
High	0.003 (<0.001–0.04)	0.03 (0.008–0.18)	0.02 (0.003–0.16)	0.05 (0.005–0.26)
Upper middle	0.08 (0.006–0.2)	0.39 (0.16–0.69)	0.21 (0.05–0.42)	0.31 (0.12–1.07)
Lower middle	0.20 (0.05–0.44)	1.60 (0.94–4.13)	0.40 (0.19–1.50)	0.58 (0.18–1.47)
Low	1.08 (0.56–2.69)	3.70 (3.34–8.47)	1.03 (0.67–1.80)	0.90 (0.31–1.88)

In Portugal, the residual risk for hepatitis B virus is 0.2 per 100,000 donations (in contrast to a residual risk of 390 per 100,000 donations in Congo) and 0.03 per 100,000 donations for hepatitis C virus (Koch and Araújo, 2013; Di Minno et al., 2016).

Thus, considering the risk associated with blood supply, it is necessary to develop strategies to mitigate this problem and increase the safety of blood supply in the world, mainly in the less developed countries.

1.1.3 Blood products disinfection techniques

Blood products disinfection techniques aim to eliminate microorganisms in the blood to avoid a possible transmission of pathogens for the transfusion receptor and should ensure the blood products viability (Bihl et al., 2007; Dean et al., 2018). The main criteria that define blood products disinfection techniques were described in 2003 by Epstein and Vostal, so, these treatments should target a wide range of microorganisms and safeguard the viability of blood and the safety of the receptor (Epstein and Vostal, 2003; Salunkhe et al., 2015). In 2013, Seghatchian and Putter supplemented these criteria by adding observations on the cost-effectiveness and accessibility of treatment (Seghatchian and Putter, 2013; Salunkhe et al., 2015). However, approved conventional blood disinfection techniques are mainly intended for plasma due to the damage that can cause in cellular fractions, such as platelets and especially erythrocytes (Wainwright, 2002; Casteel et al., 2004; Salunkhe et al., 2015; Marciel et al., 2017). Furthermore, the most of these procedures were mainly developed for viruses, mostly for enveloped viruses (Wainwright, 2002; Casteel et al., 2004; Marciel et al., 2017). Nevertheless, these treatments may not be equally effective against all microorganisms, for instance, enveloped viruses are more easily inactivated than non-enveloped viruses (such as hepatitis A virus or picornavirus) (Schneider et al., 2004; Klein, 2005; Di Minno et al., 2016).

In relation to plasma, heat treatments may be used, such as wet heat treatment (pasteurization), which consists in subjecting the plasma to temperatures of 60 °C for 10 hours or dry heat treatment, that is based on sterilization at 100 °C for 1 hour (Seghatchian and Allain, 2001; Salunkhe et al., 2015). Heat treatments have been in use since 1948 and have already inactivated HIV, hepatitis B virus and hepatitis C virus (Salunkhe et al., 2015). However, these techniques are not effective against all microorganisms since non-enveloped viruses are not easily destroyed and can cause damage to the plasma proteins (Burnouf and Radosevich, 2000; Seghatchian and Allain, 2001; Salunkhe et al., 2015; Marciel et al., 2017).

Nanofiltration and chromatographic techniques can also be used for the plasma disinfection. Nanofiltration is based on the different particle sizes and uses filters with pores of similar size to the viral particles (Salunkhe et al., 2015). However, high molecular weight proteins may hinder the removal of smaller particles (Wainwright, 2002; Marciel et al., 2017). Chromatographic techniques use specific antibodies adsorbed, but these antibodies may bind non-specifically to the cell membranes, hence, this technique cannot be used in platelets and red blood cells (Wainwright, 2002; Marciel et al., 2017).

In some European countries and USA, there is a conventional blood disinfection technique which is mostly used for plasma disinfection, the solvent-detergent method (SD method) (Bihl et al., 2007; Salunkhe et al., 2015). SD method was approved by the FDA in 2013 (Dean et al., 2018). This technique consists in combination of an organic solvent (1% tri-*n*-butyl phosphate) with a detergent (1% Triton X-100) and this treatment is applied for 1.5-4 hours at 30 °C. After this period, these chemicals must be removed to avoid potential damage to plasma proteins (Burnouf and Radosevich, 2000; Wainwright, 2002; Salunkhe et al., 2015; Marciel et al., 2017). SD method allows breaking the lipid membrane of enveloped virus and thus preventing replication and compromising their integrity. Yet, this technique is not effective against all microorganisms such as non-enveloped viruses and may affect clotting factors (Hellstern, 2004; Bihl et al., 2007; Salunkhe et al., 2015; Dean et al., 2018).

However, in addition to conventional techniques, alternative methods for blood products disinfection are required, such as antimicrobial photodynamic therapy (aPDT) (see section 1.2.5, page 15) (Marciel et al., 2017). So, photoinactivation by methylene blue (**MB**) in the presence of visible light is used in plasma disinfection (Bihl et al., 2007; Salunkhe et al., 2015). **MB**-light method is based on the photodynamic inactivation and it was produced for clinical use in 1992 (Lambrecht et al., 1991; Seghatchian et al., 2011). This technique can inactivate enveloped viruses but is less efficient in the non-enveloped viruses or in protozoa (Salunkhe et al., 2015). Besides that, **MB** may interact with clotting factors, however, no relevant side effects were detected in patients transfused with **MB**-treated plasma (Bryant and Klein, 2007; Salunkhe et al., 2015).

Regarding to platelets, treatment with ultraviolet (UV) light is used and this method is applied in plasma and platelet disinfection. The irradiation causes damage to the microbial genetic material that impairing replication (Wainwright, 2002; Di Minno et al., 2016; Marciel et al., 2017). As in the **MB**-light method, combination of irradiation UV with photochemicals, such as riboflavin and amotosalen (a synthetic psoralen), is also utilized (see section 1.2.5, page 15) (Marschner and Goodrich, 2011; Salunkhe et al., 2015; Di Minno et al., 2016). Riboflavin or vitamin B2 is a naturally photochemical which is added to plasma or platelets concentrates. In the UV (UVB) light presence, this compound generates free radicals that break down the genetic material, leading to the irreversible damage in the pathogen (Salunkhe et al., 2015). Although these radicals can be harmful to plasma proteins or platelets, the advantage of this technique is that riboflavin is considered safe by the FDA, because it may be present in natural foods or even in blood (Bryant and Klein, 2007; Marschner and Goodrich, 2011; Salunkhe et al., 2015). This method is already applied in many countries and was approved in 2008 (Schlenke, 2014; Salunkhe et al., 2015). Amotosalen, a synthetic psoralen, creates an irreversible crosslinking in nucleic acid chains of the pathogen in the presence of UV (UVA) light, preventing their replication (Hanson, 1992; Bihl et al., 2007; Salunkhe et al., 2015). This method was approved in 2014 by the FDA for plasma and

platelets disinfection in USA but has been used in Europe since 2006 (Schlenke, 2014; Salunkhe et al., 2015; Dean et al., 2018).

In Portugal, the first blood disinfection technique was SD method which is directed to plasma. Later, the amotosalen with UVA light (directed to plasma and platelets) was first used in Lisbon Regional Blood Centre and started being applied in different hospitals in the country in 2010 (Sousa, 2014).

In relation to red blood cells and whole blood, there is a great difficulty in developing pathogen elimination treatments due to the complexity and sensitivity of the matrix, so, there are no approved treatments for erythrocytes and whole blood. The technique that seems most promising is S-303, which is still under study. S-303 is a positively charged synthetic alkylating agent which could disrupt the pathogen genetic material. Glutathione is also added to mitigate protein damage that may be caused by interactions of S-303 with other blood proteins (Benjamin et al., 2005; Bihl et al., 2007; Salunkhe et al., 2015; Cancelas et al., 2017).

In summary, the blood products disinfection techniques more used in the world are described in Table 4. Therefore, there is still a long way to go in this area, and despite advances, further developments in blood disinfection for transfusion are needed. Thus, this work will focus on aPDT for the blood disinfection.

Table 4 – Blood products disinfection techniques more used in the different blood components. Adapted from Salunkhe et al. [2015].

BLOOD COMPONENT	BLOOD PRODUCTS DISINFECTION TECHNIQUES
Plasma	• SD-method • **MB**-light • Riboflavin-UVB • Amotosalen-UVA
Platelets	• Riboflavin-UVB • Amotosalen-UVA
Red blood cells	• S-303 (in study)

1.2 Antimicrobial photodynamic therapy (aPDT)

Photodynamic therapy was discovered in 1900 by Oscar Raab in a study on the toxicity of dye acridine in *Paramecium* spp. (Raab, 1900; Cieplik et al., 2018). Later, this therapy was first applied by Tappeiner and Jesionek in a study with skin cancer patients (Jesionek and von Tappeiner, 1905; Wainwright, 1998; Kashef et al., 2017; Cieplik et al., 2018). So, this therapy was first used in cancer cases and demonstrated efficacy in the treatment of several tumours. Photodynamic therapy is based on the exposure of a photosensitizer (PS) to light of an appropriate wavelength and, consequently, produces reactive oxygen species (ROS) that generate damage in the pathogen (Kashef et al., 2017; Cieplik et al., 2018).

Later, this technique was applied in the treatment of infectious diseases (antimicrobial photodynamic therapy - aPDT), emerging as an alternative to antibiotics to combat antimicrobial resistance and is currently used in some countries for blood products sterilization.

The antibiotic era started with the discovery of penicillin in 1929 and lasted for many years due to success in controlling bacterial infections. However, there has been an increasing rate of microbial resistance, specifically bacterial resistance to antibiotics and viruses to antivirals, derived from their excessive and inappropriate use in medicine, veterinary and food production. In addition, the mechanism of action is directed to a specific target (Nakonieczna et al., 2018). Furthermore, this resistance is spread among the microorganisms through mechanisms such as mutations and horizontal gene transfer (conjugation, transformation or transduction) (Andersson and Hughes, 2010). Mutations can occur in microorganisms and may confer advantages over an antibiotic. In addition, bacteria multiply rapidly and this characteristic is disseminated in the bacterial population, which contributes to the increase of resistance (Jori et al., 2006; Tavares et al., 2010; Mesquita et al., 2018). Conjugation is based on the cell to cell transmission of genetic material, transformation refers to the acquisition of exogenous genetic material and transduction is a bacteriophage-mediated process (Andersson and Hughes, 2010). Some of the microorganisms that have developed resistance are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and some species of the *Enterobacteriaceae* family, such as *Escherichia coli* (Bassetti et al., 2013). Antimicrobial resistance leads to a constant need to introduce new antibiotics into the pharmaceutical market. However, this resistance is spreading faster than the introduction of new drugs into the market, which further aggravates this problem (Ling et al., 2015). Therefore, antibiotic resistance is a worldwide growing problem, both in medicine and in other areas, resulting in incurable infections, deaths, and higher health costs. Currently, most infections are caused by multiresistant microorganisms, which do not allow treatment only with the use of antibiotics (Davies and Davies, 2010). Although the resistance is more associated with bacteria, it is also frequent in viruses, fungi or other microorganisms. For example, Cytomegalovirus has already acquired resistance to some antivirals, such as ganciclovir (Strasfeld and Chou, 2010). An example for fungi is the *Candida krusei*, that is resistant to fluconazole (Wiederhold, 2017).

Therefore, the major advantage of aPDT in relation to conventional antimicrobials is the formation of ROS, which acts on nonspecific molecular targets of the microbial structure, such as proteins, lipids and nucleic acids, whereas antibiotics acts on specific and unique cellular targets. Thus, the probability of the treated microorganism developing resistance is improbable. Several studies have proved that the different microorganisms such as *Actinobacillus actinomycetemcomitans*, *Peptostreptococcus micros*, *Vibrio fischeri* and *Escherichia coli* did not develop resistance to this therapy after several cycles of photodynamic inactivation (Lauro et al., 2002; Pedigo et al., 2009; Tavares et al., 2010; Costa et al., 2011; Cieplik et al., 2018). Relatively to viruses, there are few studies about the potential development of resistance after aPDT, but in accordance with Costa et al., 2011, this therapy can efficiently inactivate viruses studied without development of resistance after ten cycles of aPDT.

On the other hand, aPDT has already proved to inactivate multidrug resistant strains (Cieplik et al., 2018; Nakonieczna et al., 2018). In addition, aPDT can act on bacteria, viruses, fungi and other parasites (Wainwright, 1998; Alves et al., 2009).

Overall, aPDT has a broad spectrum of action, low probability of mutagenicity, independent efficacy of antibiotic resistance profile, allows repetitive application, among other aspects (Jori et al., 2006; Kashef et al., 2017).

In relation to PS administration, this is injected intravenously in cancer cases, but in infectious diseases treatment, PS can be applied locally in affected area by topic application to the skin, aerosols or interstitial injection (Hamblin and Hasan, 2004; Almeida et al., 2011; Kashef et al., 2017).

1.2.1 Mechanisms of action

The principle of aPDT is the interaction of three components, a photosensitising agent, oxygen and light (Fig. 4). The combined action of these components results in the production of ROS that generates biomolecular damage, leading to microbial death (Melo et al., 2013; Alves et al., 2015; Marciel et al., 2017).

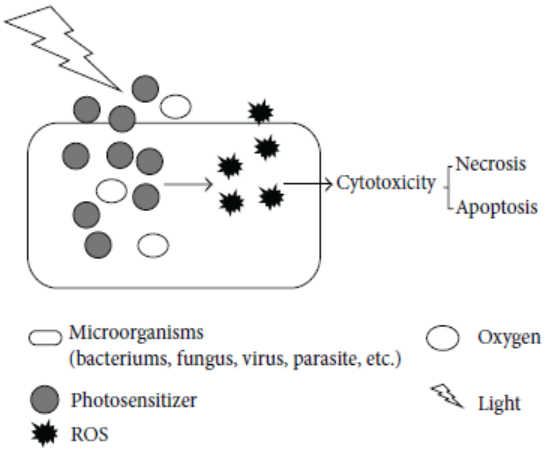

Figure 4 – Schematic representation of the aPDT mechanism. Retrieved from Fu et al. [2013].

The mechanism of action is based on the ability of the PS to absorb energy from light and transfer it to oxygen or to a substrate (Fig. 5). The PS is stable in the dark because its electronic configuration is in the ground state. However, when PS is irradiated at the appropriate wavelength, the absorption of photons makes it unstable (the lifetime of this state diverges from 10^{-9} to 10^{-6} s). So, the excited PS may decay to the ground state or can continue to receive light and attained the excited triplet state, which has a longer lifetime (10^{-3} a 10 s). At this stage, PS can also reach the ground state or react in two different ways that will define the type of mechanism by which aPDT will act. In the type I mechanism, PS triggers the formation of free radicals through electron transfer to substrates, whereas in the type II mechanism, PS acts by energy transfer to the molecular oxygen, leading to the singlet oxygen formation (Schmidt-Erfurth and Hasan, 2000; Via and Magno, 2001; DeRosa and Crutchley, 2002; Jori and Brown, 2004; Costa et al., 2012).

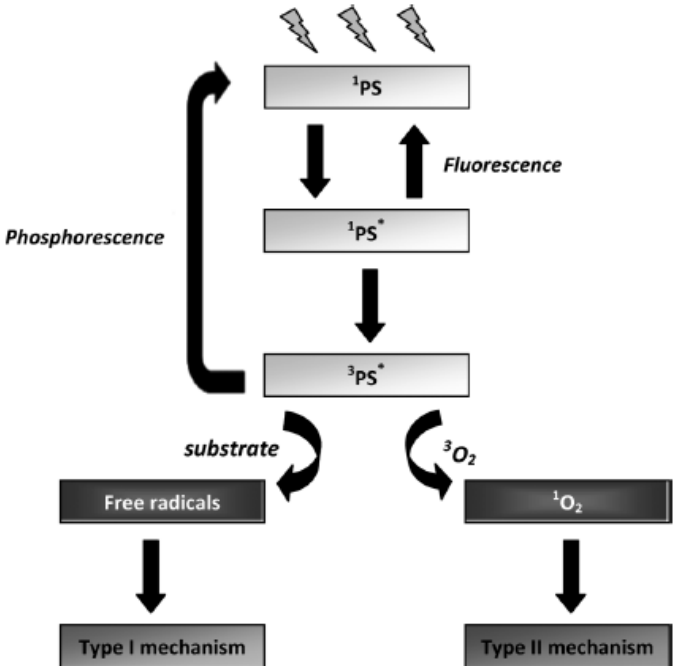

Figure 5 – aPDT process diagram. Retrieved from Costa et al. [2012], adapted from Wainwright, [1998].

Type I mechanism starts with chemical reactions between hydrogen bound to the substrate and PS (Fig. 6). In this reaction, one of the hydrogen atoms remains connected to substrate and another one associates to PS (Equation 1). This type of mechanism can also start with electron transfer between the excited PS and a substrate, producing free radicals (Equation 2). These radicals can react with oxygen to form ROS, such as superoxide radical (Equation 3). This radical, when protonated, is capable to produce hydrogen peroxide and oxygen (Equations 4 and 5) or hydroxyl radicals (Equations 6, 7 and 8) (Bonnett, 2000; Costa et al., 2012).

In relation to type II mechanism, molecular oxygen ($^3O_2$) receives the excess of energy from excited triplet state of the PS ($^3PS^*$), that returns to its ground state (Fig. 6). This reaction produces excited singlet oxygen ($^1O_2$) (Equation 1) that interacts with several enzymes and biomolecules and generates oxidized products (Equation 2) that are involved in the microbial death (Bonnett, 2000; DeRosa and Crutchley, 2002; Calin and Parasca, 2009; Costa et al., 2012). However, although singlet oxygen to be highly reactive with proteins, it has a very short lifetime, which decreases its diffusion distance (Fu et al., 2013).

Type I mechanism

$$SubstrateH_2 + PS \rightarrow PSH^{\bullet} + SubstrateH^{\bullet} \quad (1)$$

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

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

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

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

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

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

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

Type II mechanism

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

$$Biomolecules + {}^1O_2 \rightarrow oxidative\ products \quad (2)$$

Figure 6 – Chemical reactions that occur in type I and type II mechanisms of the aPDT. Retrieved from Costa et al. [2012].

These mechanisms can occur simultaneously or exclusively, depending on the PS used and substrate and oxygen concentrations. Thus, it is the competition between substrate and molecular oxygen by $^3PS^*$ that will determine the predominant type of mechanism during aPDT process (Via and Magno, 2001; Min and Boff, 2002; Costa et al., 2012).

1.2.2 Photosensitizers

PS is usually an aromatic molecule that is crucial in photodynamic therapy, because it initiates the formation of cytotoxic species through the light absorption (Fu et al., 2013).

A PS is considered appropriate for aPDT if it has determinate characteristics, such as high capacity to produce ROS, a minimal risk of resistant strains development and mutagenic processes in the host, absorption capacity at the wavelength corresponding to region of the light spectrum emitted by source, selective for target cells, should be a pure compound with a stable composition and safe for healthy cells (Jori and Brown, 2004; Jori et al., 2006; Engelhardt et al., 2010; Kashef et al., 2017).

Most of the PSs used in aPDT are derived from tetrapyrrolic macrocycles, such as porphyrins, phthalocyanines or chlorines. These PSs are involved in important biological functions, such as respiration (heme group) and photosynthesis (chlorophyll). Thus, from these compounds it is possible to develop several synthetic analogues to be used as PSs by modifying its chemical structure or optimizing its photodynamic properties (Almeida et al., 2011; Costa et al., 2012; Wainwright et al., 2017; Cieplik et al., 2018). For example, there are compounds which are transformed into cationic entities by introduction of positively charged substituents at the peripheral positions of the tetrapyrrolic macrocycle (*meso* positions), that may improve the PS effectiveness (Jori et al., 2006; Alves et al., 2009).

Beyond tetrapyrrolic macrocycles, the non-tetrapyrrolic derivatives or synthetic dyes (such as bengal rose or **MB**) are also used in aPDT (Castano et al., 2004; Almeida et al., 2011; Costa et al., 2012).

In this study, four different PSs was tested (Fig. 7), a synthetic dye (**MB**) and three cationic porphyrins (**Tri-Py(+)-Me**, **Tetra-Py(+)-Me** and **Tetra-S-Py(+)-Me**).

Porphyrins are already applied in oncology and in the photoinactivation of microorganisms but are not yet used in blood disinfection. However, there are several studies showing their potential in aPDT (Almeida et al., 2011) and even in blood disinfection (Marciel et al., 2017; Sousa et al., 2019).

The **Tri-Py(+)-Me** is a porphyrin with three positive charges and has already been shown to be effective in photoinactivation of *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* in plasma and whole blood (Marciel et al., 2017; Sousa et al., 2019).

The **Tetra-Py(+)-Me**, with four positive charges, was not yet tested in blood disinfection, but has been already used in several aPDT studies on other matrices. This PS has proven its efficiency in the photoinactivation of bacteria (Bartolomeu et al., 2017), viruses (Costa et al., 2008), fungi (Beirão et al., 2014) and bacterial endospores (Oliveira et al., 2009).

Another porphyrinic PS with four positive charges in study, **Tetra-S-Py(+)-Me**, has never been tested in blood, but its effectiveness has been demonstrated in the photoinactivation of *Penicillium chrysogenum* conidia (Gomes et al., 2011).

MB was the first approved PS in the clinical field for application in oncology. Later, this PS began to be applied in the treatment of infectious diseases and is currently approved for plasma disinfection (Miclescu and Wiklund, 2010; Marciel et al., 2017; Cieplik et al., 2018).

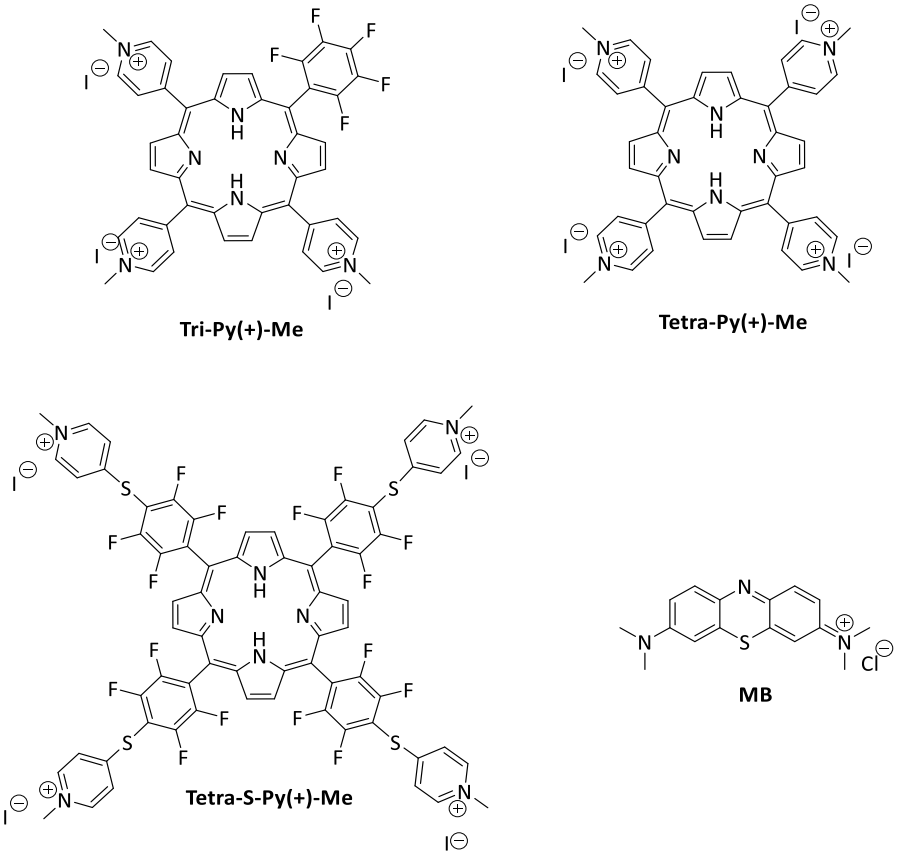

Figure 7 – Chemical structures of the PSs used in this study. [**Tri-Py(+)-Me**] 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide. [**Tetra-Py(+)-Me**] 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide. [**Tetra-S-Py(+)-Me**] 5,10,15,20-tetrakis[2,3,5,6-tetrafluoro-4-(1-methylpyridinium-4-ylsulfanyl)phenyl]porphyrin tetra-iodide. [**MB**] 3, 7-bis (dimethylamino)-phenazathionium chloride tetramethylthionine.

1.2.3 Molecular targets

Regardless of the involved mechanism to be type I or II, oxidative products will be formed, which have several molecular targets and produces irreversible damage in the pathogenic microorganisms (Costa et al., 2012).

Normally, the molecular targets for all kinds of microorganisms can be outer structures, such as cell membrane or virus envelope, proteins or enzymes and nucleic acids (Hamblin and Hasan, 2004; Almeida et al., 2015; Kashef et al., 2017). In general, damage generated by ROS may be the increase of ionic permeability, cell lysis, loss of DNA repair, inhibition of respiration and DNA replication, among others (Wainwright, 1998).

Some of the damages in the nucleic acids can be repaired by DNA repair systems, but there are microorganisms (such as *Deinococcus radiodurans*) which are easily inactivated by aPDT and have an efficient DNA repair mechanism (Maisch, 2009; Cieplik et al., 2018). So, nucleic acids are not the major aPDT target. Thus, although damage in the material genetic occurs, external structures of the microorganisms are the main aPDT target. The membrane rupture is the first morphologic

alteration and causes loss of cellular content and inactivation of enzymes and membrane transport systems (Hamblin and Hasan, 2004; Jori et al., 2006; Tavares et al., 2010; Almeida et al., 2011; Alves et al., 2013; Kashef et al., 2017). Specific and suitable PS adhesion to outer structures is generally considered enough for target cell inactivation or destruction (Winckler, 2007; Tavares et al., 2010). In addition, genetic material can only be affected when external structures have already been destroyed by aPDT (Almeida et al., 2015; Cieplik et al., 2018).

In relation to nucleic acids and proteins, there are several functional damages which can occur. For example, aPDT can affect or inhibit protein synthesis and metabolic processes, such as glucose transport and can also cause loss of enzymatic activities. Thus, ROS may have a mutagenic effect on microorganisms (Jori et al., 2006; Costa et al., 2012; Kashef et al., 2017). There are several factors that can be involved in the effect caused on nucleic acids, such as PS structure and mechanism of action, PS cell localization and the PS adherence to microorganism (Alves et al., 2013). In addition, ROS also triggers lipid peroxidation reactions that leading to loss of fluidity and increased permeability of the membrane and can affect neighbouring proteins and other molecules (Wainwright, 1998; Bonnett, 2000; Costa et al., 2012).

1.2.4 Factors that interfere in aPDT

The aPDT demonstrated efficacy in the destruction of Gram-positive and Gram-negative bacteria, as well as in viruses, fungi and parasites (Costa et al., 2008; Costa et al., 2012; Alves et al., 2015; Bartolomeu et al., 2017). However, this inactivation relies on the microorganisms and the PS used (Sobotta et al., 2015; Marciel et al., 2018).

The number and distribution of positive charges in the PS have a crucial role in aPDT, because the positive charge promotes an electrostatic bond to outer cell surface, leading to initial damage that guides the PS to critical spots for microorganism stability (Costa et al., 2008; Alves et al., 2009; Marciel et al., 2018). Several studies indicated that cationic porphyrins are more efficient than anionic or neutral against bacteria and can be used at lower doses (Simões et al., 2016; Marciel et al., 2018). Furthermore, the PS affinity to the microorganism raises with the amphiphilic character of the PS, which depends on the number of charges and the relative position in molecule (Spesia et al., 2005; Marciel et al., 2018). The PS concentration and irradiation time are also relevant parameters that must be considered (Costa et al., 2012). Wavelength is another important aspect since it should be suitable to the PS absorption level and biological material. Therefore, if the material complexity is higher, the wavelength used must also be greater (Kashef et al., 2017).

In relation to type of microorganisms, the main difference is in the cellular structure and these dissimilarities influence the interaction of PS with cellular components, leading to differences in the photoinactivation profile of the various microorganisms (Kashef et al., 2017).

Relatively to bacteria, Gram-positive bacteria are effectively easier to inactivate than Gram-negative bacteria, because the outer cell wall of peptidoglycan that surround a cytoplasmic membrane is an ineffective permeability barrier. Conversely, Gram-negative bacteria present an outer membrane to protect the thinner peptidoglycan layer, which hampers the diffusion of PS, mainly with negative or neutral charge (Fu et al., 2013; Kashef et al., 2017; Mesquita et al., 2018).

Bacteria physiological state also interferes in aPDT, because the cells are more susceptible to the photoinactivation in the logarithmic phase of growth than in the stationary phase. Cell density also affects the process, because the bacteria compete for attaching to PS and for reaction with ROS (Demidova and Hamblin, 2005a; Jori et al., 2006).

Regarding to viruses, the main difference is in the lipid envelope. Enveloped viruses are more easily inactivated because the lipid envelope destruction leading to the loss of virus infectivity, unlike non-enveloped viruses (Käsermann and Kempf, 1998; Costa et al., 2012). Furthermore, lipids and proteins of the envelope can be PS binding sites allowing greater adhesion to the viral particles (Käsermann and Kempf, 1998; Kashef et al., 2017).

Yeasts and fungal pathogens have a variable external structure and its permeability is intermediate between Gram-positive and Gram-negative bacteria, respectively (Kashef et al., 2017). However, these pathogens are larger in size when compared to the small bacterial cell, so, a greater amount of ROS is required to photoinactivate fungi than to photoinactivate bacterial cells. Thus, the fungi are less susceptible to aPDT treatment than bacteria and viruses (Demidova and Hamblin, 2005b; Kashef et al., 2017).

1.2.5 aPDT in blood

Currently, there are three PSs which are already approved in some countries to disinfect plasma or platelets (Mundt et al., 2014; Marciel et al., 2017). **MB** is used in combination with visible light, psoralen with UVA and riboflavin with UVB. All these PSs are applied in plasma disinfection, but psoralen and riboflavin are also used in platelets (Salunkhe et al., 2015). However, there is no aPDT treatment to red blood cells or whole blood, nonetheless there are several PSs in study for blood disinfection (Marciel et al., 2017; Sousa et al., 2019).

MB is a phenothiazine dye that has a natural affinity for nucleic acids. After exposure to visible light are generated ROS which targets the guanine residues. These residues are oxidized by ROS, leading to complications in the pathogen replication (Bihl et al., 2007; Solheim, 2008; Seghatchian et al., 2011; Salunkhe et al., 2015). Furthermore, other structures are also strongly affected, because an experiment with HIV 1 has shown that lipid envelope, core proteins and reverse transcriptase enzyme are also **MB** targets (Bachmann et al., 1995; Seghatchian et al., 2011). The current method in use (Theraflex) was developed from the improvement of the original method (Springe) (Table 5) (Seghatchian et al., 2011).

The Springe process (Table 5) requires freezing and thawing of plasma to disrupt the possible suspended leukocytes. Subsequently, an amount of **MB** solution is added to the plasma to reach a final concentration of 1 µmoL/L. Plasma is then irradiated with fluorescent lamps without temperature control and no **MB** removal (Seghatchian et al., 2011). The current improved Theraflex method (Fig. 8) does not need freezing/thawing of plasma but requires a leucodepletion phase through filtration membranes which removes suspended leukocytes and other aggregates or microparticles (Chabanel et al., 2003; Seghatchian et al., 2011). A dry **MB** pill of 85 µg that is integrated into the bag systems is dissolved in the filtered plasma, providing a final concentration of 1 µmoL/L for a volume of plasma between 235 and 315 mL. The irradiation is performed using a device with sodium lamps which ensures the temperature control (approximately 22 °C). Then,

over 90% of the residual **MB** is removed by a filter attached to the bag systems (Wainwright, 2000; Seghatchian et al., 2011; Gravemann et al., 2018).

Table 5 – Comparison of the processing steps between original (Springe) and current (Theraflex) method of the **MB** treatment. Adapted from Seghatchian et al. [2011].

PROCESSING STEPS	SPRINGE	THERAFLEX
1. Freezing/thawing	yes	no
2. Leucodepletion	no	yes (membrane filter with 0.65 µm)
3. Addition of MB	50 µmoL/L **MB** solution volume adjusted to 1 µmoL/L	85 µg of **MB** dry pill
4. Illumination	one side fluorescent lamps no temperature controls with agitation	two side sodium lamps temperature monitoring (≤ 22°C) with agitation
5. Removal of MB	no	yes

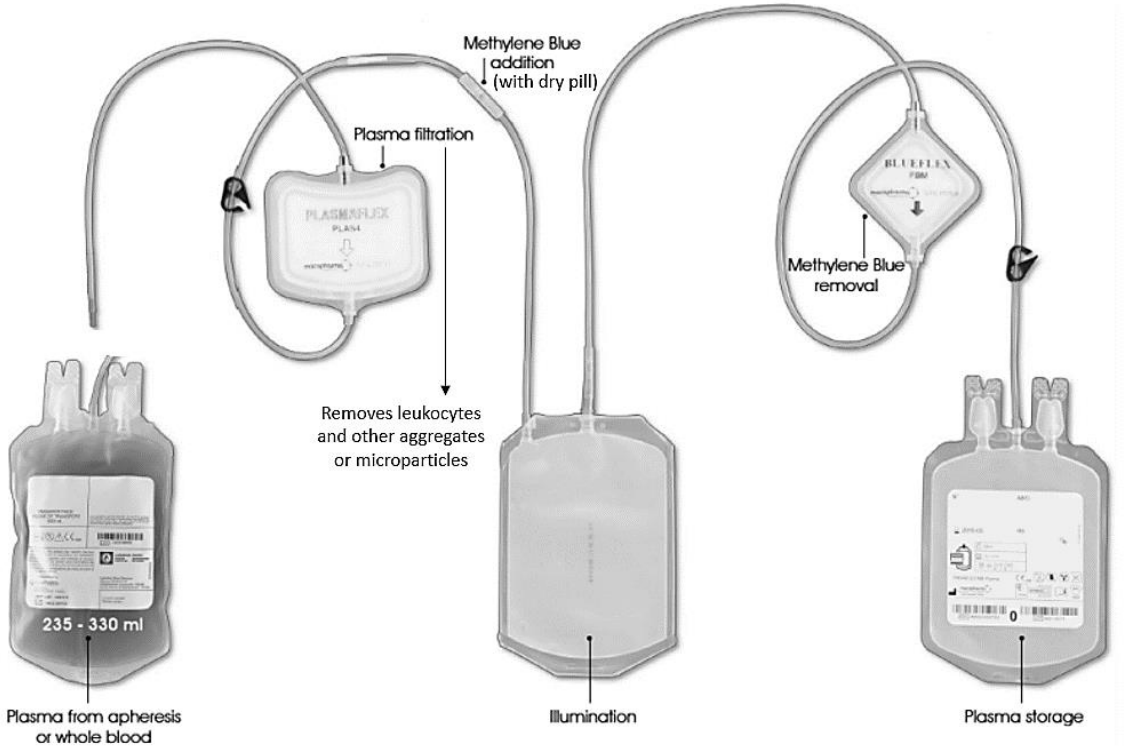

Figure 8 – Schematic illustration of the Theraflex method bag systems. Adapted from Gravemann et al. [2018].

This method has already shown efficacy in the photoinactivation of the several enveloped viruses, such as HIV or West Nile virus (Mohr et al., 2004; Seghatchian et al., 2011). Regarding to non-enveloped viruses, the effectiveness is lower. Although **MB** is not effective against hepatitis A virus (Mohr et al., 1995; Seghatchian et al., 2011), it has demonstrated efficiency in the photoinactivation of parvovirus B19 (Mohr et al., 1997; Seghatchian et al., 2011). Nonetheless, there are studies that shown an efficient photoinactivation of hepatitis A virus with synthetic porphyrins (Casteel et al., 2004), although these PSs are not yet approved for clinical use. **MB** has also demonstrated to be effective against *Trypanosoma cruzi*, a protozoan that causes Chagas disease (Girones et al., 2006). The adverse effects of this therapy have been evaluated over the last years and haemovigilance plays a crucial role in this process. An 11-year study demonstrated the long-term safety and quality of **MB**-treated plasma and a low rate of adverse events in transfused patients (Politis et al., 2014).

Psoralen and riboflavin are also PSs used in the blood products disinfection, such as plasma and platelets (Salunkhe et al., 2015).

Amotosalen is a synthetic psoralen that establishes a covalent bond to pyrimidine and forms a crosslinking in nucleic acids of the microorganism in the light presence. In addition, it can also bind to lipids and proteins of the pathogen (Solheim, 2008; Salunkhe et al., 2015). This compound is added to plasma or platelets in order to achieve a final concentration of 150 μmoL/L and the irradiation is performed with UVA light under stirring. Afterward, residual amotosalen is removed by an adsorption device. The complete process is realized in a closed integrated disposable set with sterile storage bags (Irsch and Lin, 2011; Mundt et al., 2014).

This technique has been effective in the photoinactivation of several enveloped viruses, bacteria and protozoa, yet its efficacy is lower in non-enveloped viruses (Solheim, 2008). For example, this method was effective against HIV or hepatitis B virus, *Escherichia coli* or *Treponema pallidum* (syphilis) and *Plasmodium falciparum* (malaria). Nevertheless, its efficacy is more reduced for parvovirus B19, which is a non-enveloped virus (Irsch and Lin, 2011).

Haemovigilance studies demonstrated high levels of safety and a low frequency of acute transfusion reactions in the plasma and platelets treated with amotosalen (Cazenave et al., 2010; Irsch and Lin, 2011). The major advantage of this method is that amotosalen has a weak interaction with proteins or other cellular components, so, the functional properties of the blood components are conserved (Wollowitz, 2001; Irsch and Lin, 2011). However, this procedure is not applied in erythrocytes, because these cells absorbs some of the UV light through haemoglobin (Solheim, 2008; Sobral et al., 2012).

Riboflavin or vitamin B2 is a naturally photochemical that causes irreversible damages in the pathogen nucleic acids after exposure to UVB light. This compound induces break down of pathogen nucleic acids through electron transfer reactions between light-activated riboflavin and the DNA/RNA chain, leading to complications in the mechanisms of replication (Kumar et al., 2004; Marschner and Goodrich, 2011; Salunkhe et al., 2015). Riboflavin is added to plasma or platelets in order to reach a final concentration of 50 μmoL/L and the irradiation is performed with UVB light under stirring. This whole process is performed in a closed disposable kit with sterile storage bags. After treatment, riboflavin does not need to be removed as it is considered safe by the FDA since it is naturally present in foods and human body (Marschner and Goodrich, 2011; Salunkhe et al., 2015).

This method is effective against enveloped viruses and some non-enveloped viruses, bacteria and protozoa. For example, riboflavin demonstrated efficacy in the photoinactivation of West Nile virus or hepatitis C virus (both enveloped viruses), *Staphylococcus aureus* or *Escherichia coli* and *Babesia microti*. Regarding to non-enveloped viruses, riboflavin was the only approved PS which demonstrated efficacy in the photoinactivation of hepatitis A virus, that is very resistant to chemical and heat inactivation (Solheim, 2008; Marschner and Goodrich, 2011).

Despite the safety attributed to riboflavin, several toxicity tests were realized to ensure the security of treatment. None of these trials detected significant toxic effects and haemovigilance studies reported no adverse events following a transfusion (Marschner and Goodrich, 2011).

Riboflavin was also tested in whole blood and demonstrated efficacy in the photoinactivation of some clinically relevant pathogens (Marschner and Goodrich, 2011; Mundt et al., 2014; Yonemura et al., 2017). For example, a clinical trial in the malaria-endemic country has showed that the risk of transfusion-transmitted malaria was minimized by action of riboflavin and UVB light in the whole blood. After treatment and storage of treated blood, there were no significant differences in the haemolysis percentage and incidence of adverse reactions between patients who received treated whole blood with those receiving untreated whole blood (Allain et al., 2016; Yonemura et al., 2017). However, other studies have shown that this treatment influences the red blood cells quality and can cause morphological and biochemical alterations in these cells and prolonged storage of treated blood increases the erythrocyte susceptibility to programmed cell death (Qadri et al., 2017). Therefore, the riboflavin is only used in plasma and platelets and not in whole blood due to the ambiguity in adverse effects that may occur in transfused patients.

The difficulty in the development of an aPDT protocol for erythrocytes or whole blood, stems from sensibility of these cells, as red blood cells can also be photosensitized in this process (haemoglobin absorbs some of the light). So, erythrocytes are important study models to evaluate the possible toxicity of photodynamic therapy in red blood cells concentrates and whole blood (Kaestner et al., 2004).

1.3 Other applications of photodynamic therapy

Photodynamic therapy was first used in the oncology to damage cancer cells, but it was later applied in the treatment of infectious diseases. Nowadays, aPDT is increasingly studied to combat the antimicrobial resistance problem.

Currently, the main application of aPDT is in the blood products sterilization, aiming to increase the transfusion safety (Wainwright, 1998; Almeida et al., 2011). In addition, this therapy can also be used in disinfection of infected skin, treatment of oral cavity infections, surgical material disinfection, treatment of wounds, among other applications (Hamblin and Hasan, 2004; Jori et al., 2006; Almeida et al., 2011; Cieplik et al., 2018).

The application of aPDT is very useful to treat infections located in easily accessible body sites, but through endoscopes and fibber-optic devices, the local application of the PS and irradiation becomes easier. Thus, it is possible to treat infections in the less accessible body locations, such as gastrointestinal tract, ear or lungs (Wainwright et al., 2017; Cieplik et al., 2018).

In the clinical field, photodynamic therapy is not only used in the treatment of infections, so, it can also be applied in dermatology (psoriasis treatment) or even ophthalmology (treatment of age-related macular degeneration) (Bressler and Bressler, 2000; Maisch et al., 2004; Jori et al., 2006).

Beyond medical scope, this therapy is also applicable in the environmental area, such as in the wastewater treatment, drinking water disinfection, elimination of foodborne pathogens and in the pisciculture (Almeida et al., 2011; Luksiene and Brovko, 2013; Alves et al., 2015; Bartolomeu et al., 2017; Marciel et al., 2018).

1.4 Objectives

aPDT is a method already used in the blood products sterilization to reduce the risk of transfusion-transmitted infections, however, only three PSs (riboflavin, amotosalen and **MB**) are approved for plasma and platelets disinfection and there are no applications of aPDT for erythrocytes and whole blood.

Therefore, the main objective of this study was to evaluate the aPDT effect of cationic porphyrinic PSs in the virus inactivation in plasma and whole blood.

For this purpose, T4 bacteriophage was used as a model of mammalian viruses and three cationic porphyrinic PSs were tested (**Tri-Py(+)-Me**, **Tetra-Py(+)-Me** and **Tetra-S-Py(+)-Me**). **MB** was also included as a standard, because this PS is approved to plasma disinfection.

In order to determine the most appropriate and safe PS concentration and to pave the aPDT application in the clinic, it was necessary to evaluate the possible side effects of aPDT with the tested PSs on erythrocytes. Thus, erythrocyte osmotic fragility tests and erythrocytes count before and after aPDT treatment were performed.

The photodynamic activity of these PSs in the virus inactivation was first evaluated in PBS (Phosphate-Buffered Saline) and later in plasma. Given the matrix complexity, only the most effective PS in plasma was tested in the whole blood.

1.5 References

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CHAPTER 2

Viruses inactivation in blood by photodynamic treatment with tetrapyrrolic photosensitizers

ABSTRACT

The therapeutic methods applied in plasma disinfection can induce damages in other blood components. Antimicrobial photodynamic therapy (aPDT) represents a promising approach and is approved for plasma and platelet disinfection using non-porphyrinic photosensitizers (PSs), such as methylene blue (**MB**). In this study, it was evaluated the photoinactivation efficiency of three cationic porphyrinic PSs (**Tri-Py(+)-Me**, **Tetra-Py(+)-Me** and **Tetra-S-Py(+)-Me**) in the photoinactivation of viruses and the results were compared with the efficacy of **MB**. None of the PSs caused haemolysis at the isotonic conditions. All porphyrinic PSs were more effective than **MB** in the photoinactivation of T4-like phage in plasma, mainly **Tetra-S-Py(+)-Me**, although its effectiveness has decreased in whole blood. Porphyrins can be considered promising PSs to inactivate viruses in plasma.

KEYWORDS

Viruses; Transfusion; Blood; Plasma; Antimicrobial photodynamic therapy; Cationic porphyrins; Methylene blue; Erythrocytes.

1. Introduction

Blood has vital functions for maintenance and survival of the organism, such as transport of oxygen and other substances, pathogen identification/elimination, wound healing, among other functions (Wainwright, 2002; WHO, 2002). However, blood is a resource whose availability is heterogeneous in the world, because it depends on the geographical location and economic concerns of the region (Wainwright, 2002).

Whole blood transfusions were common until the 70s, nevertheless, processed blood transfusions are currently more used. Thus, whole blood can be processed (by centrifugation) in different products (red blood cells, platelets and plasma) and transfused according to the needs of each patient (Hardwick, 2008; Ben-Hur and Goodrich, 2011; Marciel et al., 2017). The plasma and platelets can also be obtained by apheresis, a process that allows to collect these constituents directly from the donor and not by whole blood processing (WHO, 2002). Red blood cells are used in hypoxia cases caused by bleeding after trauma, surgery or anaemia and can be stored at 2-6 °C for 35 days in preservation solutions (Wainwright, 2002; Hardwick, 2008; Liumbruno et al., 2009). Platelets are adequate to patients with platelet dysfunction or for treatment of bleeding disorders originated by thrombocytopenia and can be stored for 5 days at 22 °C to maintain platelet function (WHO, 2002; Shander and Goodnough, 2007; Wainwright et al., 2007; Hardwick, 2008). Plasma is indicated to the treatment of coagulopathies or in autoimmune disorders and has a

shelf life of 5 years if stored at -18 °C, so it is called fresh frozen plasma (Wainwright, 2000; Wainwright, 2002; Hardwick, 2008).

In accordance with Food and Drug Administration (FDA), transfusion transmissible infections (TTIs) can be defined as infections caused by a pathogen that is potentially transmissible through the blood supply and causes severe damage in the organism (Dean et al., 2018). For ensuring the quality and safety of transfusion, haemovigilance systems have been implemented in many countries (Politis et al., 2014). Screening tests to the presence of Human Immunodeficiency Virus (HIV), hepatitis B virus, hepatitis C virus and the bacterium *Treponema pallidum* (causative agent of syphilis) are recommended for all blood donations (Salunkhe et al., 2015; Di Minno et al., 2016).

Nevertheless, despite the safety of donor blood products being the main worry of medical community, the emerging infectious diseases remain to be a danger to the blood supply (Salunkhe et al., 2015; Di Minno et al., 2016; Dean et al., 2018). The "immunological window" still intensifies this problem, because it refers to the period where pathogenic agents are not detectable, but are present (WHO, 2002; Seghatchian and Sousa, 2006; Bihl et al., 2007; Marciel et al., 2017). Many infectious agents can be transmitted through transfusion, such as viruses, bacteria, protozoa, fungi or other parasites (Brecher and Hay, 2005; Ludlam et al., 2006; Di Minno et al., 2016; Wairimu et al., 2016; Aubron et al., 2017; Udvardy, 2018). In the European Union, 111 TTIs were reported between 2010 and 2013, of which 66% bacterial, 32% viral and about 3% parasites (Domanović et al., 2017). So, disinfection of blood and blood derivates assumes great importance.

Although disinfection techniques are applied to donate blood to ensure the transfusion safety, approved conventional disinfection techniques are mainly intended for plasma (specially against viruses) due to the damage that can cause in cellular fractions, such as platelets and especially erythrocytes (Wainwright, 2002; Casteel et al., 2004; Salunkhe et al., 2015). Moreover, the approved treatments have not the same efficacy against all microorganisms, for instance, enveloped viruses are more easily inactivated than non-enveloped viruses (such as hepatitis A virus or picornavirus) (Schneider et al., 2004; Klein, 2005; Di Minno et al., 2016).

Heat treatments, such as wet heat treatment (pasteurization) or dry heat treatment, are some of the conventional approved disinfection techniques for plasma (Seghatchian and Allain, 2001; Salunkhe et al., 2015). These heat techniques have inactivated HIV or hepatitis B virus but are not effective against non-enveloped viruses and can cause damage to the plasma proteins (Burnouf and Radosevich, 2000; Seghatchian and Allain, 2001; Salunkhe et al., 2015). Nanofiltration is based on the different particle sizes and uses filters with pores of similar size to the viral particles (Salunkhe et al., 2015). However, high molecular weight proteins may hinder the removal of smaller particles (Wainwright, 2002; Marciel et al., 2017). Immunoaffinity chromatographic techniques can also be applied to treat plasma, but the used antibodies may bind non-specifically to the cell membranes. Hence, this technique cannot be used in platelets and red blood cells (Wainwright, 2002; Marciel et al., 2017). In some European countries and USA, there is a conventional disinfection technique which is mostly used in plasma disinfection, the solvent-detergent method (SD method) (Bihl et al., 2007; Salunkhe et al., 2015). SD method was approved by the FDA in 2013 (Dean et al., 2018) and consists in the combination of an organic solvent (1% tri-*n*-butyl phosphate) with a detergent (1% Triton X-100). After treatment, these chemicals must be removed to avoid potential damage to plasma proteins (Burnouf and Radosevich, 2000;

Wainwright, 2002; Salunkhe et al., 2015). Yet, this technique is not effective against non-enveloped viruses and may affect clotting factors (Hellstern, 2004; Bihl et al., 2007; Salunkhe et al., 2015; Dean et al., 2018).

Overall, in addition to the conventional approved techniques, alternative methods for blood disinfection are needed and the antimicrobial photodynamic therapy (aPDT) approach can be a potential alternative for whole blood and erythrocytes disinfection procedures. aPDT is based on the application of a photosensitizer (PS), which upon exposure to light of an adequate wavelength and in the presence of molecular oxygen, is able to produce cytotoxic reactive oxygen species (ROS) responsible by the pathogen inactivation (Kashef et al., 2017; Cieplik et al., 2018). Briefly, the light activated PS after an intersystem crossing process can promote the formation of highly oxidative species (*e.g.* H_2O_2, $OH^{\bullet}$, $O_2^{-\bullet}$) through electronic transference to substrates (type I mechanism) and/or the formation of the highly cytotoxic species, the singlet oxygen ($^1O_2$), through energy transfer to molecular oxygen (type II mechanism) (Cieplik et al., 2018; Nakonieczna et al., 2018). aPDT is a multitarget approach and it is directed mainly to outer structures, affecting lipids and proteins, but can also target nucleic acids (Hamblin and Hasan, 2004; Costa et al., 2012; Alves et al., 2013; Alves et al., 2014; Almeida et al., 2015; Kashef et al., 2017). Consequently, the development of resistance to this therapy is highly improbable (Costa et al., 2011; Cieplik et al., 2018).

aPDT can act on bacteria (even in multiresistant strains), viruses, fungi and other parasites (Wainwright, 1998; Alves et al., 2009). Nevertheless, the photoinactivation effectiveness relies on the microorganisms (Sobotta et al., 2015; Marciel et al., 2018). For example, aPDT is considered more effective against Gram-positive bacteria than Gram-negative bacteria due to dissimilarities in the cell wall permeability (Fu et al., 2013; Kashef et al., 2017; Mesquita et al., 2018). Regarding to viruses, enveloped viruses are inactivated more easily than non-enveloped viruses (Käsermann and Kempf, 1998; Costa et al., 2012; Kashef et al., 2017).

aPDT is currently one of the most promising alternative methods in blood products disinfection and there are currently three non-porphyrinic PSs which are already approved in some countries to disinfect plasma or platelets (Mundt et al., 2014; Marciel et al., 2017). Methylene blue (**MB**) is activated by visible light, psoralen by UVA and riboflavin by UVB. All these PSs can be applied in plasma disinfection, but psoralen and riboflavin are also used in platelets (Salunkhe et al., 2015).

MB-light method is based on the photodynamic action and it was produced for clinical use in 1992 (Lambrecht et al., 1991; Seghatchian et al., 2011). **MB** is a phenothiazine dye that has a natural affinity for nucleic acids and affects proteins and lipids, under exposure to visible light (Bihl et al., 2007; Solheim, 2008; Seghatchian et al., 2011; Salunkhe et al., 2015). **MB** is added to plasma after leucodepletion phase through filtration membranes (Chabanel et al., 2003) and irradiation is performed using sodium lamps. Then, over 90% of the residual **MB** is removed by a filter attached to the bag systems (Wainwright, 2000; Seghatchian et al., 2011; Gravemann et al., 2018). This method has already shown efficacy in photoinactivation of the several enveloped viruses, such as HIV or West Nile virus (Mohr et al., 2004; Seghatchian et al., 2011) and the protozoan *Trypanosoma cruzi* (Girones et al., 2006). Regarding to non-enveloped viruses, the effectiveness is slightly lower and although it is not effective against hepatitis A virus (Mohr et al., 1995), it was efficient in the photoinactivation of the non-enveloped parvovirus B19 (Mohr et al., 1997; Seghatchian et al., 2011). The adverse effects of **MB** have been evaluated over the last

years through haemovigilance studies. An 11-year study demonstrated the long-term safety and quality of **MB**-treated plasma and a low rate of adverse events in transfused patients (Politis et al., 2014).

Amotosalen, a synthetic psoralen, prevents the pathogen replication under UVA light presence, affecting also the lipids and proteins of the microorganism (Hanson, 1992; Bihl et al., 2007; Salunkhe et al., 2015). This method was approved in 2014 by FDA for the plasma and platelets disinfection in USA and since 2006 have been used in Europe (Schlenke, 2014; Salunkhe et al., 2015; Dean et al., 2018). After irradiation, residual amotosalen is removed by an adsorption system (Irsch and Lin, 2011; Mundt et al., 2014). This technique has been effective in the photoinactivation of several enveloped viruses, bacteria and protozoa, yet its efficacy is lower in non-enveloped viruses (Solheim, 2008; Irsch and Lin, 2011). Haemovigilance studies demonstrated high levels of safety and low frequency of acute transfusion reactions in the plasma and platelets treated with amotosalen (Cazenave et al., 2010; Irsch and Lin, 2011). The major advantage of this method is that amotosalen has a weak interaction with proteins or other cellular components, so, the functional properties of the blood components are conserved (Wollowitz, 2001; Irsch and Lin, 2011). However, this procedure cannot be applied in erythrocytes because these cells absorbs some of the UV light through haemoglobin (Solheim, 2008; Sobral et al., 2012).

Riboflavin or vitamin B2 is a natural photochemical that causes irreversible damage in the pathogen under UVB light presence. After irradiation, riboflavin does not need to be removed as it is considered safe by FDA because it is naturally present in foods and human body (Bryant and Klein, 2007; Marschner and Goodrich, 2011; Salunkhe et al., 2015). This method is already being applied in many countries and it was approved in 2008 (Schlenke, 2014; Salunkhe et al., 2015). Riboflavin has been demonstrated efficacy in the photoinactivation of hepatitis C virus, *Staphylococcus aureus* and *Babesia microti*. This technology was the only one which demonstrated efficacy in the photoinactivation of hepatitis A virus (Solheim, 2008; Marschner and Goodrich, 2011). Despite the safety attributed to riboflavin, several toxicity tests were realized to ensure the security of treatment. None of these trials detected significant toxic effects and haemovigilance studies reported no adverse events after a transfusion (Marschner and Goodrich, 2011). Riboflavin was also tested in whole blood and demonstrated efficacy in the photoinactivation of *Plasmodium falciparum* (Marschner and Goodrich, 2011; Mundt et al., 2014; Allain et al., 2016; Yonemura et al., 2017). However, this treatment influences the red blood cells quality (Qadri et al., 2017). Therefore, the riboflavin with UV light is only used in plasma and platelets and not for whole blood due to the ambiguity in adverse effects that may occur in transfused patients.

Although there is no approved treatment to red blood cells or whole blood, there are several PSs in study for blood disinfection by aPDT (Marciel et al., 2017; Sousa et al., 2019). The difficulty in the development of an aPDT protocol for erythrocytes or whole blood, stems from the sensibility of these cells, as red blood cells can also be photosensitized in this process (haemoglobin absorbs some of the light) (Kaestner et al., 2004).

Thereby, the aim of this study was to evaluate the aPDT effect of the porphyrinic PSs (Fig. 1) **Tri-Py(+)-Me**, **Tetra-Py(+)-Me** and **Tetra-S-Py(+)-Me** in the viruses inactivation in plasma and whole blood and evaluate the possible side effects of aPDT with these PSs on erythrocytes. For

comparison, the photodynamic effect of **MB** (already approved to treat plasma) to photoinactivate viruses was also considered.

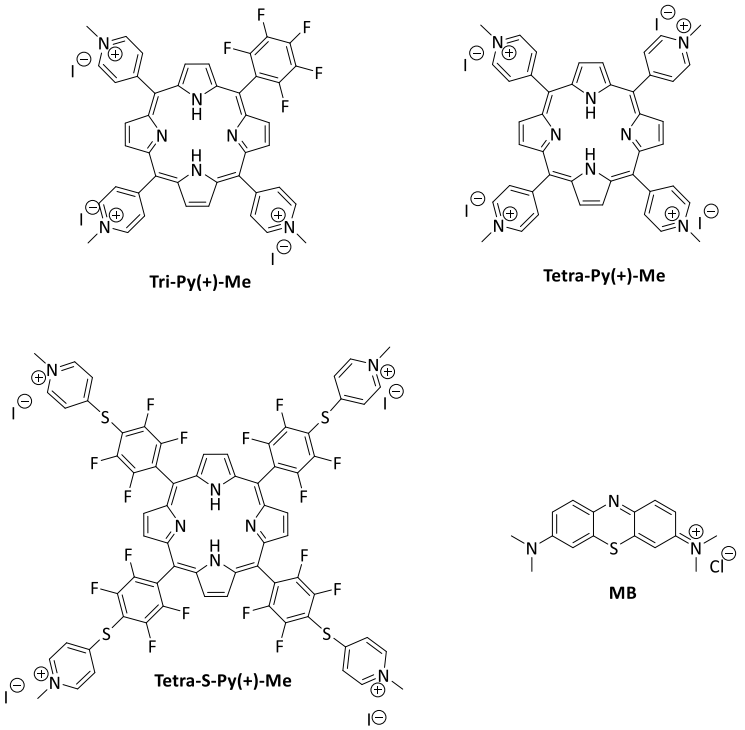

Figure 1 – Chemical structures of the PSs used in this study.

2. Materials and Methods

In order to assess the efficacy of the aPDT approach in the photoinactivation of viruses in plasma and whole blood, T4-like phage was used as the model of mammalian viruses (Costa et al., 2012).

Osmotic fragility tests and the aPDT effect on erythrocytes before and after photodynamic treatment were performed to evaluate the possible side effects on red blood cells in order to identify the most appropriate and safe PS concentration for aPDT assays.

The first aPDT assays were performed in Phosphate-Buffered Saline (PBS) to evaluate and compare the photodynamic efficacy of **Tri-Py(+)-Me**, **Tetra-Py(+)-Me**, **Tetra-S-Py(+)-Me** and **MB** to inactivate viruses. Subsequently, these PSs were tested in plasma and only the most effective one was used to perform the whole blood assays.

2.1 Blood samples

Blood samples tested were supplied by a clinical laboratory. The samples consisted in blood test tubes containing whole blood and anticoagulant $EDTAk_3$, 5.4 mg with a final volume of 3 mL (BD Vacutainer®, Becton Dickinson, Plymouth, UK). The samples were used within a period from

up to 5 days after harvest. The whole blood samples were centrifuged (Heraeus Megafuge 16R Centrifuge, Thermo Scientific) at 2195 × *g* for 5 min to obtain the plasma and the erythrocytes. The plasma was then separated from red blood cells and used in plasma aPDT assays. For whole blood assays, the centrifugation step was not required.

2.2 Bacterial strain and growth conditions

The bacterial strain *Escherichia coli* ATCC 13706 was used as T4-like phage host. The bacterial culture was stored in Tryptic Soy Agar (TSA) at 4 °C. Before each assay, some isolated colonies were transferred to 30 mL of Tryptic Soy Broth (TSB) and grown overnight for about 18 h at 37 °C with stirring (about 120 rotations per minute (rpm)). Subsequently, 0.3 mL of fresh culture was transferred to 30 mL of fresh TSB and incubated overnight under the same conditions as above in order to reach the stationary phase, corresponding approximately to a concentration of 10^8-10^9 colony forming units per mL (CFU/mL) (which is equivalent to an optical density of 0.8 at 600 nm).

2.3 Phage stock preparation and quantification

A wastewater sample from a secondary-treated sewage plant of Aveiro was used to select the somatic bacteriophages of *Escherichia coli* C (ATCC 13706) (T4-like phage) (Costa et al., 2008). For phage stock preparation, a morphologically representative phage plate was isolated and then added to an *E. coli* culture (mentioned in section 2.2). This mixture was incubated at 37 °C for about 5 h with slow agitation. Subsequently, the suspension was centrifuged (Heraeus Megafuge 16R Centrifuge, Thermo Scientific) at 13000 x *g* for 10 min to remove non-infected bacteria and bacterial cell residues. The supernatant with the phage particles was kept at 4 °C after the addition of 1% chloroform to prevent the growth of bacteria (Costa et al., 2008; Valério et al., 2017).

The quantification of bacteriophages was determined, in duplicate, by the agar double layer technique (Adams, 1959). A serial dilution (1/100) of the phage suspension in PBS was prepared. Subsequently, 0.2 mL of *E. coli* bacterial host and 0.5 mL of serially diluted samples were added to a tube with 5 mL of soft TSA growth medium. The contents of the tube were mixed and then immediately poured into a Petri plate with a previously prepared confluent TSA monolayer. The plates were incubated upside-down at 37 °C in dark for about 18 h. After incubation, the number of lysis plaques was counted at the most convenient dilution (with around 30-300 lysis plaques per plate) and the number of plaque forming units per mL (PFU/mL) was calculated. The phage title of T4-like phage was ~10^9 PFU/mL.

2.4 Photosensitizers stock solutions

The PSs 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide [**Tri-Py(+)-Me**], 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide [**Tetra-Py(+)-Me**]

and 5,10,15,20-tetrakis[2,3,5,6-tetrafluoro-4-(1-methylpyridinium-4-ylsulfanyl)phenyl]porphyrin tetra-iodide [**Tetra-S-Py(+)-Me**] were synthetized according to the literature (Carvalho et al., 2010; Gomes et al., 2011; Simões et al., 2016). Their ^{1}H NMR and UV–vis spectra were consistent with the literature data. Their purity was confirmed by thin layer chromatography and ^{1}H NMR [**Tri-Py(+)-Me**: ^{1}H NMR (DMSO-d_6): δ -3.13 (s, 2H, *N*H), 4.72 (s, 9H,CH_3), 8.99–9.47 (m, 20 H, Py-H, β-H). UV–vis (DMSO) λ_{max} (log ε): 422 (5.48), 485 (3.85), 513 (4.30), 545 (3.70), 640 (3.14) nm. **Tetra-Py(+)-Me**: ^{1}H NMR (DMSO-d_6): δ -3.12 (s, 2H, *N*H), 4.73 (s, 12H, CH_3), 9.00 (d, *J* = 6.5 Hz, 8H, Py-*o*-H), 9.22 (s, 8H, β-H), 9.49 (d, *J* = 6.5 Hz, 8H, Py-*m*-H). UV–vis (DMSO) λ_{max} (log ε): 425 (5.43), 516 (4.29), 549 (3.77), 588 (3.84), 642 (3.30) nm. **Tetra-S-Py(+)-Me**: ^{1}H NMR: δ -3.08 (s, 2H, *N*H), 4.36 (12H, CH_3), 8.46 (d, *J* = 5.5, 8H, Py-*o*-H), 8.96 (d, *J* = 5.5, 8H, Py-*m*-H), 9.65 (s, 8H, β-H). ^{19}F NMR: δ -160.29 (dd, *J* = 9.9 and 28.2, 8F, Ar-*o*-F), -155.35 (dd, *J* = 9.9 and 28.2, 8F, Ar-*m*-F). UV–vis (DMSO) λ_{max} (log ε): 416 (5.50), 507 (4.37), 581 (3.95), 632 (3.40). The methylene blue (**MB**) was purchased from Acros Organics and was used as received.

Stock solutions of **Tri-Py(+)-Me**, **Tetra-Py(+)-Me** and **Tetra-S-Py(+)-Me** were prepared at 500 μM in dimethyl sulfoxide (DMSO) and stored in the dark. **MB** was prepared at 500 μM in PBS and stored also in the dark. All these stock solutions were sonicated (ultrasonic cleaner, Nahita 0.6 L, 40 kHz) for 15 min at room temperature before each experiment. For biological assays, a specific volume of the PS stock solutions was used in order to reach the selected final concentrations.

2.5 Irradiation conditions

The photodynamic effect of the selected PSs in PBS was evaluated by exposing the samples and controls to white light provided by a LED projector (EL®MARK, power, voltage and frequency of 20 W, ~230 V and ~50 Hz, respectively) at an irradiance of 25 mW/cm^2, for a maximum irradiation period of 60 min.

The photodynamic effect of the PSs in plasma and whole blood was analysed by exposing the samples and controls to white light (400-800 nm) supplied from a compatible fibber optic probe attached to a 250 W quartz/halogen lamp (LUMACARE model 122, USA) with an irradiance of 150 mW/cm^2, for a maximum irradiation period of 270 min.

All the irradiances were measured with a Power Meter Coherent FieldMaxII-Top combined with a Coherent PowerSens PS19Q energy sensor.

2.6 Evaluation of aPDT effect on erythrocyte osmotic fragility

The erythrocyte osmotic fragility allows to infer the percentage of haemolysis and it was analysed according to the method described in literature (Azeez et al., 2012). These experiments were realized using concentrations of 5.0, 10 and 20 μM of each PS under study and the haemolysis was evaluated before (incubation time) and after aPDT treatment. For this, light controls (whole blood without PS), samples and dark controls (whole blood with PS) were incubated for 30 min in the dark (incubation time) and then samples and light controls were irradiated at 150 mW/cm^2. Subsequently, 0.02 mL of blood samples and controls were collected in

the initial and final time of treatment and were added to different tubes containing 1.98 mL of sodium chloride (NaCl) solution at pH 7.4 with different concentrations (0.0, 0.1, 0.3, 0.5, 0.7 and 0.9% NaCl). The tubes were softly mixed and incubated at 25 °C for 30 min under stirring. Subsequently, the content of each tube was centrifuged (Gyrozen 1730R) at 1155 x *g* for 10 min and the supernatant removed for analysis. Lastly, optical density of the supernatant was measurement spectrophotometrically (Multiskan GO, Thermo Scientific) at 540 nm (the appropriate wavelength to quantify haemoglobin in solution) through a 96-well plate which allows to perform multiple readings at once. Thus, haemolysis in each tube was expressed in percentage, considering that the value in distilled water (0% NaCl) is 100%. In order to evaluate the effect of DMSO in erythrocyte membrane, similar procedure was performed using different percentages of this solvent (1, 2 and 4%). Three independent assays to each condition were done.

2.7 Evaluation of aPDT effect on erythrocytes

The evaluation of aPDT effect on erythrocytes was performed before (incubation time) and after aPDT treatment and using concentrations of 5.0, 10 and 20 µM of each PS under study. For this, light controls (whole blood without PS), samples and dark controls (whole blood with PS) were incubated for 30 min in the dark (incubation time) and then samples and light controls were irradiated at 150 mW/cm^2. Subsequently, an aliquot of blood samples and controls were collected in the initial and final time of treatment and then diluted in PBS at 1/500. Then, 0.01 mL of the diluted samples and controls were collected and thus the number of cells was determined using a Neubauer chamber in an optical microscope at 40× magnification (Nikon Eclipse Ni). Three independent assays to each condition were done.

2.8 Photodynamic inactivation assays with T4-like phage in PBS, plasma and whole blood

Before each experiment, the phage suspension was diluted in PBS (pH 7.4), plasma or whole blood in order to reach 10^8 PFU/mL. For the plasma assays, the blood samples were centrifuged as described in section 2.1 but this step was not necessary for whole blood assays. So, the phage suspension was equally distributed in sterilized 12-well plate. The appropriate volume of the PS was added to achieve a final concentration of 10 µM and the suspensions were kept under magnetic stirring. During the assays, light and dark controls were included. In the light control, the phage suspension in the absence of PS was exposed to light, while in dark control, the sample (phage suspension + PS) was protected from light. Then, the samples and controls were protected with aluminium foil and incubated for 30 min (T_0) in dark under stirring (~ 100 rpm), in order to promote the PS binding to phage particles. Afterward, the samples and controls were irradiated with white light and depending of the experiment with an irradiance of 25 mW/cm^2 for PBS assays and 150 mW/cm^2 for plasma and whole blood assays. As it was mentioned, the dark control was protected from light with aluminium foil. During the experiments, the samples and controls were kept under stirring and the temperature was controlled and maintained at 25 °C.

The photodynamic effect was evaluated through phage quantification, in duplicate, by the agar double layer technique (Adams, 1959). For this purpose, an aliquot of sample and controls was collected in the established times of light exposure (0, 15, 30 and 60 min for PBS assays; 0, 15, 30, 60, 90, 180 and 270 min for plasma and whole blood assays) and then serially diluted in PBS. Subsequently, 0.2 mL of bacterial host (*E. coli* overnight culture) was added to a tube with 5 mL of soft TSA growth medium. The contents of the tube were mixed and then immediately poured into a Petri plate with a previously prepared confluent TSA monolayer. After the plate dried for a few seconds, plating was performed by the drop method. The Petri plates were kept in the dark following plating for about 18 h at 37 °C. After incubation, the number of phage plaques was counted in the most convenient series of dilution and then the number of PFU/mL was calculated. At least, three independent assays to each condition were done.

2.9 Statistical analysis

The statistical analysis was performed with GraphPad Prism 7.04. Normal distributions were checked by Kolmogorov–Smirnov test and homogeneity of variances was assessed by Levene's test. Ordinary two-way ANOVA and Tukey's multiple comparison tests were applied to assess the significance of the differences between the tested conditions. The significance of the differences verified for phage concentration was evaluated by comparing the results of each experimental treatment with that of control samples. A value of $p < 0.05$ was considered significant. Three independent assays, with two replicates, to each condition were done.

3. Results

3.1 Evaluation of aPDT effect on erythrocyte osmotic fragility

The assays concerning the erythrocyte osmotic fragility were useful to evaluate in which conditions aPDT causes lysis of erythrocytes (haemolysis). These fragility tests were performed for all PSs under study at concentrations 5.0, 10 and 20 μM in order to select the most appropriate and safe PS concentration for the aPDT assays. For this purpose, the assays were performed with the whole blood in the same photoinactivation assays conditions and the haemolysis was determined under different osmotic conditions (pH 7.4) before and after aPDT treatment (under white light at an irradiance of 150 mW/cm^2) by the haemoglobin quantification in solution. The PS concentrations and the irradiation conditions were selected according previous results of the research group (Marciel et al., 2017; Sousa et al., 2019).

The principle of these tests is based on the induction of osmotic stress by increasing the concentration of NaCl in solution. Therefore, haemolysis values are supposed to be elevated at low NaCl concentrations (0 to 0.3%) due to the creation of a hypotonic medium outside the erythrocyte cells and a hypertonic medium within the cell. So, the water enters in the erythrocytes by osmosis, causing their lysis. On the other hand, it is expected that there will be no

significant haemolysis at higher NaCl concentrations (0.5 to 0.9%), because the medium becomes increasingly isotonic (0.9% NaCl is the isotonic condition) (Al-Akhras, 2006; Hai et al., 2015).

The results obtained of erythrocyte osmotic fragility are summarized in Figs. 2-5 for **Tetra-Py(+)-Me**, **Tetra-S-Py(+)-Me**, **MB** and DMSO. The results had shown that no haemolysis was observed for light control in nonstress (isotonic) conditions, which means that the white light at an irradiance of 150 mW/cm^2 does not cause haemolysis.

For **Tetra-Py(+)-Me** (Fig. 2) and **Tetra-S-Py(+)-Me** (Fig. 3) at 5.0 μM, no significant differences (ANOVA, $p > 0.05$) were detected between the sample and the light and dark controls for 0.5-0.9% NaCl conditions. This fact proves that these PSs are not toxic to erythrocytes at this concentration. At 10 μM, no significant alterations (ANOVA, $p > 0.05$) were observed between the sample and controls for 0.7 and 0.9% NaCl to these PSs. However, it was observed a high erythrocyte haemolysis rate before (60%) and after (70%) aPDT treatment being these results significantly different (ANOVA, $p < 0.05$) of the light control to 0.5% NaCl for **Tetra-Py(+)-Me**. At the same NaCl concentration, **Tetra-S-Py(+)-Me** also causes high haemolysis values before (67%) and after (73%) aPDT treatment being significantly different (ANOVA, $p < 0.05$) from the light control. In the study of the erythrocyte osmotic fragility promoted by **Tri-Py(+)-Me** reported by Marciel et al., 2017, it was showed that **Tri-Py(+)-Me** did not cause significant erythrocytes haemolysis after aPDT treatment at 5.0 and 10 μM for isotonic conditions (0.9% NaCl). However, at 20 μM, erythrocytes haemolysis occurred even for 0.9% NaCl (isotonic conditions). As in the case of **Tri-Py(+)-Me**, **Tetra-Py(+)-Me** and **Tetra-S-Py(+)-Me** also promoted haemolysis at 20 μM at the isotonic conditions. For 0.9% NaCl, a haemolysis of 53% and 55% were detected for **Tetra-Py(+)-Me** and 40% e 54% for **Tetra-S-Py(+)-Me** before and after treatment, respectively, proving to be significantly different (ANOVA, $p < 0.05$) of the light control haemolysis rate.

For these three porphyrins, no significant differences (ANOVA, $p > 0.05$) were observed between the dark controls and samples at all tested concentrations.

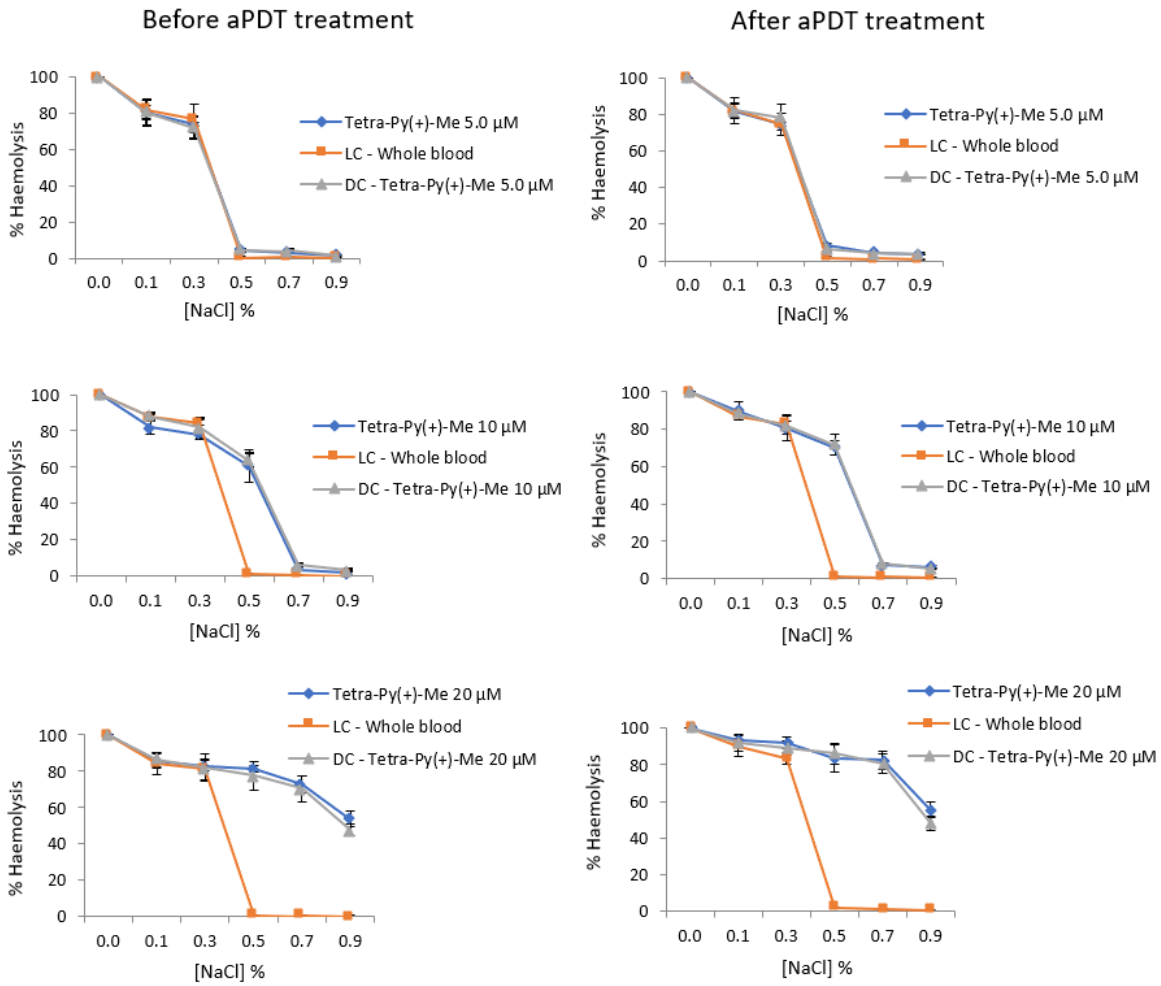

Figure 2 – Erythrocyte osmotic fragility before and after aPDT treatment with **Tetra-Py(+)-Me** (5.0, 10 and 20 µM) and under white light irradiation at an irradiance of 150 mW/cm^2. All values represent the average of three independent assays. Error bars indicate the standard deviation. LC - Light control (whole blood under light); DC - Dark control (whole blood incubated with PS without light).

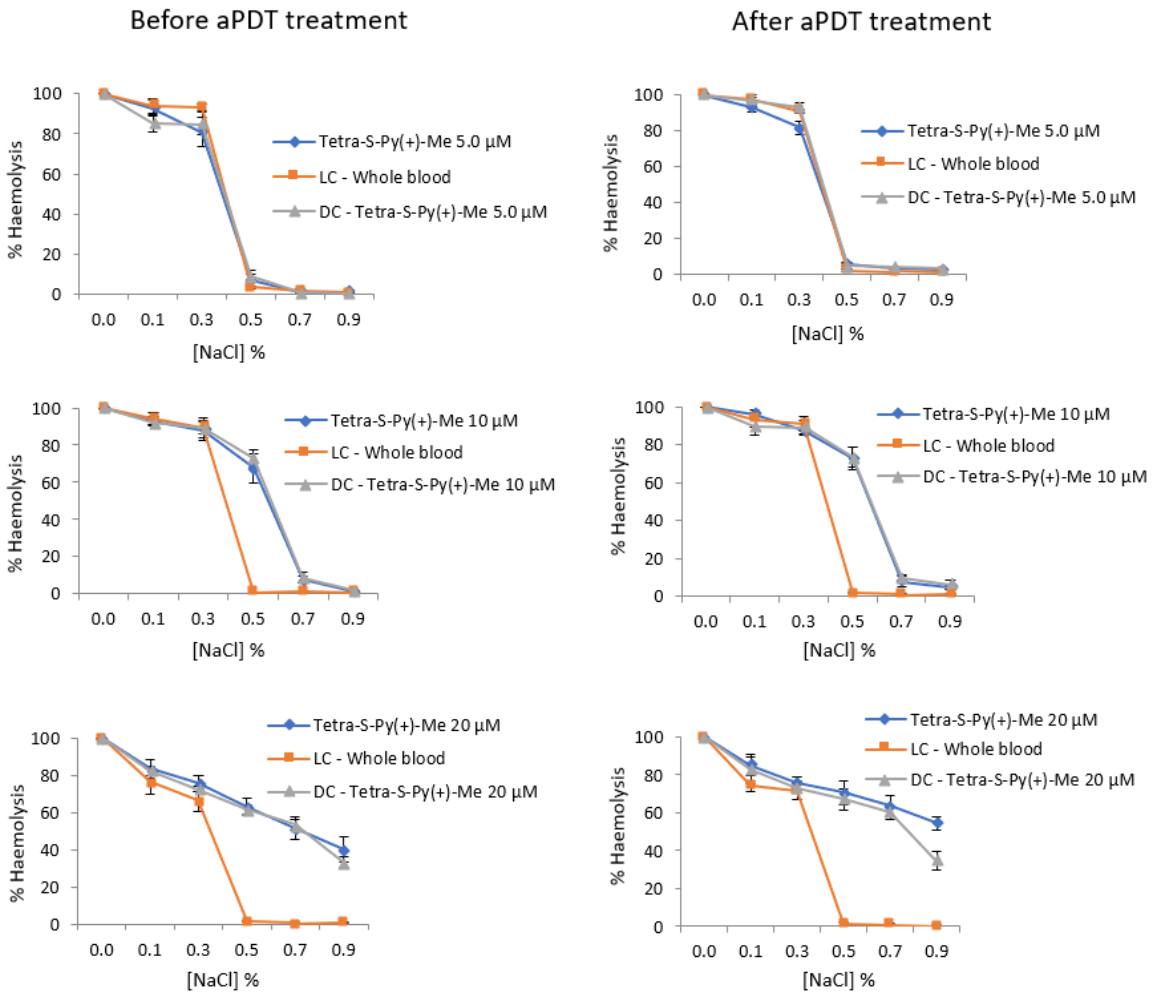

Figure 3 – Erythrocyte osmotic fragility before and after aPDT treatment with **Tetra-S-Py(+)-Me** (5.0, 10 and 20 µM) and under white light irradiation at an irradiance of 150 mW/cm^2. All values represent the average of three independent assays. Error bars indicate the standard deviation. LC - Light control (whole blood under light); DC - Dark control (whole blood incubated with PS without light).

The results of the osmotic erythrocyte fragility tests for **MB** at 5.0, 10 and 20 µM (Fig. 4) demonstrated a very distinct erythrocyte fragility profile from porphyrins. No significant differences (ANOVA, $p > 0.05$) were observed between the blood samples and light control at all tested concentrations. In addition, dark controls were also demonstrated no significant differences (ANOVA, $p > 0.05$) regarding to light control. Therefore, **MB** does not cause toxicity and further haemolysis in erythrocytes at 5.0, 10 and 20 µM.

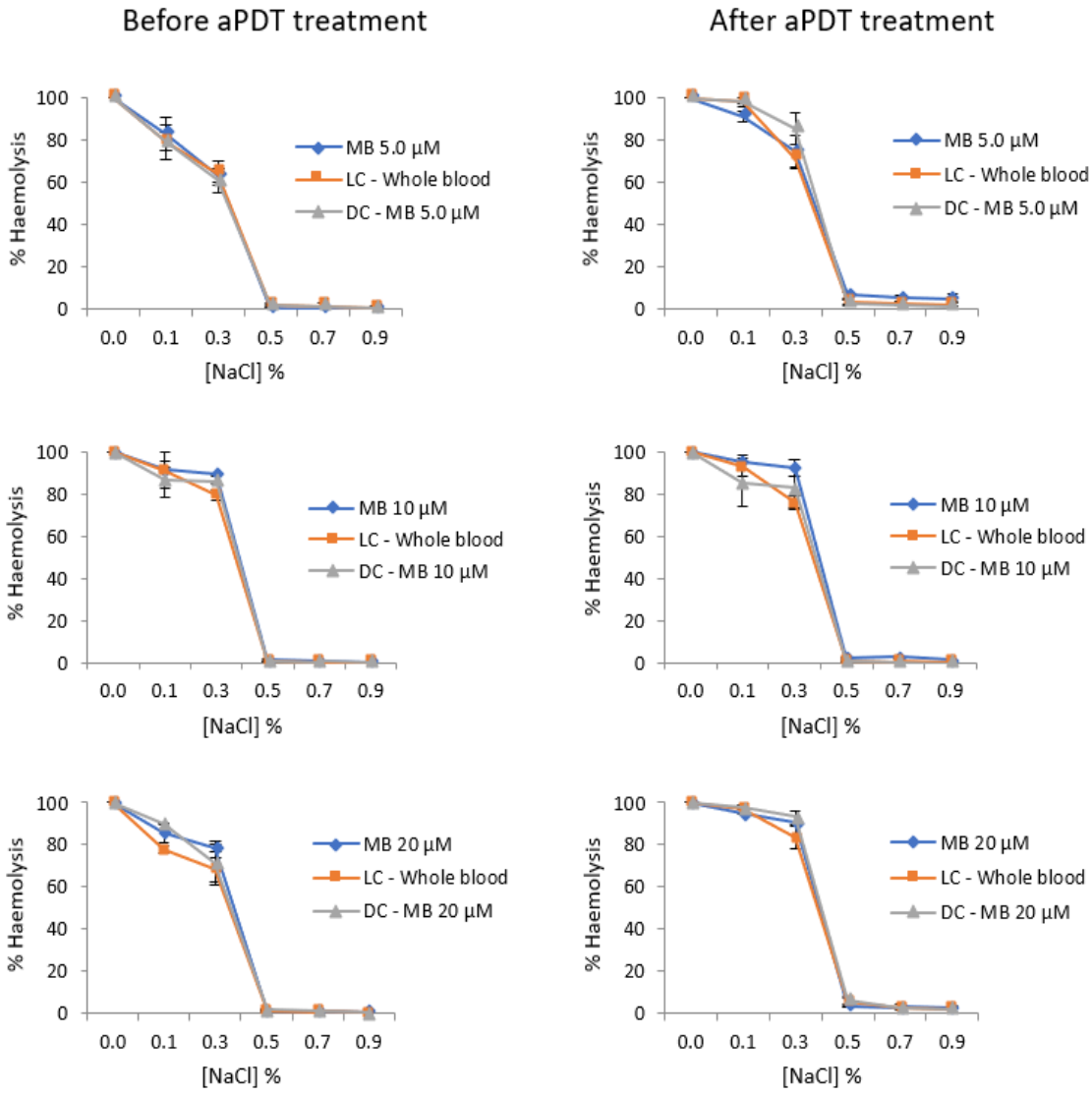

Figure 4 – Erythrocyte osmotic fragility before and after aPDT treatment with **MB** (5.0, 10 and 20 µM) and under white light irradiation at an irradiance of 150 mW/cm^2. All values represent the average of three independent assays. Error bars indicate the standard deviation. LC - Light control (whole blood under light); DC - Dark control (whole blood incubated with PS without light).

Such significant differences between the porphyrinic PSs and **MB** led to the doubt that DMSO used to prepare stock solutions could be responsible for the high haemolysis rates found for **Tri-Py(+)-Me**, **Tetra-Py(+)-Me** and **Tetra-S-Py(+)-Me**. In fact, the stock solutions of these PSs were prepared in DMSO, due to their limited solubility in aqueous solutions. On the other hand, **MB** stock solution was prepared in PBS. Thus, additional erythrocyte osmotic fragility tests were performed using only DMSO (without any PS) in the same percentages used in the assays and these results are summarized in Fig. 5. The solution containing DMSO at 1% (that corresponds to a PS concentration of 5.0 µM) did not promote significant haemolysis (ANOVA, $p > 0.05$) for 0.7 and 0.9% NaCl before and after the aPDT treatment, but significant differences (ANOVA, $p < 0.05$)

were observed between sample and control for 0.5% NaCl before (14%) and after (40%) aPDT treatment. The solution containing DMSO at 2% (that corresponds to a PS concentration of 10 µM) did not promote significant haemolysis (ANOVA, $p > 0.05$) for 0.7 and 0.9% NaCl before and after the aPDT treatment, but significant differences (ANOVA, $p < 0.05$) were also observed between sample and control for 0.5% NaCl before (22%) and after (45%) aPDT treatment. In the solution containing DMSO at 4% (that corresponds to a PS concentration of 20 µM), the haemolysis was high in all NaCl solutions, being significantly different (ANOVA, $p < 0.05$) regarding to the control. Although no haemolysis was observed before the aPDT treatment at the 0.9% NaCl, a high haemolysis rate (45%) was detected (ANOVA, $p < 0.05$) after the aPDT treatment at the isotonic conditions.

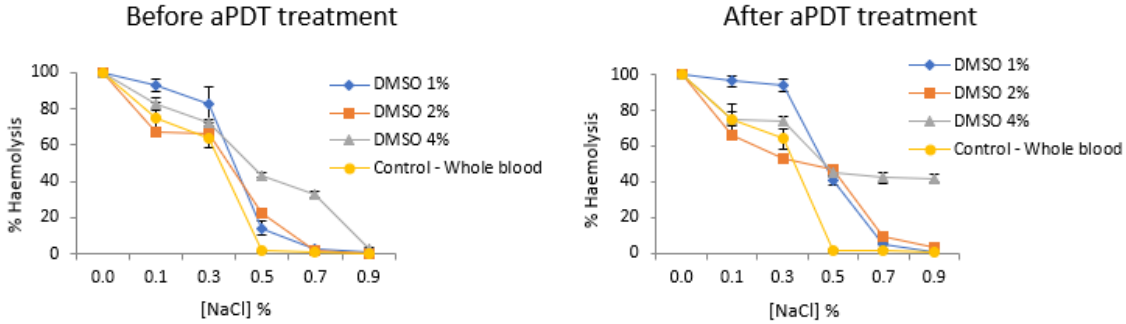

Figure 5 – Erythrocyte osmotic fragility before and after aPDT treatment with DMSO (1, 2 and 4%) and under white light irradiation at an irradiance of 150 mW/cm^2. All values represent the average of three independent assays. Error bars indicate the standard deviation. Control (whole blood without DMSO under light).

3.2 Evaluation of aPDT effect on erythrocytes

The erythrocyte counting tests are useful to evaluate whether aPDT caused erythrocyte weakening and membrane damage promoting haemolysis (Marciel et al., 2017). These tests were performed with all PSs under study at 5.0, 10 and 20 µM. For this purpose, the number of erythrocytes was determined before and after aPDT treatment.

The results are summarized in Fig. 6 and showed that no significant differences (ANOVA, $p > 0.05$) between the erythrocytes number in light control before and after aPDT treatment, which means that only white light at an irradiance of 150 mW/cm^2 does not affect the number of erythrocytes.

For **Tri-Py(+)-Me**, **Tetra-Py(+)-Me**, **Tetra-S-Py(+)-Me** and **MB**, no significant alterations (ANOVA, $p > 0.05$) were observed between the erythrocytes number in whole blood samples before and after aPDT treatment. In addition, the erythrocytes number in blood samples before and after aPDT treatment is not significantly different (ANOVA, $p > 0.05$) of the light and dark controls. Therefore, aPDT treatment with these PSs does not affect the erythrocytes concentration.

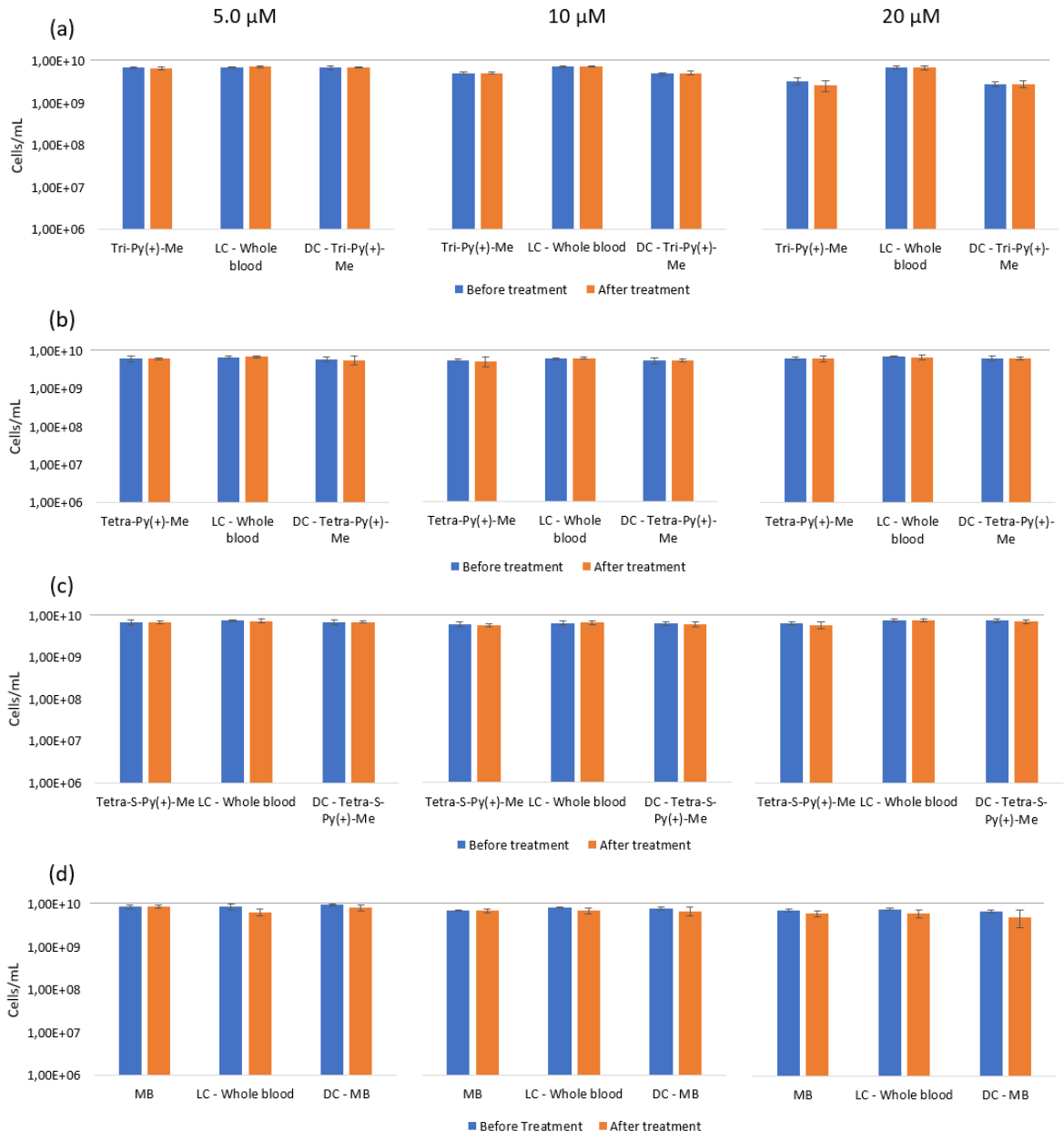

Figure 6 – Effect of aPDT treatment with (a) **Tri-Py(+)-Me**, (b) **Tetra-Py(+)-Me**, (c) **Tetra-S-Py(+)-Me** and (d) **MB** (5.0, 10 and 20 µM) on erythrocytes and under white light irradiation at an irradiance of 150 mW/cm^2. All values represent the average of three independent assays. Error bars indicate the standard deviation. LC - Light control (whole blood under light); DC - Dark control (whole blood incubated with PS without light).

3.3 Photodynamic inactivation of T4-like phage in PBS

After the erythrocyte osmotic fragility tests, it was observed that the PS concentrations of 5.0 and 10 µM did not promote haemolysis at the isotonic conditions. Therefore, the PS concentration of 10 µM was chosen in accordance with identical studies performed in our group to photoinactivate bacteria (Marciel et al., 2017) and fungi (Sousa et al., 2019) and considering its further application in plasma and blood assays since complex matrices require higher PS concentrations. Thus, all PSs were tested in PBS at 10 µM with an irradiation of 25 mW/cm^2 for 60 min.

The results obtained are summarized in Fig. 7 and show that all PSs efficiently inactivated the T4-like phage till reaching the detection limit of the method, although at different irradiation times. **Tetra-S-Py(+)-Me** was the most effective PS (ANOVA, $p < 0.05$) since it was able to achieve the detection limit of the method at 15 min of irradiation. **Tri-Py(+)-Me** and **MB** attained this limit at 30 min of irradiation. **Tetra-Py(+)-Me** was the least effective in PBS, because the detection limit was reached only after 60 min of irradiation.

Light and dark controls demonstrated no significant discrepancy (ANOVA, $p > 0.05$) in the T4-like phage photoinactivation for all PSs. These observations suggest that the viability of T4-like phage was not affected by the irradiation in the PS absence (light control) and by the PSs in the light absence (dark control). So, just the combination of light with the PS was responsible by the toxic effect during the aPDT procedure.

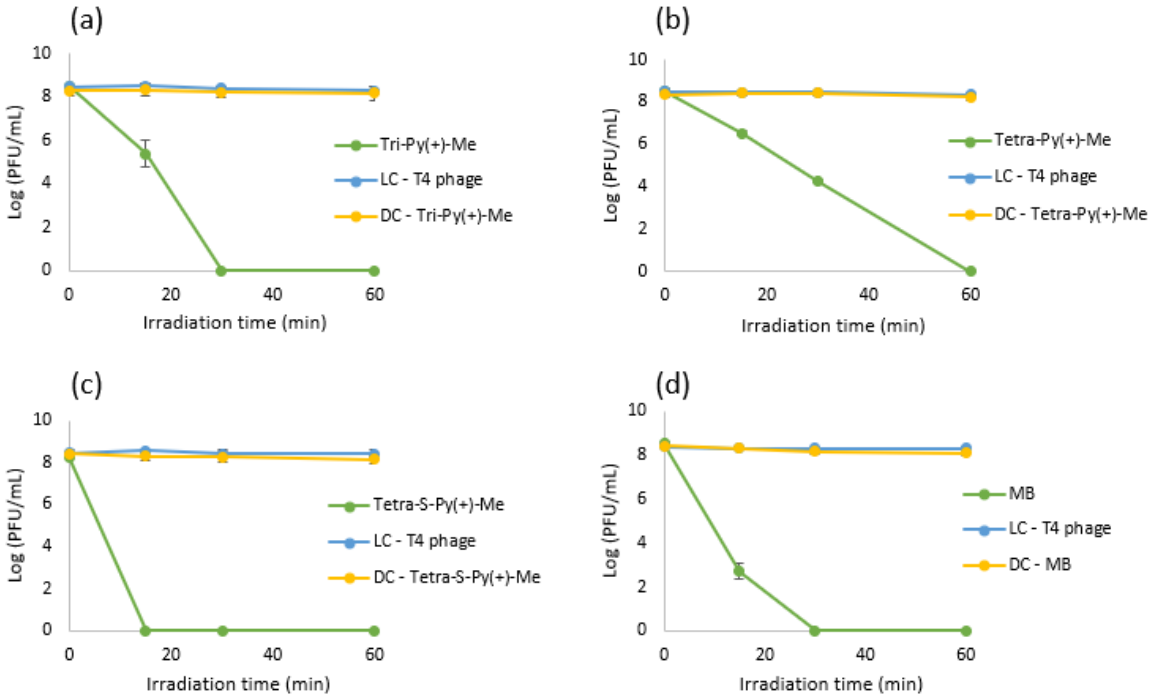

Figure 7 – T4-like phage photoinactivation in PBS in the presence of (a) **Tri-Py(+)-Me**, (b) **Tetra-Py(+)-Me**, (c) **Tetra-S-Py(+)-Me** and (d) **MB** at 10 µM and under white light irradiation at an irradiance of 25 mW/cm^2. All values represent the average of three independent assays in duplicate. Error bars indicate the standard deviation. Lines were used to connect the data points. LC: Light control; DC: Dark control.

3.4 Photodynamic inactivation of T4-like phage in plasma

All PSs under study were tested in plasma at 10 μM with an irradiation of 150 mW/cm^2. Considering the high complexity of the biological matrix in relation to PBS, it was necessary to increase the light irradiance to 150 mW/cm^2 in plasma and in whole blood assays. For the same reason, the irradiation time was also increased to 270 min. These conditions were also chosen in accordance with similar studies performed in our group to photoinactivate bacteria (Marciel et al., 2017) and fungi (Sousa et al., 2019).

The results obtained are summarized in Fig. 8 and demonstrate large differences when compared to the PBS assays. For the three porphyrinic PSs, T4-like phage photoinactivation was only observed after 90 min of irradiation. After this period, significant differences (ANOVA, $p < 0.05$) between the samples and controls were observed at 180 and 270 min of irradiation. Furthermore, only **Tetra-S-Py(+)-Me** reached the detection limit of the method after 270 min of irradiation (causing a reduction of 8 $\log_{10}$ PFU/mL (ANOVA, $p < 0.05$)). A reduction of 5.1 $\log_{10}$ PFU/mL (ANOVA, $p < 0.05$) in the phage survival was observed for **Tri-Py(+)-Me** and 4.4 $\log_{10}$ PFU/mL (ANOVA, $p < 0.05$) for **Tetra-Py(+)-Me** after 270 min of irradiation. Regarding to **MB**, this approved PS revealed a different profile when compared to porphyrinic derivatives. A reduction in phage viability of 1.5 $\log_{10}$ PFU/mL occurred after 15 min of irradiation (ANOVA, $p < 0.05$). After 30 min of irradiation, **MB** caused a decrease of 3 $\log_{10}$ PFU/mL (ANOVA, $p < 0.05$) in the phage concentration and no further reduction was detected in the following irradiation period between 30 and 270 min. All PSs demonstrated significant differences (ANOVA, $p < 0.05$) among themselves after aPDT treatment (at 270 min of irradiation).

Light and dark controls indicated no significant differences (ANOVA, $p > 0.05$) in the T4-like phage photoinactivation, which means that only irradiation or PS in the dark do not interfere with T4-like phage viability.

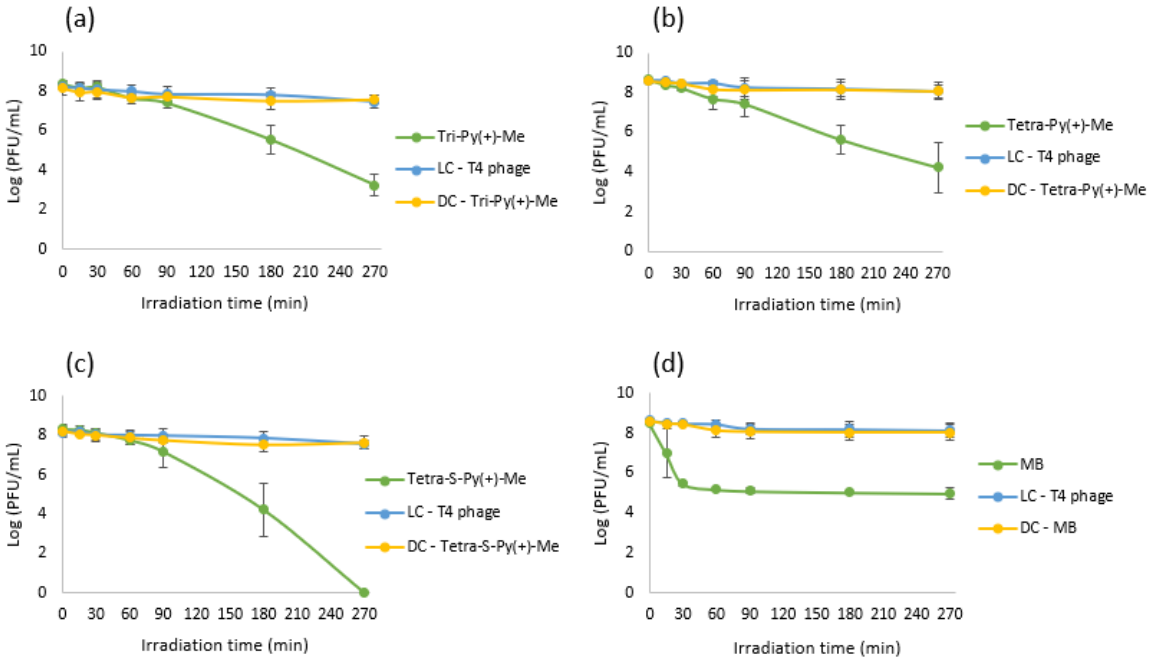

Figure 8 – T4-like phage photoinactivation in plasma in the presence of (a) **Tri-Py(+)-Me**, (b) **Tetra-Py(+)-Me**, (c) **Tetra-S-Py(+)-Me** and (d) **MB** at 10 µM and under white light irradiation at an irradiance of 150 mW/cm^2. All values represent the average of three independent assays in duplicate. Error bars indicate the standard deviation. Lines were used to connect the data points. LC: Light control; DC: Dark control.

3.5 Photodynamic inactivation of T4-like phage in whole blood

Since **Tetra-S-Py(+)-Me** was the most effective PS in plasma, it was used for the photoinactivation of T4-like phage in whole blood at the same PS concentration (10 µM), using also white light at an irradiance of 150 mW/cm^2.

The results obtained are summarized in Fig. 9 and demonstrate significant differences when compared to PBS and plasma assays. Although **Tetra-S-Py(+)-Me** reaches the detection limit of the method in PBS (after 15 min of white light irradiation at 25 mW/cm^2) and plasma (after 270 min of white light irradiation at 150 mW/cm^2) assays, this PS caused a decrease of 1 $\log_{10}$ PFU/mL (ANOVA, $p < 0.05$) in the T4-like phage concentration in whole blood after 270 min of white light irradiation and there are no significant differences (ANOVA, $p > 0.05$) between the sample and controls in the previous irradiation times.

Light and dark controls proved that the viability of T4-like phage was not affected by light and PS in the dark (ANOVA, $p > 0.05$), as it was verified in PBS and plasma assays.

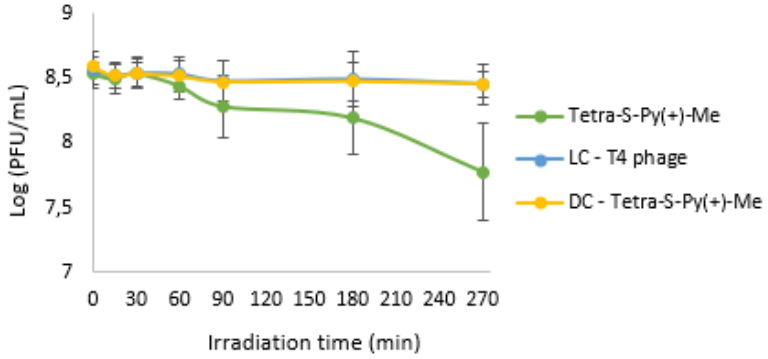

Figure 9 – T4-like phage photoinactivation in whole blood in the presence of **Tetra-S-Py(+)-Me** at 10 µM and under white light irradiation at an irradiance of 150 mW/cm^2. All values represent the average of three independent assays in duplicate. Error bars indicate the standard deviation. Lines were used to connect the data points. LC: Light control; DC: Dark control.

4. Discussion

Despite the improvement in blood safety, there is still an associated risk with the transmission of infections by blood supply. Furthermore, blood treatments are currently only available for plasma and platelets and are still under study for erythrocytes and whole blood (Solheim, 2008; Politis et al., 2014; Salunkhe et al., 2015; Marciel et al., 2017). Consequently, the development of new blood disinfection alternatives is essential to increase the safety and quality of blood supply.

aPDT is an alternative method and three non-porphyrinic PSs are already approved to treat plasma and platelets, but not to treat erythrocytes and whole blood (Bihl et al., 2007; Salunkhe et al., 2015). However, recently it has been shown that aPDT with tetrapyrrolic PSs is a promising technique to inactivate bacteria (Marciel et al., 2017) and fungi (Sousa et al., 2019), not only in plasma but also in the whole blood. Therefore, the aim of this study was to evaluate the effectiveness of three porphyrinic PSs (**Tri-Py(+)-Me**, **Tetra-Py(+)-Me** and **Tetra-S-Py(+)-Me**) and to compare their efficiency with the **MB** efficacy (an already approved PS to disinfect plasma) to photoinactivate viruses in plasma and whole blood. Viruses are usually transmitted by blood-to-blood contact and, hence, present a significant risk of blood-borne infection. Consequently, viruses screening for the presence of HIV, hepatitis B virus and hepatitis C virus for all blood donations is recommended (Salunkhe et al., 2015; Di Minno et al., 2016). However, as viral quantification techniques are time-consuming and labour-intensive processes or detect only viral nucleic acid and do not determine infectivity, when new PSs compounds are initially evaluated, bacteriophages can be useful as surrogates of mammalian viruses (Costa et al., 2012). Thus, in this study, the effectiveness of the different PSs on inactivation of viruses was evaluated using the T4-like phage as a model of mammalian viruses (Costa et al., 2008; Costa et al., 2012).

None of the PS approved currently to disinfect plasma and platelets is approved for concentrated erythrocytes and whole blood because of the side effects observed in these cells after aPDT procedures (Wainwright, 2000; Marciel et al., 2017). Therefore, considering that aPDT

treatment must not affect the blood elements, the erythrocyte osmotic fragility tests were performed to determine a safe PS concentration. In addition, it is important to evaluate the possible side effects of aPDT on erythrocytes after a transfusion with aPDT treated blood derivates in order to pave its application in the clinic. The results demonstrated that **Tri-Py(+)-Me** (Marciel et al., 2017), **Tetra-Py(+)-Me** (Fig. 2) and **Tetra-S-Py(+)-Me** (Fig. 3) did not promote significant haemolysis for 0.5-0.9% NaCl at 5.0 μM. At concentration of 10 μM, despite an increase in haemolysis values for 0.5% NaCl, no significant haemolysis was detected at 0.7 and 0.9% NaCl. So, these PSs did not induce haemolysis at the nonstress and isotonic conditions (0.9% NaCl), which means that these PSs concentrations are safe for disinfection of blood and blood products. The evaluation of aPDT effect on erythrocytes concentration confirms the results of the membrane fragility tests. No significant alterations were detected for **Tri-Py(+)-Me**, **Tetra-Py(+)-Me**, **Tetra-S-Py(+)-Me** and **MB** (Fig. 6) at all tested concentrations after aPDT treatment.

However, all the porphyrinic derivatives tested in this study seems to fragilize the erythrocyte membrane at concentrations of 20 μM even for the isotonic conditions (0.9% NaCl). In fact, earlier studies have reported that porphyrins affected the erythrocyte membrane at higher concentrations (Beaton et al., 1995; Bolodon et al., 1996). However, for the three porphyrinic PSs tested, it is important to note that similar osmotic fragility results were obtained for the dark controls and for the samples and there are no significant differences in erythrocyte membrane fragility before and after aPDT treatment. This seems to indicate that the osmotic stress in erythrocytes is caused by the porphyrin concentration at 20 μM and not by the irradiation treatment and/or the presence of ROS resulting from PS activation (Marciel et al., 2017).

Unlike porphyrins, **MB** (Fig. 4) does not cause toxicity and further haemolysis in erythrocytes for 0.5-0.9% NaCl at all concentrations tested, even at 20 μM. Stock solutions of all porphyrinic PSs were prepared in DMSO whereas **MB** was prepared in PBS, suggesting that DMSO can be related to the high haemolysis rates when porphyrins were used. Erythrocyte osmotic fragility tests with DMSO and without PS (Fig. 5) demonstrated that this solvent presents a similar haemolysis profile to porphyrins at 5.0, 10 and 20 μM. DMSO at 1 and 2% (corresponding to the DMSO percentage used when the PS concentration of 5.0 and 10 μM are used, respectively) caused an increase in haemolysis values for 0.5% NaCl, but no significant haemolysis was detected for 0.7 and 0.9% NaCl. For DMSO at 4% (corresponding to the DMSO percentage used when the PS concentration of 20 μM is used), a high haemolysis rate at the isotonic conditions (0.9% NaCl) after the aPDT treatment was detected (such as in the porphyrinic PSs). For example, **Tetra-Py(+)-Me** at 20 μM and DMSO at 4% promoted 55% and 45% of haemolysis at the isotonic conditions after the aPDT treatment, respectively, which means that this solvent contributes to haemolysis caused on the erythrocytes when the porphyrinic PSs at 20 μM are used. Therefore, new solubilizing systems for stock solutions preparation of porphyrinic compounds should be considered (Wikene et al., 2016). Furthermore, drug-delivery systems, such as micelles or liposomes, can also be an alternative for blood disinfection by aPDT (Mesquita et al., 2018).

Nonetheless, as already mentioned, it is important to emphasize that these porphyrins did not induce haemolysis at 5.0 and 10 μM at the isotonic conditions. Therefore, the PS concentration of 10 μM was chosen considering that plasma and whole blood are complex matrices with large amount of organic matter and require higher PS concentrations for an effective photoinactivation.

Buffered solutions, such as PBS, are useful to evaluate the behaviour and efficacy of the PSs in a medium without organic matter and cell interference in order to select the best PS and the more appropriate aPDT conditions. However, as the composition of test matrix is important in aPDT, in order to pave the clinical application, it is required to test the PSs in clinically relevant setting such as in plasma and whole blood, which is the case of this study.

According to the literature, the photoinactivation of microorganisms effectiveness in blood derivates decreases with the increase of sample complexity and the presence of organic matter (Orlandi et al., 2012; Marciel et al., 2017; Corrêa et al., 2019). Plasma contains various proteins, clotting factors and metabolic products in suspension, while whole blood has more organic matter because it includes the blood figurative elements (red blood cells, leukocytes and platelets) (WHO, 2002). Thus, the efficacy of aPDT is expected to be lower in plasma and whole blood in relation to PBS. In fact, the results obtained in this study (Figs. 7, 8 and 9) demonstrated that the efficiency of aPDT to inactivate the T4-like phage decreased with the increase of sample complexity (PBS, plasma and whole blood, respectively). The decrease of aPDT efficacy with the increase of organic matter can be related to the possible nonspecific binding of PS to proteins of plasma and whole blood and to the coating elements of the cell membranes. Light penetration can also be hampered by the presence of organic matter (Solheim, 2008; Mundt et al., 2014; Marciel et al., 2017; Kim et al., 2018).

The efficacy of all the PSs tested decreased in the plasma assays regarding to PBS. **Tri-Py(+)-Me** inactivated the T4-like phage until the detection limit of the method (reduction of 8 $\log_{10}$ PFU/mL) after 30 min of irradiation in PBS (Fig. 7) and caused a T4-like phage inactivation of 5.1 $\log_{10}$ PFU/mL after 270 min of irradiation in plasma (Fig. 8). **Tetra-Py(+)-Me** inactivated the T4-like phage until the detection limit of the method (reduction of 8 $\log_{10}$ PFU/mL) after 60 min of irradiation in PBS (Fig. 7) and caused a T4-like phage inactivation of 4.4 $\log_{10}$ PFU/mL after 270 min of irradiation in plasma (Fig. 8). **Tetra-S-Py(+)-Me** was the most effective PS, inactivating the T4-like phage until the detection limit of the method not only in PBS (Fig. 7) but also in the plasma (Fig. 8), with a reduction of 8 $\log_{10}$ PFU/mL after 15 and 270 min of white light irradiation, respectively.

Although **Tetra-Py(+)-Me** and **Tetra-S-Py(+)-Me** are both symmetric porphyrins with four positive charges, their effectiveness to photoinactivate the T4-like phage was significantly different. Though the singlet oxygen ($^1O_2$) production is an important factor to have in account in the damage of pathogenic microorganisms (Costa et al., 2012), **Tetra-S-Py(+)-Me** produces less amounts of singlet oxygen than **Tetra-Py(+)-Me** (Gomes et al., 2011; Simões et al., 2016). So, the highest efficiency of **Tetra-S-Py(+)-Me** in PBS and plasma is not related to $^1O_2$ production, which means that the PSs structures can explain their different efficacy. These two cationic PSs have different peripheral substituents decorating the macrocycle core, resulting in different PS physicochemical properties. In **Tetra-Py(+)-Me**, the cationic methylpyridinium units are directly linked to the *meso* positions of macrocycle core, while in **Tetra-S-Py(+)-Me**, there is a spacer between the porphyrin core and the less rigid methylpyridinium-4-sulphanyl subunit (Gomes et al., 2011). The higher efficacy of viral inactivation by the **Tetra-S-Py(+)-Me** in relation to **Tetra-Py(+)-Me**, mainly in plasma, can be due to a higher rotational mobility in **Tetra-S-Py(+)-Me** of the cationic unit allowing a better targeted adhesion to the phage particles.

The increase in the number of positive charges bearing by porphyrin PSs may also increase the aPDT efficiency (Costa et al., 2008; Alves et al., 2009; Marciel et al., 2018). However, the **Tri-Py(+)-Me** (bearing three positive charges) demonstrated to be significantly more effectiveness in the T4-like phage photoinactivation in PBS and plasma than **Tetra-Py(+)-Me** (four positive charges). The higher efficacy of the **Tri-Py(+)-Me** when compared to **Tetra-Py(+)-Me** is in accordance with previous results from the group (Costa et al., 2008; Alves et al., 2009). This may be due to the asymmetric charge distribution at the periphery of **Tri-Py(+)-Me**, that increases the amphiphilic character of porphyrin and, consequently, improves its affinity for microorganism targets. In fact, asymmetric PSs are significantly more effective than symmetric PSs, such as **Tetra-Py(+)-Me** (Alves et al., 2009; Simões et al., 2016). Nevertheless, the **Tetra-S-Py(+)-Me** (a symmetric porphyrin with four positive charges) proved to be significantly more effectiveness in T4-like phage photoinactivation in PBS and plasma than **Tri-Py(+)-Me**. Considering that **Tetra-S-Py(+)-Me** produces less amount of $^1O_2$ than **Tri-Py(+)-Me** (Gomes et al., 2011; Simões et al., 2016), the higher photodynamic efficiency in complex samples, such as plasma, besides the positive charges number and PS symmetry, the higher mobility of the peripherical substituent groups of PS can be an important factor to be taking into account in the PS selection (Gomes et al., 2011).

As opposed to the T4-like phage inactivation in PBS, in plasma it is remarkable the efficiency of porphyrins in viruses photoinactivation when compared to the efficacy of **MB**, that is already approved for plasma disinfection. The T4-like phage inactivation with **MB** reached the detection limit of the method (reduction of 8 $\log_{10}$ PFU/mL) after 30 min of irradiation in PBS (Fig. 7) and caused a T4-like phage inactivation of 3 $\log_{10}$ PFU/mL after 270 min of irradiation in plasma (Fig. 8). Although the **MB** efficiency in the inactivation of microorganisms is highly dependent on the pH of solution, PBS (pH 7.4) and plasma (pH 7.35 to 7.45) (Waugh and Grant, 2007) have a similar pH, which indicates that other factors can be responsible for the high difference of aPDT effectiveness between PBS and plasma. The high organic content of plasma can explain the significantly lower T4-like phage inactivation with **MB** in plasma, but also the **MB** photobleaching due to the high irradiance used (150 mW/cm^2) in the photoinactivation plasma assays. In fact, after 30 min of treatment, the T4-like phage concentration remains constant, which seems to indicate that no more **MB** is available to produce ROS responsible for the T4-like phage inactivation.

Therefore, cationic porphyrins, mainly **Tetra-S-Py(+)-Me**, can be promising PSs for future clinical application in the inactivation of viruses in plasma. Furthermore, there are other studies that also demonstrated the effectiveness of cationic porphyrins in virus photoinactivation (Casteel et al., 2004; Zupán et al., 2008).

As **Tetra-S-Py(+)-Me** was the most effective PS to inactivate the T4-like phage in plasma, it was tested in whole blood. Despite the promising results in PBS and plasma, the photoinactivation rate of T4-like phage in whole blood (Fig. 9) was limited (1 $\log_{10}$ PFU/mL after 270 min of irradiation). Identical studies using porphyrins as PS to inactivate other microorganisms in whole blood showed also lower inactivation (Marciel et al., 2017; Corrêa et al., 2019; Sousa et al., 2019). This can be due to the large amount of organic matter present in whole blood, but also by the different location of microorganisms in the both matrices. In plasma the pathogens may only be in suspension whereas in whole blood they can also be associated with cells (Wainwright, 2002; Marciel et al., 2017) and thus may hinder the PS action. Moreover, another reason may be based

on the action of haemoglobin. In fact, it is possible that the heme group (the iron(II) complex of protoporphyrin IX) present in haemoglobin and responsible by the oxygen transport, acts as oxygen scavenger and consequently less molecular oxygen is available to generate the required ROS for an efficient photodynamic action (Sil et al., 2004; Kufner et al., 2005; Montellano, 2008; Costa et al., 2012). Given this fact, the PSs effectiveness can be affected and consequently reduced. Furthermore, haemoglobin absorbs light comprising a large fraction of the visible light and this can also contribute to the PS reduced efficacy in whole blood (Kim et al., 2018). Thus, haemoglobin can also prejudice the aPDT efficacy by absorbing the required light for PS activation.

Since there are only three PSs approved for plasma disinfection, the results obtained in this study suggest that the tested porphyrinic PSs, mainly **Tetra-S-Py(+)-Me**, are promising alternative PSs for photoinactivation of viruses in plasma. Nevertheless, considering that PS concentration should be as low as possible for clinical application, further studies should be done at concentrations below 10 μM. In order to increase the effectiveness of photoinactivation of T4-like phage in whole blood, it is required to block the effects of haemoglobin, mainly testing PS with absorptions profile far from the haemoglobin one (Kim et al., 2018). So, a different light dose and other light sources must also be tested.

5. Conclusions

As conclusion, the effective photoinactivation of T4-like phage with cationic porphyrinic PSs in plasma indicates that this class of compounds can be successfully used on aPDT, mainly the **Tetra-S-Py(+)-Me**. The three porphyrins can be promising PSs for virus inactivation in plasma, allowing a higher and fast viruses inactivation than the already approved **MB**.

The established protocol is not so effective for whole blood due to the aPDT blocking effects caused by the complexity of the matrix and by the presence of haemoglobin, which can trap molecular oxygen and to absorb part of the light irradiation available to the photodynamic process. Consequently, improvements relatively to light dose and light source may be considered.

As the DMSO used to prepare porphyrinic stock solutions demonstrated to affect the erythrocytes integrity at 4%, new solubilizing systems must be considered.

6. Future perspectives

aPDT is already a technique used in many countries around the world to disinfect plasma and platelets and there are three approved PSs for this purpose. Though, there is no approved PSs for application in erythrocyte concentrates and whole blood due to possible side effects for red blood cells. Therefore, further studies involving other light sources and light doses are needed to optimize this approach and the effectiveness of viral photoinactivation in whole blood. It is also important to develop safer PSs solubilizers and delivery systems, such as micelles or liposomes. The development of PSs removal methods after aPDT treatment is also needed to prevent non-target cells damage. Studies to evaluate a possible injury in erythrocytes are also required.

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APPENDIX

An insight into the synthesis of cationic porphyrin-imidazole derivatives and their photoinactivation efficiency against *Escherichia coli*

ABSTRACT

New cationic porphyrin-imidazole derivatives were synthesized from Radziszewski reaction between 2-formyl-tetraphenylporphyrin **1** and several 1,2-diones, followed cationization affording the promising photosensitizers **3a-d**. Singlet oxygen studies have demonstrated that all the cationic porphyrin-imidazole conjugates **3a–d** were capable to produce cytotoxic species. These photosensitisers were able to photoinactivate *Escherichia coli* and its inactivation profile was improved in the KI presence.

1. Introduction

In the last years, antimicrobial resistance problem has been intensified and it is considered a global threat to human health. Infections caused by resistant bacteria may not be treated with antibiotics, are a serious threat to public health, with an increased risk of morbidity and mortality, increasing the healthcare costs.[1] The spread of drug resistant bacteria and the lack of new antibiotics, requires that new alternative therapies to antibiotics have to be developed urgently.

Antimicrobial Photodynamic Therapy (aPDT) is a promising alternative to antibiotic treatment and involves the use of a photosensitizer (PS) which in the presence of light and oxygen produces reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), causing microbial inactivation.[2,3] aPDT is a multitarget therapy, acting mainly on the outer structures, affecting also nucleic acids.[4,2] Consequently, the development of resistance to this therapy is highly improbable.[5,3] aPDT has already proved its efficiency against Gram-positive and Gram- negative bacteria, viruses, fungi and other parasites.[6]

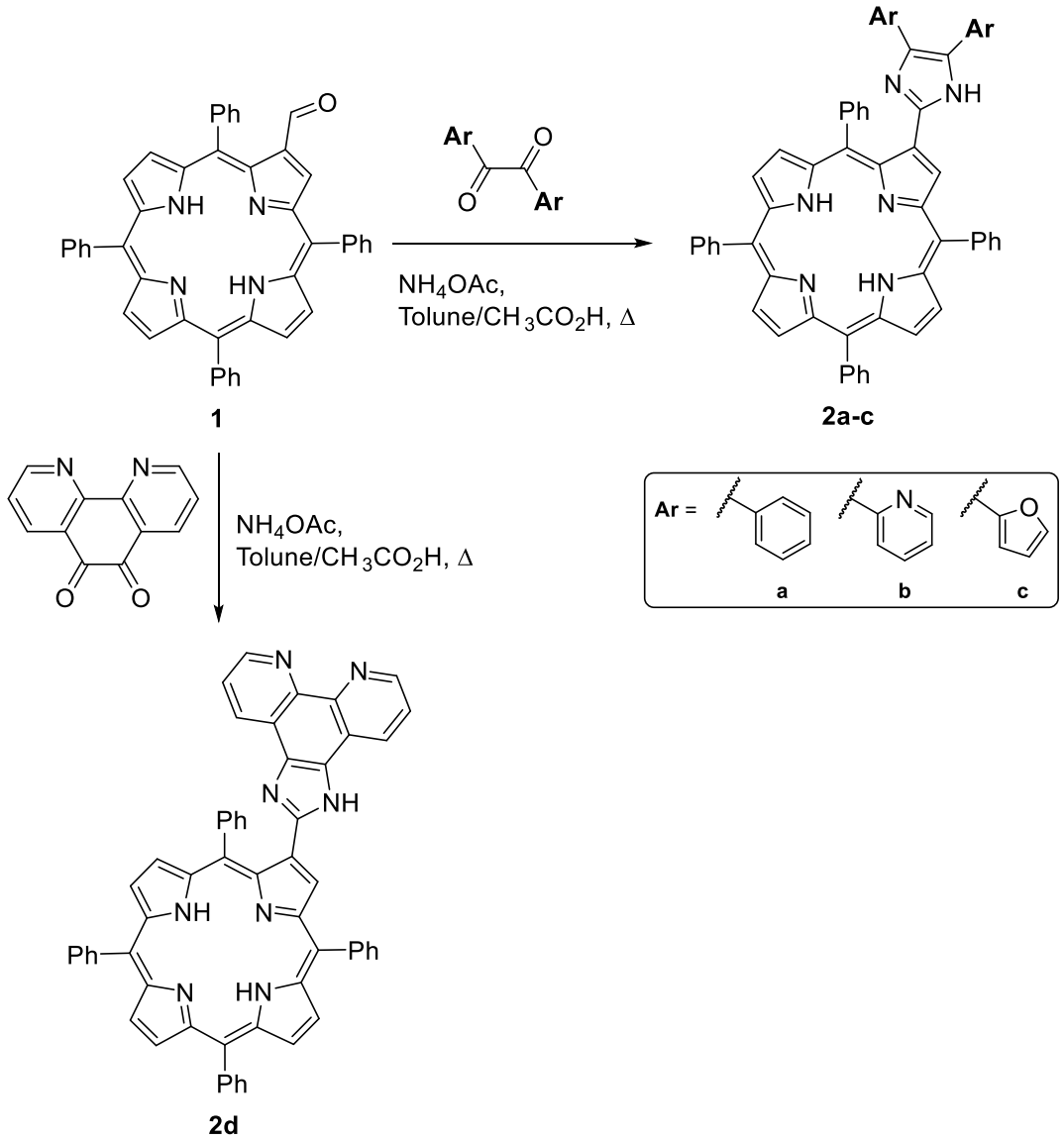
Among the PSs studied in the photoinactivation of microorganisms by aPDT, porphyrins are, unquestionably, the most considered class of compounds. Nowadays, several research groups are involved in the study of new synthetic methodologies for porphyrin derivatives with appropriate features for their application in aPDT.[7,8] One of the strategies used consists of coupling different compounds with well-established pharmacological activities. In this context, porphyrins can be linked to other biologically active molecules, aiming to increase their biological efficacy.[7,9] Having in mind the already known photodynamic effect of porphyrins and the antimicrobial, fungicidal and cytotoxic activity of imidazoles,[10] in this work we reported the synthesis and photodynamic activity of novel cationic porphyrin-imidazole derivatives. The synthesis of other porphyrin/imidazole conjugates has already been reported by our group and proved to be efficient in the photoinactivation of *Escherichia coli*.[11] The potentiation of aPDT efficiency with these new cationic porphyrin-imidazole derivatives by potassium iodide was also accessed.[12] The KI reacts with $^1O_2$, affording free iodine (I_2/I_3^-), hydrogen peroxide (H_2O_2) and iodine radicals ($I_2^{\cdot-}$), that are extremely bactericidal.[12-13] In fact, several in vitro and in vivo studies have shown the potentiation of porphyrins, fullerenes, rose bengal and methylene blue by KI towards several

microorganisms such as *E. coli*, *Acinetobacter baumannii*, *P. aeruginosa*, C*. albicans* and methicillin-resistant *S. aureus*.[12-13, 13e]

2. Results and Discussion

2.1 Synthesis and photophysical properties of porphyrin-imidazole derivatives

Mono-cationic porphyrin derivatives **3a**-**d** were prepared from the correspondent neutral derivatives **2a**-**d**, which were synthesized by a methodology involving a Radziszewski reaction from 2-formyl-5,10,15,20-tetraphenylporphyrin **1** and as outlined in scheme 1.



Scheme 1. Radziszewski reaction between 2-formyl-tetraphenylporphyrin **1** and a series of 1,2-diones.

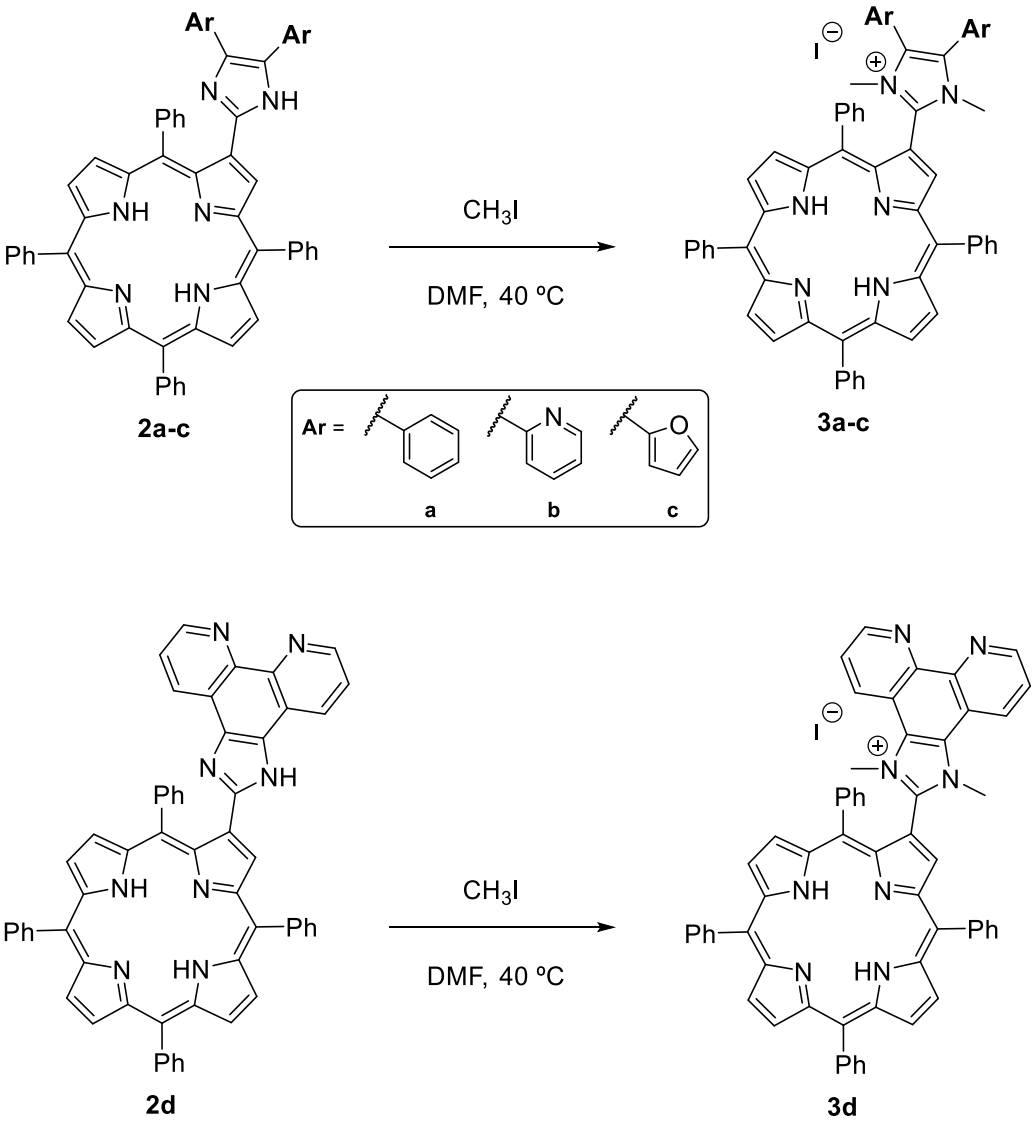
The reaction of 2-formyl-tetraphenylporphyrin **1** and a series of 1,2-diones (benzil, 2,2´-pyridil and α-furil) was performed in the presence of ammonium acetate in refluxing toluene. The mixture was stirred for 1-3 hours, after this period the reaction control by TLC showed the full consumption of the starting material **1**. After the workup and purification by column chromatography, the corresponding porphyrin-imidazole derivative **2a**-**c** were isolated in excellent yields, ranging from 79 to 88 % (Table 1).

Table 1. Yields of compounds **2a-d** obtained from the Radziszewski reaction between 2-formyl-tetraphenylporphyrin **1** and a series of 1,2-diones.

Entry	Compound	Time (h)	Yield (%)
1	**2a**	3	79
2	**2b**	3	84
3	**2c**	1	88
4	**2d**	2	99

The reaction was extended to 1,10-phenanthroline-5,6-dione under the same reactional conditions, after 2 hours the expected porphyrin-imidazo-phenanthroline derivative **2d** was isolated in almost quantitative yield (99%).

The cationization/alkylation of the imidazole moiety of derivatives **2a**-**d** was performed in the presence of an excess of methyl iodide, as the alkylating agent, in *N*,*N*´-dimethylformamide (DMF) for 24 h at 40 ºC as is outlined in Scheme 2. After the workup, the TLC control confirmed the conversion of all the starting porphyrin-imidazole derivatives **2a**-**d** into more polar compounds. These new derivatives were identified as the mono-cationic products **3a**-**d** and were obtained pure in excellent yields (81-97%), directly from crystallization in CH_2Cl_2/hexane.



Scheme 2. Alkylation/cationization reaction of compounds **2a-d**.

It is worth to refer that when the cationization/alkylation of compounds **2b** and **2d** was performed, only the mono-cationic derivatives and no products from the additional quaternization of the nitrogens from the pyridine and phenanthroline units were detected.

The structural elucidation of all the compounds involved the use of 1D (^{1}H and ^{13}C spectra) and 2D (^{1}H/^{1}H COSY) NMR techniques, HRMS and UV-Vis spectroscopy. The ^{1}H NMR spectra of the neutral derivatives **2a**-**d** clearly shows the formation of the imidazole ring and are consistent with β-substituted porphyrins, showing a prominent singlet in the range from δ 13.35 ppm to δ 12.25 ppm due to the resonance of the N-*H* proton from the imidazole ring and the resonance of the corresponding H-3 as a singlet at *ca.* δ 9.2 ppm or under the multiplet at *ca.* δ 8.9 ppm due to the resonances of the other β-pyrrolic protons. The resonances of the protons from the *meso*-phenyl groups appear between *ca.* δ 8.3 ppm and *ca.* δ 7.7 ppm and the *meta* and *para* protons of one of the phenyl rings are deprotected by the presence of the new imidazole core and the signals of their resonances ranging from δ 7.26 ppm and *ca.* δ 6.60 ppm.

The most relevant features of the ^{1}H NMR of the mono-cationic derivatives **2a-d** are the absence of the signal due to the resonance of the imidazole N-*H* proton and the appearance of a new distinctively singlet in the aliphatic region ranging from δ3.82 ppm and *ca.* δ3.12 ppm due to the resonances of the protons from the methyl groups.

In both, neutral (**2a-d**) and cationic derivatives (**3a-d**), the ^{1}H NMR presents the characteristic singlet at *ca.* δ-2.7 ppm due to the resonances of the inner N-*H* protons, confirming that all the new derivatives synthesised are in the *free-base* form.

The analysis of the HRMS-ESI(+) spectra shows the presence of a peak with *m/z* corresponding to the $[M+H]^+$ and $[M]^+$ ions, for the neutral derivatives (**2a-d**) and mono-cationic derivatives (**3a-d**), respectively.

The electronic spectra of the neutral and mono-cationic derivatives were recorded and the ability of the cationic derivatives to produce oxygen singlet was measured. For a better evaluation of the influence of the different substituents in the photophysical parameters, all the studies were performed in the same solvent, dimethylformamide (DMF).

The Figure 1 shows the absorption spectra in DMF solutions at a concentration of 3.0 x 10^{-6} M recorded at 298K of compound **2a** and the correspondent mono-charged derivative **3a**, as a representative example for both series of compounds synthesised.

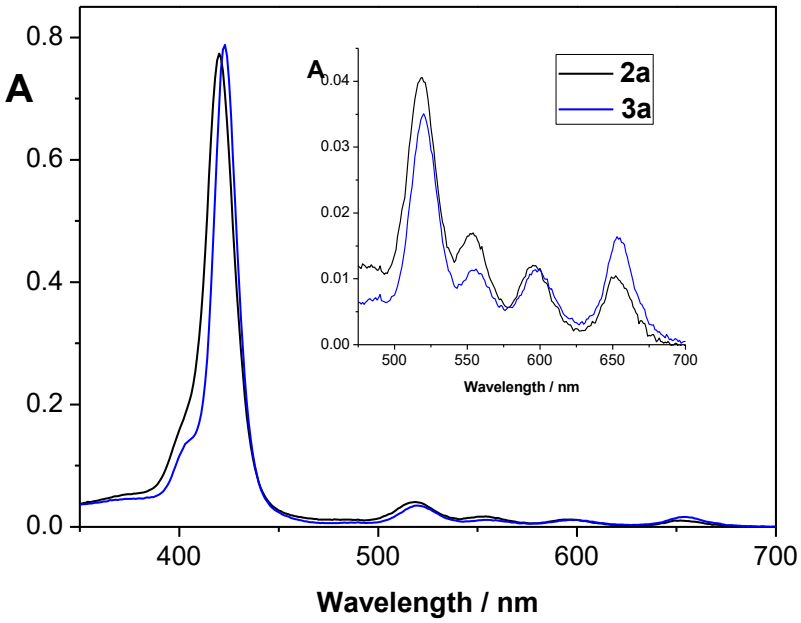


Figure 1. Absorption spectra of compounds **2a** (black line) and **3a** (blue line) at a concentration of 3.0 x 10^{-6} M in DMF at 298K. The inset shows the absorption at the Q bands region.

In general, the electronic spectra shows the typical features of free-bases *meso*-tetraarylporphyrin substituted at beta position, with a strong *Soret* band centred at *ca.* 420 nm and well-defined four Q bands between 518 and 652 nm due to π–π* transitions.[14] When compared with the corresponding neutral derivative, the absorption *Soret* band of the products resulting from the cationization/alkylation of the imidazole moiety is red-shifted 3 nm; however,

no significant changes were observed into the Q bands zone. In both series, the sharp *Soret* band observed, allowing to conclude that the porphyrinic derivatives are not aggregated in DMF.[14c]

It is well established that one of the most relevant features of a photosensitizer is their ability to generate reactive oxygen species (ROS), namely singlet oxygen ($^1O_2$), which is strongly related with the efficiency of the photodynamic process.[15]

The ability of the new synthesised mono-charged porphyrin-imidazole derivatives to generate singlet oxygen ($^1O_2$) was quantitatively assessed by monitoring the decomposition of 1,3-diphenylisobenzofuran (DPiBF) during irradiation with a red light at 630 ± 20 nm.[16] The first order decay observed for the yellow DPiBF at 415 nm results from its reaction with $^1O_2$, generated by the combined action of light, PS and dissolved oxygen, *via* a [4 + 2] cycloaddition affording a colourless *o*-dibenzoylbenzene.

The results obtained with the cationic porphyrins (**3a-d**) are represented in Figure 2 and confirm that all the studied porphyrins are able to induce the decay of DPiBF absorption within 10 min when irradiated with light at an irradiance of 11.0 mW cm^{-2}. As a reference, it was used 5,10,15,20-tetraphenylporphyrin (**TPP**) which is known to be a good oxygen generator.[17]

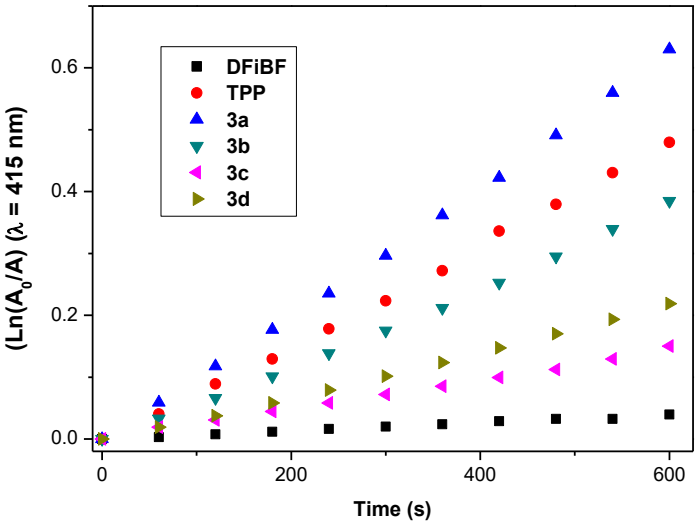


Figure 2. Time-dependent photodecomposition of DPiBF (50 µM) photosensitized by derivatives **3a-d** and TPP at 0.5 µM in DMF upon irradiation with red light LEDs (654 nm ± 20 nm) with an irradiance of 11.0 mW cm^{-2}.

The $^1O_2$ generation assessment results obtained with the cationic porphyrins **3a-d** confirm that all the studied mono-charged porphyrinic induce the decay of the DPiBF absorption when irradiated with light. It is worth to refer that the absorbance of DPiBF, when irradiated in the absence of a porphyrin derivative, remains almost unchanged.

The most efficient $^1O_2$ producer was derivative **3a** with 0.5 time-fold higher than the reference **TPP**. Compound **3b** present approximately the same ability to generate $^1O_2$ then the reference used in this study. However, the remaining derivatives **3c** and **3d** show a remarkable lower ability to generate $^1O_2$ than the one observed with **TPP**. These results show that the presence of heteroatoms at the substituents into the positions 4 and 5 of the imidazole moiety reduce the

capability to produce $^1O_2$. Nevertheless, the high ability of the compounds to generate $^1O_2$, namely for compound **3a**, after being exposed to light and oxygen, allowed to envisage their potentiality to be used as PSs in the photodynamic inactivation of microorganisms.

2.2 Photodynamic inactivation of *E. coli* using cationic porphyrin-imidazole derivatives 3a-d as PSs and KI

The photodynamic efficiency of the cationic porphyrin-imidazole derivatives **3a-d** was tested against a bioluminescent *E. coli* strain as a bacterial model. The bioluminescence approach provides a sensitive and innocuous procedure to detect the viability of microorganisms. The major advantage of this methodology being used in aPDT is the possibility to monitor the process in real-time, providing a sensitive and cost-effective methodology to evaluate the photodynamic effect. Moreover, the strong correlation between CFU and bioluminescent signal of the bioluminescent *E. coli* used in this work has already been proved and described.[18]

In order to evaluate the effect of PS on the *E. coli* photoinactivation efficiency, the compounds were tested at different porphyrin concentrations (5.0 and 20.0 μM) in the absence and in the presence of 100 mM of KI. The KI concentration was chosen in accordance with similar studies, which demonstrated that higher concentrations can limit the application in clinic area due to osmotic stress.[12] The photodynamic experiments were carried out under white light (380–700 nm) irradiation at an irradiance of 25 $mW.cm^{-2}$ for 90 min. The results obtained are summarized in Fig. 3 and had shown that the inactivation profile of bioluminescent *E. coli* with cationic porphyrin-imidazole derivatives **3a-d** is improved in the KI presence (Figure 3). In all cases, light control, KI control and dark controls did not promote a decrease in the bioluminescence *E. coli* signal, showing that the viability of recombinant bioluminescent *E. coli* was not affected by irradiation, by the presence of PS in the dark and by the presence of KI.

Porphyrin-imidazole derivative **3a** had promoted a decrease of 2.38 $\log_{10}$ RLU (ANOVA, $p<0.05$) and of 3.85 $\log_{10}$ RLU (ANOVA, $p<0.05$) in the bioluminescence of *E. coli* at 5 μM and 20 μM, respectively, after 90 min of irradiation (Figure 3 A). For both concentrations of derivative **3a**, the addition of KI at 100 mM had potentiated the photodynamic effect, causing a decrease of the *E. coli* viability till the detection limit of the method (decrease of 4.3 $\log_{10}$ RLU) after 75 min and 45 min of irradiation, for combination of porphyrin-imidazole derivative **3a** at 5.0 μM + KI and **3a** at 20 μM + KI, respectively.

In what concerns to the results achieved with porphyrin-imidazole **3b** it was possible to observe that this compound is capable to induce a reduction of 1.86 and 3.01 $\log_{10}$ RLU (ANOVA, $p<0.05$) in the viability of *E. coli* after 90 min of irradiation, at 5.0 μM and 20 μM, respectively (Figure 3 B). The combination of this PS with KI had drastically potentiated the photodynamic effect of this PS, since, for both tested concentrations inactivation to the detection limit of the method (decrease of 4.3 $\log_{10}$ RLU) was reached after 15 minutes of irradiation.

Porphyrin-imidazole derivative **3c** revealed to be the less efficient PS in the photoinactivation of bioluminescent *E. coli*, causing a decrease of 0.63 $\log_{10}$ RLU (ANOVA, $p<0.05$) in the viability of the bacterium, after 90 min of irradiation, for both used concentrations (Figure 3C). However, when this compound was used combined with KI the reduction of bioluminescent signal begins after 15 minutes of irradiation and the inactivation to the detection limit of the method (decrease

of 4.3 $\log_{10}$ RLU) was reached after 75 minutes of irradiation for 5 µM of **3c** and after 45 minutes of irradiation for 20 µM of **3c**.

The photodynamic profile of porphyrin-imidazole derivative **3d** was also more accentuated when this compound was combined with KI (Figure 3 D). In this case, the inactivation to the detection limit of the method (decrease of 4.3 $\log_{10}$ RLU) was reached after 75 min for combination of derivative **3d** at 5.0 µM + KI and after 30 minutes of irradiation of derivative **3d** at 20 µM + KI. When acting alone, porphyrin-imidazole derivative **3d** caused a decrease of 1.10 and 1.44 $\log_{10}$ RLU (ANOVA, $p<0.05$) in the viability of *E. coli*, after 90 min of irradiation, for 5.0 and 20 µM, respectively.

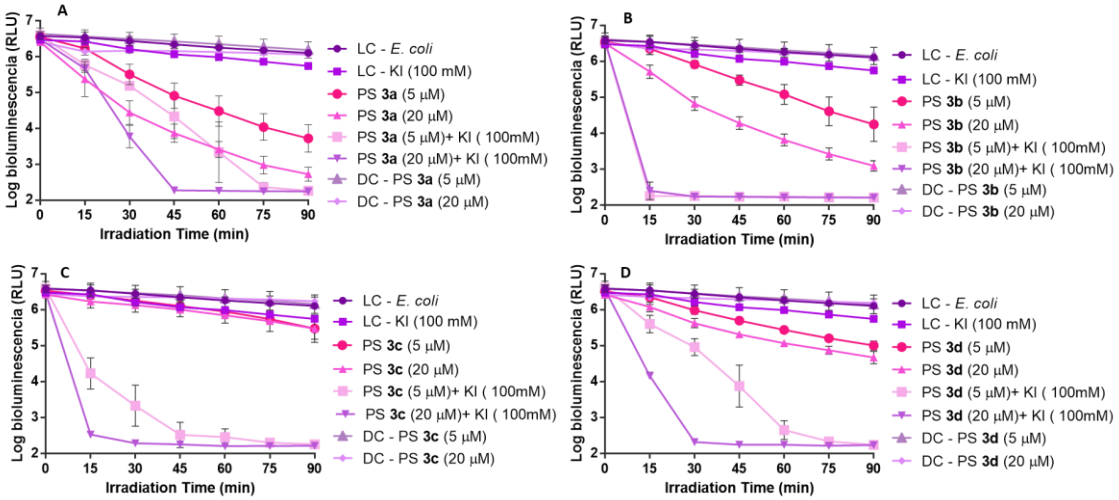


Figure 3. Survival of bioluminescent *E. coli* during aPDT assays in the presence of derivatives **3a** (A), **3b** (B), **3c** (C) and **3d** (D) at at 5.0 µM and 20 µM alone and combined with KI at 100 mM. The values are expressed as the three independent experiments; error bars indicate the SD.

The photodynamic efficiency of each PS was also correlated with the singlet oxygen generation. The capability of $^1O_2$ generation of cationic porphyrin-imidazole derivatives **3c** and **3d** was lower than the one achieved for derivatives **3a** and **3b**, which can justify the poor ability to photoinactivate bioluminescent *E. coli* of these PSs when acting alone. Even when combined with KI, these two PSs presented the slower curve of photoinactivation, attaining the detection limit latter than combinations of derivatives **3a** and **3b** with KI. Porphyrin-imidazole derivative **3a**, when acting alone, revealed to be the most promising PS achieving higher rates of inactivation of bioluminescent *E. coli*, which is in accordance with the $^1O_2$ generation studies. It is also important to highlight the results achieved with combination of derivative **3b** + KI, where the inactivation curve suffer a sharp decrease in the first 15 min of irradiation. Besides this PS was the second most capable to generate $^1O_2$, the combination with KI highly potentiated its photodynamic effect. Due to the abrupt decrease in the *E. coli* survival rate profile, it is possible to infer that the mechanism of action of the combination porphyrin-imidazole derivative **3b** + KI is probably related to the preferential decomposition of the peroxyiodide into free iodine (I_2/I_3^-).[12, 13d]

According to the American Society for Microbiology, cationic porphyrin-imidazole derivatives **3a** and **3b** at 20 µM and combinations of all cationic porphyrin-imidazole derivatives **3a-d** with KI

can be considered effective antimicrobial agents for *E. coli* inactivation, since promoted a reduction of at least 3 $\log_{10}$ in the viability of this bacterium.[19]

3. Experimental section

3.1 General remarks

^{1}H and ^{13}C solution NMR spectra were recorded on Bruker Avance 300 (300.13 and 75.47 MHz, respectively), 500 (500.13 and 125.76 MHz, respectively) and 700 (700.13 MHz) spectrometers. $CDCl_3$ and DMSO-d_6 were used as solvent and tetramethylsilane (TMS) as the internal reference; the chemical shifts are expressed in δ (ppm) and the coupling constants (*J*) in Hertz (Hz).

Unequivocal ^{1}H assignments were made using 2D COSY ($^{1}H/^{1}H$), while ^{13}C assignments were made on the basis of 2D HSQC ($^{1}H/^{13}C$) and HMBC (delay for long-range *J* C/H couplings were optimized for 7 Hz) experiments. Electrospray ionization mass spectra were acquired with a Micromass Q-Tof 2 (Micromass, Manchester, UK), operating in the positive ion mode, equipped with a Z-spray source, an electrospray probe and a syringe pump. Source and desolvation temperatures were 80 ºC and 150 ºC, respectively. Capillary voltage was 3000 V. The spectra were acquired at a nominal resolution of 9000 and at cone voltages of 30 V. Nebulisation and collision gases were N_2 and Ar, respectively. Porphyrin solutions in methanol were introduced at a 10 µL min^{-1} flow rate. Mass spectra HRMS were recorded on a LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) using methanol as the solvent. The absorption spectra were recorded on a UV-2501PC Shimadzu spectrophotometer using DMF as solvent. Preparative thin-layer chromatography was carried out on 20×20 cm glass plates coated with silica gel (0.5 mm thick). Column chromatography was carried out using silica gel (Merck, 35-70 mesh). Analytical TLC was carried out on precoated sheets with silica gel (Merck 60, 0.2 mm thick).

All the chemicals were used as supplied. Solvents were purified or dried according to the literature procedures.[20]

3.2 Synthesis

3.2.1 Synthesis of the porphyrin precursor TPP-CHO

The 2-formyl-5,10,15,20-tetraphenylporphyrin **TPP-CHO** was prepared from 5,10,15,20-tetraphenylporphyrinatocopper(II), *N,N´*-dimethylformamide (DMF) and phosphorus oxychloride ($POCl_3$), according to literature procedure.[21]

3.2.2 Synthesis of neutral derivatives 2a-d.

To a solution of 2-formyl-5,10,15,20-tetraphenylporphyrin **1** (25 mg, 3.9 $x10^{-5}$ mol) in a toluene:acetic acid 5:1 mixture (1.5 mL) was added 1.5 equiv. of the appropriate dione and ammonium acetate (10 equiv.) and the mixture was heated at reflux under magnetic stirring in the range of 1 to 3 h. It was allowed to cool to room temperature, diluted with CH_2Cl_2 (5 mL), neutralized with an aqueous $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic layer was dried (Na_2SO_4) and the solvent was evaporated under reduced pressure. The crude mixture was submitted to column chromatography (silica gel) using CH_2Cl_2 and CH_2Cl_2/MeOH (99:2) as eluents. The desired products **2a-d** were obtained pure after crystallization from CH_2Cl_2/hexane in yields

ranging from 79% to 99%. The porphyrin-imidazole derivatives obtained were fully characterized by NMR, mass and UV-Vis techniques.

2-(4,5-Diphenyl-1*H*-imidazol-2-yl)-5,10,15,20-tetraphenylporphyrin, 2a

^{1}H NMR (500.13 MHz, $CDCl_3$) = δ 9.26 (1H, s, H-3), 8.86 and 8.84 (2H, AB system, *J* = 4.9 Hz, H-β), 8.82 (1H, d, *J* = 4.8 Hz, H-β), 8.80 (1H, d, *J* = 4.8 Hz, H-β), 8.78 (1H, d, *J* = 4.9 Hz, H-β), 8.74 (1H, d, *J* = 4.9 Hz, H-β), 8.34 (1H, s, H-1´), 8.23-8.20 (6H, m, H-*o*-Ph), 8.16-8.15 (2H, m, H-*o*-Ph), 7.80-7.76 (11H, m, H-*m,p*-Ph), 7.36-7.26 (11H, m, H-*p*-Ph, H-2´,6´, H-3´,5´ and H-4´), -2.62 (2H, s, N-*H*) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) = δ 143.6, 142.11, 142.06, 141.7, 141.1, 137.3, 135.0, 134.61, 134.6, 133.6, 132.4, 131.0, 130.0, 129.9, 129.4, 129.06, 128.9, 128.8, 128.6, 128.3, 128.0, 127.9, 127.8, 127.7, 127.5, 126.9, 126.74, 126.71, 126.6, 125.2, 120.9, 120.5, 120.4, 120.3 ppm. **MS-ESI(+)**: *m/z* 833.4 $[M+H]^+$. **HRMS-ESI(+)**: *m/z* calculated to $C_{59}H_{41}N_6$ $[M+H]^+$ 833,3421; found, 833,3387. **UV-Vis** (DMF): λ_{max} (log ε) 420 (4.37), 518 (3.29), 552 (2.93), 596 (2.81), 652 (2.76) nm.

2-(4,5-Di(pyridin-2-yl)-1*H*-imidazol-2-yl)-5,10,15,20-tetraphenylporphyrin, 2b

^{1}H NMR (500.13 MHz, DMSO-d_6) = δ 12.59 (1H, s, N-*H*), 8.99-8.73 (7H, m, H-β), 8.63-8.16 (2H, m, H-6´), 8.27-8.16 (8H, m, H-*o*-Ph and H-3´), 8.03-7.99 (2H, m, H-*o*-Ph), 7.94-7.74 (11H, m, H-*m*,*p*-Ph and H-4´), 7.30-7.12 (5H, m, H-*m*,*p*-Ph and H-5´), -2.74 (2H, s, N-*H*) ppm. ^{13}C NMR (125 MHz, DMSO-d_6) = δ 166.1, 156.4, 154.4, 150.3, 149.8, 148.6, 148.5, 148.2, 143.1, 141.4, 141.3, 140.9, 139.7, 138.2, 137.7, 136.3, 136.0, 134.9, 134.3, 134.2, 132.7-130.4 (C-β), 129.5, 128.2, 127.2, 127.1, 126.5, 125.7, 123.3, 122.7, 121.8, 121.6, 120.2, 120.1, 120.0 ppm. **MS-ESI(+)**: *m/z* 835.4 $[M+H]^+$. **HRMS-ESI(+)**: *m/z* calculated to $C_{57}H_{39}N_8$ $[M+H]^+$ 835.3292; found, 835.3323. **UV-Vis** (DMF): λ_{max} (log ε) 419 (4.72), 518 (3.44), 552 (3.04), 595 (2.88), 650 (2.76) nm.

2-(4,5-Di(furan-2-yl)-1*H*-imidazol-2-yl)-5,10,15,20-tetraphenylporphyrin, 2c

^{1}H NMR (500.13 MHz, DMSO-d_6) = δ 12.26 (1H, s, N-*H*), 8.94-8.88 (3H, m, H-β), 8.83-8.74 (4H, m, H-β), 8.27-8.22 (6H, m, H-*o*-Ph), 8.04 (2H, d, *J* = 7.3 Hz, H-*o*-Ph), 7.85-7.83 (9H, m, H-*m*,*p*-Ph), 7.75 (2H, d, *J* = 22.7 Hz, H-5´), 7.32 (2H, t, *J* = 7.3 Hz, H-*m*-Ph), 7.26 (1H, t, *J* = 7.3 Hz, H-*p*-Ph), 6.78 (1H, d, *J* = 1.7 Hz, H-3´), 6.69 (1H, d, *J* = 1.7 Hz, H-3´), 6.64-6.59 (2H, m, H-4´), -2.72 (2H, s, N-*H*) ppm. ^{13}C NMR (125 MHz, DMSO-d_6) = δ 149.8, 144.94, 143.2, 142.2, 141.5, 141.4, 141.3, 140.8, 139.5, 135.0, 134.3, 134.1, 132.6-130.4 (C-β), 128.3, 128.2, 127.2, 127.1, 125.9, 121.5, 120.3, 120.2, 120.0, 119.3, 111.7, 111.3, 107.3, 106.4 ppm. **MS-ESI(+)**: *m/z* 813.3 $[M+H]^+$. **HRMS-ESI(+)**: *m/z* calculated to $C_{55}H_{37}O_2NO_6$ $[M+H]^+$ 813.2973; found, 813.3018. **UV-Vis** (DMF): λ_{max} (log ε) 420 (4.64), 519 (3.49), 552 (3.13), 595 (3.00), 651 (2.94) nm.

2-(1*H*-imidazo[4,5-*f*][1,10]phenanthrolin-2-yl)-5,10,15,20-tetraphenylporphyrin, 2d

^{1}H NMR (500.13 MHz, DMSO-d_6) = δ 13.35 (1H, s, N-*H*), 9.09 (1H, s, H-3), 9.06 (2H, dd, *J* = 4.3 and 1.7 Hz, H-6´), 8.93 (2H, s, H-β), 8.88 (1H, d, *J* = 7.3 Hz, H-4´), 8.56 (1H, d, *J* = 7.3 Hz, H-4´), 8.31-8.29 (2H, m, H-*o*-Ph), 8.27-8.23 (4H, m, H-*o*-Ph), 8.09 (2H, d, *J* = 7.3 Hz, H-*o*-Ph), 7.87-7.82 (11H, m, H-*m*,*p*-Ph and H-5´), 7.01 (2H, t, *J* = 7.4 Hz, H-*m*-Ph), 6.60 (1H, t, *J* = 7.4 Hz, H-*p*-Ph), -2.68 (2H, s, N-*H*) ppm. **^{13}C NMR** (125 MHz, DMSO-d_6) = δ 147.7, 147.5, 143.3, 141.3, 141.2, 140.8, 139.7, 135.2, 134.3, 134.2, 133.1-130.4 (C-β), 129.9, 129.4, 128.4, 128.2, 127.2, 127.1, 126.8, 126.3, 125.5, 124.0, 123.3, 123.1, 121.4, 120.5, 120.4, 120.2, 119.1 ppm. **MS-ESI(+)**: *m/z* 833.4 $[M+H]^+$. **HRMS-ESI(+)**: *m/z* calculated to $C_{57}H_{37}N_8$ $[M+H]^+$ 833.3136; found, 835.3209. **UV-Vis** (DMF): λ_{max} (log ε) 420 (4.41), 519 (3.20), 552 (2.77), 596 (2.62), 651 (2.54) nm.

3.2.3 Methylation of porphyrin-imidazole derivatives 2a-d: general procedure.

To a solution of each porphyrin-imidazole derivative **2a-d** (25.0 mg) in DMF (1 mL) in a sealed tube was added an excess of methyl iodide (60 equiv.). The mixture was kept under stirring overnight at 40 °C. After cooling, the crude mixture was precipitated with diethyl ether, filtered, washed with diethyl ether, dissolved in a mixture CH2Cl2/CH3OH (9 : 1) and the solvent was evaporated under reduced pressure. Compounds 2a-d were obtained without further purification by crystallisation from CH2Cl2/hexane in 81-97%. The compounds obtained were fully characterised by NMR, mass and UV-Vis techniques.

2-(4,5-Diphenyl-(1,3-dimethyl)-1*H*-imidazol-3-ium-2-yl)-5,10,15,20-tetraphenylporphyrin iodide, 3a

^{1}H NMR (500.13 MHz, DMSO-d_6) = δ 9.45 (1H, s, H-3), 9.05 (1H, d, *J* = 4.8 Hz, H-β), 9.01 (1H, d, *J* = 4.8 Hz, H-β), 8.95 (1H, d, *J* = 4.8 Hz, H-β), 8.80 (1H, d, *J* = 4.8 Hz, H-β), 8.76 and 8.73 (2H, AB system, *J* = 4.8 Hz, H-β), 8.37-8.34 (4H, m, H-*o*-Ph), 8.25-8.21 (4H, m, H-*o*-Ph), 7.91-7.79 (12H, m, H-*m,p*-Ph), 7.56-7.50 (6H, m, H-2´,6´and H-4´), 7.41-7.39 (4H, m, H-3´,5´), 3.48 (6H, s, -CH_3), -2.72 (2H, s, N-*H*) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$) = δ 142.5, 140.79, 140.76, 140.6, 139.3, 134.8, 134.4, 134.3, 133.4, 130.8, 130.4, 130.1, 129.2, 129.0, 128.5, 128.4, 127.2, 127.2, 127.1, 125.4, 121.9, 120.8, 120.6, 119.5, 52.8, 34.3 ppm. **MS-ESI(+)**: *m/z* 861.4 $[M]^+$. **HRMS-ESI(+)**: *m/z* calculated to $C_{61}H_{45}N_6^+$ $[M]^+$ 861.3700 found, 861.3723. **UV-Vis** (DMF): λ_{max} (log ε) 423 (5.40), 520 (4.14), 556 (3.70), 597 (3.57), 654 (3.56) nm.

2-(4,5-Di(pyridin-2-yl)-(1,3-dimethyl)-1*H*-imidazol-3-ium-2-yl)-5,10,15,20-tetraphenylporphyrin iodide, 3b

^{1}H NMR (500.13 MHz, DMSO-d_6) = δ 9.56 (1H, s, H-3), 9.38-931 (1H, m, H-6´´), 9.08-9.02 (2H, m, H-β and H-6´´), 8.97 (1H, d, *J* = 5.0 Hz, H-β), 8.88-8.81 (2H, m, H-β), 8.76 and 8.73 (2H, AB system, *J* = 4.8 Hz, H-β), 8.65-8.60 (1H, m, H-3´´), 8.49-8.46 (1H, m, H-3´´), 8.43-8.35 (3H, m, H-*o*-Ph), 8.30-8.18 (3H, m, H-*o*-Ph), 8.11-8.04 (2H, m, H-4´´), 7.94-7.80 (11H, m, H-*m,p*-Ph and H-5´´), 7.73-7.67 (2H, m, H-*m*-Ph), 7.64-7.58 (1H, m, H-*m,p*-Ph), 3.82 (3H, s, -CH_3), 3.62 (3H, s, -CH_3), -2.71 (2H, s, N-*H*) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$) = δ 151.1, 150.9, 150.5, 149.9, 147.0, 146.6, 143.1, 140.8, 140.7, 140.5, 139.6, 139.3, 138.7, 138.4, 137.7, 135.0, 134.9, 134.4, 134.3, 134.2, 134.1, 133.8, 133.4, 131.5, 130.1, 129.5, 129.2, 128.6, 128.4, 127.4, 127.3, 127.2, 126.2, 125.6, 124.8, 124.5, 122.2, 121.7, 121.0, 120.7, 119.3, 47.0, 35.9 ppm. **MS-ESI(+)**: *m/z* 863.4 $[M]^+$. **HRMS-ESI(+)**: *m/z* calculated to $C_{59}H_{43}N_8^+$ $[M]^+$. 863.3605; found, 863.3631. **UV-Vis** (DMF): λ_{max} (log ε) 423 (4.53), 520 (3.40), 554 (3.14), 597 (3.11), 653 (3.08) nm.

2-(4,5-Di(furan-2-yl)-(1,3-dimethyl)-1*H*-imidazol-3-ium-2-yl)-5,10,15,20-tetraphenylporphyrin iodide, 3c

^{1}H NMR (500.13 MHz, DMSO-d_6) = δ 9.50 (1H, s, H-3), 9.05 (1H, d, *J* = 5.0 Hz, H-β), 9.01 (1H, d, *J* = 5.0 Hz, H-β), 8.94 (1H, d, *J* = 5.0 Hz, H-β), 8.79 (1H, d, *J* = 5.0 Hz, H-β), 8.76 and 8.74 (2H, AB system, *J* = 4.8 Hz, H-β), 8.36-8.33 (2H, m, H-*o*-Ph), 8.29-8.20 (6H, m, H-*o*-Ph), 8.05 (2H, d, *J* = 1.7 Hz, H-5´), 7.92-7.81 (9H, m, H-*m,p*-Ph), 7.67-7.64 (3H, m, H-*m,p*-Ph), 7.03 (2H, d, *J* = 3.4 Hz, H-3´), 6.82 (2H, dd, *J* = 1.7 and 3.4 Hz, H-4´), 3.59 (6H, s, -CH_3), -2.74 (2H, s, N-*H*) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$) = δ 163.8, 147.7, 146.2, 144.5, 142.7, 140.8, 140.7, 140.6, 138.9, 138.152, 134.9, 134.7, 134.3, 133.0, 130. 5, 129.0, 128.4, 127.2, 127.2, 126.9, 123.3, 122.0, 120.8, 120.6, 119.4, 115.0, 112.1, 54.9, 35.0 ppm. **MS-ESI(+)**: *m/z* 841.4 $[M]^+$. **HRMS-ESI(+)**: *m/z* calculated to

$C_{57}H_{41}O_2N_7^+$ $[M]^+$ 841.3286; found, 841.3305. **UV-Vis** (DMF): λ_{max} (log ε) 423 (4.48), 520 (3.14), 557 (2.86), 598 (2.67), 653 (2.66) nm.

2-(1,3-dimethyl)-1*H*-imidazo[4,5-*f*][1,10]phenanthrol-3-ium-2-yl)-5,10,15,20-tetraphenylporphyrin iodide, 3d

^{1}H NMR (500.13 MHz, DMSO-d_6) = *δ* 8.76 (1H, s, H-3), 9.09 (1H, d, *J* = 5.0 Hz, H-β), 9.03 (1H, d, *J* = 5.0 Hz, H-β), 8.93 (1H, d, *J* = 5.0 Hz, H-β), 8.77-8.73 (3H, m, H-β), 8.40-8.21 (12H, m, H-*o*-Ph, H-4´ and H-6´), 7.97-7.84 (11H, m, H-*m,p*-Ph and H-5´), 7.22-7.12 (2H, m, H-*m*-Ph), 6.75-6.46 (1H, m, H-*p*-Ph), 3.12 (6H, s, -CH_3), -2.68 (2H, s, N-*H*) ppm. **^{13}C NMR** (125 MHz, $CDCl_3$) = *δ* 156.3, 152.36, 152.35, 151.6, 149.4, 140.81, 140.75, 140.65, 140.3, 139.8, 139.1, 137.7, 135.2, 134.6, 134.5, 133.5, 133.2, 131.9-129.3 (C-β), 128.7, 128.63, 128.60, 128.4, 127.5, 127.4, 126.9, 126.7, 126.6, 126.2, 125.8, 125.3, 124.6, 124.1, 122.6, 121.7, 121.2, 120.9, 120.4, 119.6, 119.5, 56.3, 38.2 ppm. **MS-ESI(+)**: *m/z* 861.5 $[M]^+$. **HRMS-ESI(+)**: *m/z* calculated to $C_{59}H_{41}N_8^+$ $[M]^+$ 861.3449 found, 861.3480. **UV-Vis** (DMF): λ_{max} (log ε) 422 (4.38), 518 (3.13), 552 (2.68), 597 (2.54), 651 (2.46) nm.

3.3 Singlet oxygen generation

Stock solution of each porphyrin derivative at 0.1 mM in DMF and a stock solution of 1,3-diphenylisobenzofuran (DPiBF) at 10 mM in DMF were prepared. Aliquots of 2.5 mL of a solution of each porphyrin (0.5 μmol dm^{-3}) and 1,3-diphenylisobenzofuran (DPiBF, 50 μmol dm^{-3}) in DMF were irradiated at an irradiance of 11.0 mW cm^{-2}, in a glass cuvette, at room temperature and under gentle magnetic stirring, with a homemade red LED array in order to prevent the photodegradation of DPiBF. The LED array is composed of a matrix of 5 x 5 LED that makes a total of 25 light sources with an emission peak at 630 nm and a bandwidth at half maximum of ± 20 nm. The absorption decay of DPiBF at 415 nm was measured at irradiation intervals up to 10 min. The production of singlet oxygen was evaluated qualitatively through the DPiBF, a singlet oxygen ($^1O_2$) quencher, due to the fact that DPiBF decays in a first order manner during continuous irradiation in a photosensitised experiment. The irradiation of the PS in the presence of dissolved oxygen will result in the formation of $^1O_2$, which is trapped by DPiBF resulting in colourless *o*-dibenzoylbenzene, after the Diels–Alder like reaction with $^1O_2$.[22]

3.4 Biologic evaluation studies

3.4.1 Photosensitizer stock solutions

Stock solutions of porphyrin-imidazole hybrids were prepared at 500 μM in dimethyl sulfoxide (DMSO) and stored in the dark. These solutions were sonicated for 15 minutes at ambient temperature before each experiment. For biological assays, the PS stock solutions were diluted to the final concentrations in PBS.

3.4.2 Light sources

The photodynamic effect of the cationic PSs was evaluated by exposing the bacterial suspension in the presence of each PS to white light (400-750 nm) delivered by a LED system (ELMARK – VEGA20, 20 W, 1400 lm) with fluence rate of 25 mW.cm^{-2}. All the irradiances were measured with a Power Meter Coherent FieldMaxII-Top combined with a Coherent PowerSens PS19Q energy sensor.

3.4.3 Bacterial strains and growth conditions

Escherichia coli Top10 is a recombinant bioluminescent strain which was genetically transformed in order to emit light (by luxCDABE genes of the marine bioluminescent bacterium *Allivibrio fischeri*).[18,23] The previously transformed bioluminescent *E. coli* was grown on Tryptic Soy Agar (TSA, Merck) supplemented with 50 mg mL^{-1} of ampicillin (Amp) and with 34 mg mL^{-1} of chloramphenicol (Cm), and stored at 4 °C. Before each assay, one isolated colony was aseptically transferred to 10 mL of Tryptic Soy Broth (TSB, Merck) medium previously supplemented with Amp and Cm and was grown overnight at 25 °C under stirring (120 rpm). Afterwards, an aliquot was transferred into 10 ml TSB under the same growth conditions to reach stationary growth phase. An optical density at 600 nm (OD_{600}) of 1.6 ± 0.1 corresponded to ≈10^8 colony forming units (CFU) mL^{-1}.

3.4.4 Bioluminescence versus colony forming units

The correlation between CFUs and the bioluminescent signal (in RLUs) of bioluminescent *E. coli* strain was evaluated. Therefore, the bacterium was grown under the aforementioned conditions. A fresh overnight bacterial culture was serially diluted (10^{-1} to 10^{-9}) in PBS. Non-diluted (10^0) and diluted aliquots were pour-plated on TSA medium (0.5 mL) and, simultaneously, were read on a luminometer (0.8mL) (TD-20/20 Luminometer, Turner Designs, Inc., Madison, WI, USA) to determine the bioluminescence signal.[18]

3.4.5 Antimicrobial Photodynamic Therapy (aPDT) procedure

The bacterial culture was grown overnight and was tenfold diluted in Phosphate-Buffered Saline (PBS), pH 7.4, to a final concentration of ∼10^8 CFU mL^{-1} which corresponds approximately to 10^8 relative light units (RLU). The bacterial suspension was equally distributed in 50 mL sterilized and acid-washed beakers. Afterwards, the appropriate volumes of each cationic porphyrin (**3a**, **3b**, **3c** and **3d**) were added to achieve a final desired concentration 5.0 µM and 20 µM and 100 mM of KI (total volume was 10 mL per beaker). The samples were protected from light with aluminium foil and remained in the dark for 15 min to promote the porphyrin binding to *E. coli* cells. Light and dark controls were also carried out simultaneously with the aPDT procedure: the light control (LC) comprised a bacterial suspension exposed to the same light protocol, and the dark control (DC) comprised a bacterial suspension incubated with each porphyrin at 5.0 and 20 µM protected from light. After the incubation period, the samples and LC were exposed to white light at 2.5 $W.m^{-2}$ under stirring (120 rpm). DC was maintained in the dark during the irradiation procedure. The temperature of experiment was controlled and kept at 25°C. Aliquots of 0.8 mL treated and control samples were collected at time 0 and after 15, 30, 45, 60, 75 and 90 min of light exposure and the bioluminescence signal was measured in the luminometer. Three independent experiments with two replicates were performed and the results were averaged.

3.4.6 Statistical analysis

All experiments for the cationic porphyrinic derivatives performed in triplicate with two replicates. The statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA, USA). Normal distributions were checked by the Kolmogorov–Smirnov test and the homogeneity of variance was verified with the Brown Forsythe test. ANOVA and Dunnet's multiple comparison tests were applied to assess the significance of the differences between the tested conditions. A value of $p < 0.05$ was considered significant.

4. Conclusions

The results presented herein show that porphyrinic derivatives bearing imidazole ring at *beta* position can be prepared in excellent yields through a Radziszewski reaction between 2-formyl-tetraphenylporphyrin and 1,2-diones. The neutral derivatives can be alkylated/cationized in the presence of methyl iodide affording the correspondent mono-charged porphyrin-imidazole derivatives in excellent yields. The photochemical properties of the cationic derivatives show their potential to be used as PSs in photodynamic processes.

The evaluation of the antimicrobial properties against bioluminescent *E. coli* as Gram-negative bacterial model showed that mono-charged porphyrin-imidazole derivatives **3a-d** efficiently photoinactivate this bacterium and the efficiency of the photoinactivation profile is highly improved in the presence of KI.

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