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Master's Dissertation

Photodynamic therapy in the treatment of skin-associated infections

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1. Introduction

Antimicrobials can be defined as "active substances of synthetic or natural origin which kill or inhibit the growth of microorganisms". These substances serve a key role in the prevention and treatment of infections in humans and animals. Broadly, antimicrobials refer to antibiotics, antivirals, antifungals and antiprotozoals. [1]

Since the discovery of penicillin almost a hundred years ago, antimicrobials have been widely distributed, saving millions of lives. However, their excessive and inappropriate use in the last decades has put at risk their effectiveness due to the appearance of Antimicrobial Resistances (AMR). AMR refers to the ability of microorganisms to develop an increasing resistance to antimicrobials to which they were susceptible before.

The process is described in Figure 1, at first only a few microbes are resistant (as a result of for example genetic mutation), but after antibiotic treatment only the resistant microbes will remain, which will then multiply and take over, creating a resistant population of microbes, Additionally, bacteria are able to transfer this resistance to other bacteria, further increasing this problem.[2] While the appearance of AMR is to some degree unavoidable, this process has been exacerbated by the misuse of antimicrobials in human and veterinary medicine and deficient healthcare practices which facilitate the transmission of resistant microorganisms. Over time, this makes the antimicrobials increasingly less effective until they ultimately become useless. [1, 3]

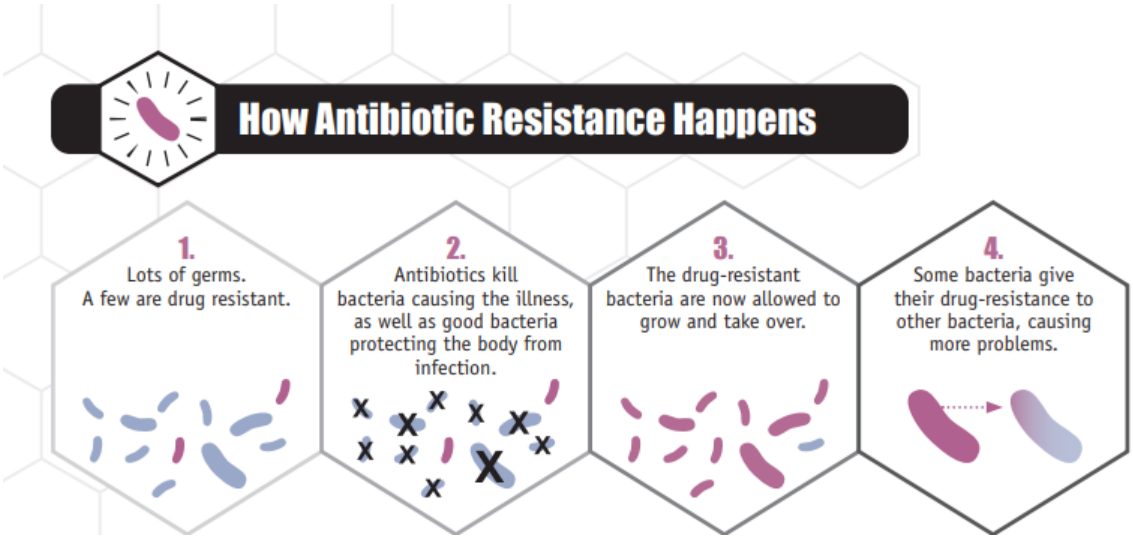

Figure 1 How antibiotic resistance takes place: graph explaining how resistant bacteria become more prevalent published by the Centers for Disease Control and Prevention (CDC)[2]

The negative effects of AMR are already well-present today. Several antibiotics have become completely ineffective for several microorganisms which they had been traditionally used for [2]. Antimicrobial-resistant infections already claim over 50.000 lives every year across Europe and the US alone, with estimates placing the global tally at several hundred thousand deaths. In less developed countries the number is frighteningly high as the lack of second, and third line treatments makes them especially vulnerable to AMR. Rich, developed countries do have access to more treatment options, but even so, the number of AMR-deaths keeps increasing in every country, and the economic cost of these resistances has been estimated to be around 34.000 million dollars per year in the US alone [1, 3- 5]

Already, the World Health Organization considers AMR as one of the emerging problems with the highest impact of the 21st century. And it is estimated that if the current trend continues, by 2050, AMR will cause around 10 million deaths per year, and the global economic loss could be over 100 billion dollars. [3]

There are 3 major steps which ought to be taken to fight this global crisis. First of all, the use of antimicrobials must be rational. Antimicrobials should only be used when truly necessary. Their use as a preventive measure can be mostly substituted by the implementation of new preventive and hygienic measures, like new cleaning and disinfection protocols, in hospitals and similar settings. [6] Secondly, vigilance programs should be set up on a global scale. The treatment of microorganism resistant to one of more drugs should be carefully monitored to reduce the spread of these resistances. [1] And third, we need new and effective antimicrobials or alternative treatment options. The need to develop new antibiotics to face the increasing number of multi-resistant bacteria has been well-known for over a decade. [7] While a few antibiotics for Gram positive bacteria have already been cleared for clinical use, none are at such a stage for Gram negative bacteria, against which only a few molecules are effective. [6,8] Moreover, the time between the introduction of a new antibiotic and the appearance of the first resistances is becoming increasingly shorter.[9]And even worse, the number of antibiotics in development is worryingly low. Developing new and effective antibiotics is becoming increasingly harder. In the meantime, generic antibiotics are becoming much more common, which makes investing in the research and development of new antibiotics even more unattractive for pharmaceutical companies. While several initiatives to help develop new antibiotics have been put in motion, [10, 11] the World Health Organization (WHO) concluded

in September, 2017 that [12] no truly new antimicrobial has been found in the last years, and present research is only focused on modifying existing options, warning about the lack of options at our disposal for the treatment of infections caused by resistant microorganisms.

The stagnation in the research of new antimicrobials has led to the focus on alternative treatment option against them. Options like vaccines, monoclonal and polyclonal antibodies or antibacterial peptides could offer new solutions to the MRA problem. [3] Amongst these options, photodynamic therapy (PDT) has emerged in the last decades as a promising non-invasive therapeutic option. [13]

2. Photodynamic therapy

2.1 What is Photodynamic therapy?

PDT is a minimally invasive therapeutic procedure based on the use of molecules known as photosensitizers (PS) that are able to absorb light energy from a certain wavelength, and in the presence of molecular oxygen can exert a cytotoxic effect.[14] When used as treatment against microorganisms it is called antimicrobial photodynamic therapy (aPDT).

We can find very early examples of PDT from more than 3000 years ago. In ancient Egypt and India, they used vegetable extract combined with sunlight to treat skin diseases like psoriasis and cancer. Similar practices were found in ancient Greece and in the roman empire, but after the fall of the later this practice was forgotten until the 20th century. During this century most studies focused on the applicability of PDT to cancer treatment, culminating in 1995, when the Food and Drug Association (FDA), approved the clinical use of the first PS for the treatment of esophageal cancer.[15] While the bactericide effect of PDT had been known since the seventies, it did not begin to spread as a viable therapy until the nineties, thanks in part to the experiments carried out by Nitzan et al[16-17]. In 1983, Nitzan et al[16] were some of the first to show the antimicrobial effect of an aPDT treatment on a culture of Gram-positive bacteria. For a decade, it was thought that aPDT by itself was not effective against Gram negative bacteria, and it would only work in combination with membrane disorganizing agents. It wasn't until 1992 that Nitzan et al[17], in another *in vitro* experiment using porphyrins as the PS, that aPDT could also be effective against Gram negative bacteria.

2.2 Components of Photodynamic therapy

PDT is composed of three main components: photosensitizer, molecular oxygen and light. When the PS is exposed to the right wavelength of light in the presence of oxygen it starts a photochemical reaction that culminates in the generation of reactive oxygen species (ROS), singlet oxygen. Depending on the generated ROS, we can broadly categorize the PS between Type I ($O_2^{\bullet-}$, $OH^{\bullet}$, H_2O_2) and Type II ($^1O_2$) reactions. While in the case of PDT we are only interested in the antimicrobial effects of ROS, in the process of reverting to its basal state, heat is also released which is the main interest of photothermal therapy[18]

2.2.1 Molecular Oxygen

Oxygen is one of the most reactive elements in the periodic table. While in its diatomic gas form it constitutes the basis for cell respiration for aerobic organisms, high concentrations may be toxic.[19]

The efficacy of a PDT treatment is directly related to capacity of the selected PS to generate ROS. But no matter how efficient this PS is, in order to generate ROS, the bioavailability of O_2 must be high. If the presence of O_2 in the infected tissue is low, for example, due to a necrosis, the effectivity of PDT will be severely limited. Without O_2 to transform into ROS, we will not be able to carry out this kind of therapy [13]

2.2.2 Light source

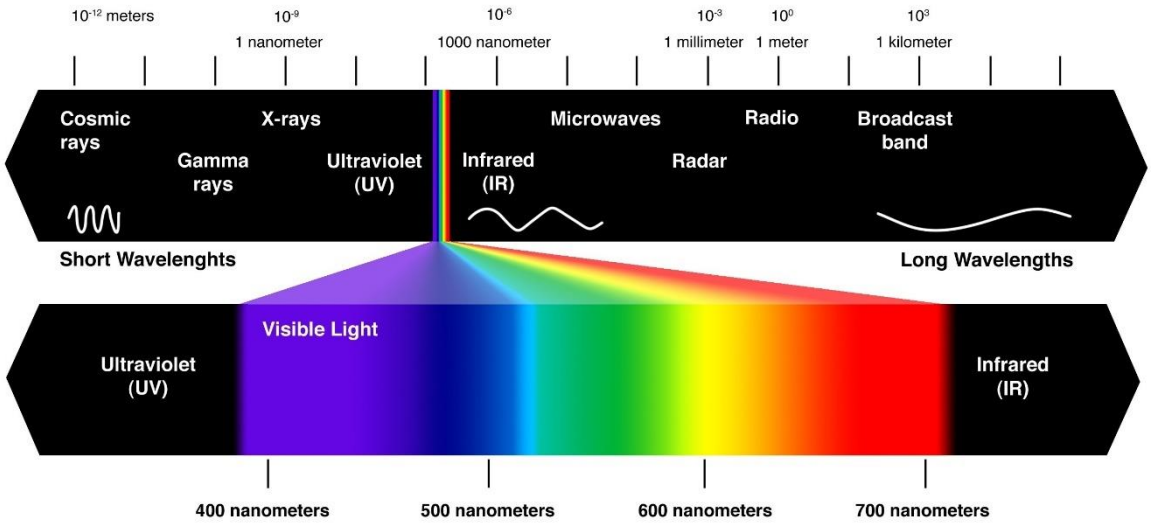

Figure 2 UV-Vis-IR light spectrum: representation of the visible area of light in reference to the electromagnetic spectrum[20]

In order to activate the PS, exposure to a source of light at a specific wavelength is necessary. Most PS are activated within the visible spectrum of the light, which approximately ranges from 400 nm to 750 nm (Figure 2). The selection of the light source will depend on the required light dose, the affected area, and the selected PS, which must be able to absorb light in the spectrum that the source of light emits.[21-22]

It is important to note that the source of light we use, together with the irradiated tissue, will be the two main factors that will determine the penetration of the light in the tissue. If we only considered the source of light, we could say that the higher the λ is, the higher the penetration. In regard to the tissue, while most of the organic compounds absorb in the visible light spectrum, tissue water absorbs at around 1200-1300 nm. This creates a range of wavelengths, from 700 to 1100 nm, known as the near-infrared (NIR) region (or water window) where light can achieve maximum penetration in tissue without harming healthy cells since neither water, blood cells, chromophores nor biological tissues can strongly absorb these wavelengths [23] (Figure 3)

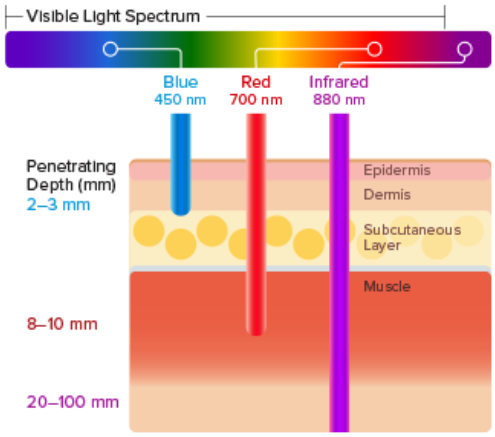

Figure 3 Light penetration in the skin. Graph depicting the skin and how the penetration of the light increases at higher λ[24]

2.2.3 Photosensitizer

PS are reagents that can interact with photons of a specific wavelength to achieve a high energy excited state and then transfer this energy when returning to the ground state to form ROS with the tissue O_2. As a consequence, the PS can suffer molecular damage caused by cleaving of covalent bonds or non-specific reactions between the PS and surrounding molecules, after which it will lose its activity in a process known as photobleaching.[25-27] The perfect PS does not exist, and so the selection of the PS has to be done on a case to case basis.

We can differentiate between four major groups of PS based on their origin and structure: synthetic dyes, tetra-pyrrole structures, natural PSs, and nanostructures. The first generation of PS that were investigated for cancer PDT were phenothiazinium derivates, part of the synthetic dyes group. These polycyclic aromatic molecules posse an intrinsic cationic charge which makes them effective against both Gram positive and Gram-negative bacteria.[27] Since then, many studies have been done on the use of PS from this group such as methylene blue and toluene blue in aPDT. [28-31]

As mentioned, most PSs absorb within the visible light spectrum. One exception is indocyanine green, a cyanine dye that absorbs in the NIR regions. Traditionally it was used as a diagnostic tool for measuring cardiac output, hepatic function, and to study the anatomy of retina vessels.[32] Its ability to produce ROS had only been reported as a negative side effect.[33] In recent years, its potential to produce ROS has been reevaluated, and now several studies exist showing its potential as a PS in both PDT and aPDT. [34-42]

2.3 Photodynamic reaction

The process in which the three components of PDT, light, molecular oxygen and PS, intervene is known as the photodynamic reaction. A source of light that emits in the same λ as the PS absorbs is necessary. This light will convert the PS from its ground state singlet PS to the singlet energy state S°, and then into the excited singlet stage S1 due to the photon absorption. Part of this energy is now radiated as a quantum of fluorescence. The remaining energy then converts the photosensitizer into the excited triplet state T1. From here, it will revert to its basal state. During this process, the energy that the PS releases will react with the molecular oxygen leading to the formation of reactive active species, which have the desired antimicrobial effect (Figure 4). [18] As mentioned, depending on the generated ROS we can divide the PS between Type I and Type II reactions.

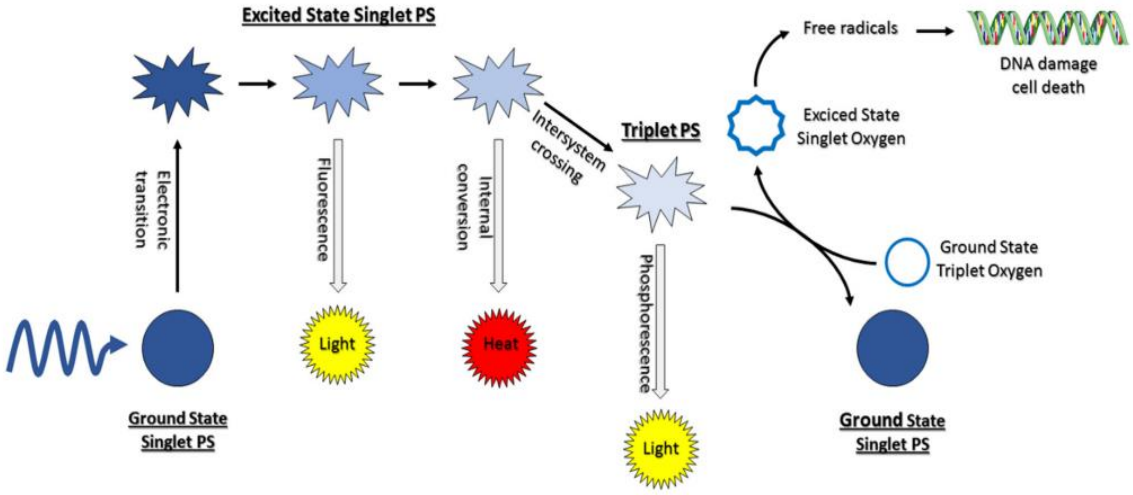

Figure 4 Graph showing the mechanism of the Photodynamic Reaction. From S. Kwiatkowski et al.[18]

2.3.1 Mechanism of photodynamic reaction type I

In type I reactions, the photosensitizer, in the excited triplet state T1, can transfer energy to its surrounding biomolecules. Between the photosensitizer in the T1 state and the target cells (substrate), a hydrogen or electron is transferred, which leads to the formation of free radicals of the photosensitizer and the substrate. These free radicals will rapidly react with oxygen, which will remain in its basic energetic state, to produce ROS. First, in the form of the superoxide anion radical, which then creates additional generation of ROS (hydroxyl radicals or hydroxide peroxide radicals) inside the cells. This cascade of reactions can produce oxidative stress in its target cells, which will, in turn, finally lead to their death.[18]

2.3.2 Mechanism of photodynamic reaction type II

In type II reactions, as a result of the photosensitizer's transition into the excited triplet stage, there is a direct energy transfer between the photosensitizer and the oxygen molecule. In this case, direct energy transfer between molecules is possible because they have the same spins. Excited oxygen particles, called singlet oxygens ($^1O_2$) are thus generated. These $^1O_2$ have extremely strong oxidizing properties, with very short lifespans and an action radius of a few nanometers. [24, 18, 43]

The basal state of most organic compounds is in the basic singlet state. Oxygen molecules are one of the exceptions as they are characterized by their basal triplet state and are then excited into the singlet state. Due to this, photosensitizer molecules do not directly interact with organic cell structures and will only react with oxygen molecules dissolved in cytoplasm.

The efficacy of a PS is usually measured in its capacity to generate a high number of PS molecules in the triplet excited state, and even though it is generally accepted that type II reactions are usually more relevant, both mechanisms can be important. For example, in cases where the concentration of oxygen is low, type I reactions tend to be predominant.

In aPDT, studies have shown that photooxidative effects caused by most of the PS in microorganisms have multiple targets, such as DNA, membrane integrity, protease activity and lipopolysaccharides. Different PSs have different degrees of permeability for each cell and different affinity for the cellular structures. This multiplicity represents one of the most important advantages of PS vs traditional antimicrobials, since PS do not have one single specific target, the risk of developing any kind resistance is highly unlikely because the probability of having multiple genetic mutations decreases.[24] ROS are produced in several cell structures, having several pathways that lead to the destruction of the cell, helping support the hypothesis that aPDT is a viable alternative to antimicrobials.[24, 25, 43-44]

2.4 Shortcomings of aPDT

aPDT will surely become a valuable tool in the fight against AMR, but, for now, it suffers from several shortcomings that limit the clinical effectivity of aPDT. A selection of the most relevant ones is:

a) Limited application in disseminated or deep infections: the limited penetration of most light sources used today complicates its application for non-superficial infections. Moreover, the localized nature of PDT makes it a bad choice for disseminated infections as it is not feasible to cover the whole affected area. [45-46]

b) Treatment is limited to the time the PS is bathed with the source of light. While this means that it is possible to achieve a bactericide effect much faster than with antibiotics, if enough microorganisms remain after treatment, infections may reappear in a few days. [45-46]

c) As of now, there are no standardized clinical protocols for aPDT, which greatly limits its potential applications. There are many PS that have shown great effectivity in animal models but have yet to be tried on humans. [45-46]

d) Photobleaching: defined as a process by which the repeated cycling of a chromophore, in this case the PS, between its ground and excited states eventually leads to molecular damage,

with a gradual decrease in its functionality over time. In aPDT this might affect the effectivity of its bactericide effect, as its ability to generate ROS decreases over time. [42, 47]

e) Most PSs have poor solubility in aqueous solutions and tend to easily form aggregates after administration. This is due to their π–π stacking and hydrophobic interactions that produce the lack of stability. [48]

3. Nanotechnology + aPDT

As one of the most promising technologies of the 21st century, it is not a surprise to hear about the possible applications of nanotechnology in aPDT. So far, most of the research has been focused on a passive application of nanoparticles, using them as vehicles or carriers for traditional PS, making use of the high selectivity that is achievable with nanoparticles (NPs). [49-50] The use of biocompatible nanoparticles as carrier to deliver the PS into microorganisms can help enhance the antimicrobial performance of many PS.[51] In the work by Pietra et al[52] they were able to use polymeric poly(lactic-co-glycolic) acid (PLGA) NPs as a carrier for curcumin. This PS had shown potential as PS for aPDT as a low cost, easy to use, highly effective option, but had been hindered by its poor aqueous solubility and fast hydrolytic degradation at physiological pH values.[52-54] In combination with PLGA NPs, its biological stability improved and its solubility in water was increased, making it a viable choice for future clinical applications.[52]

The focus of this work is nonetheless placed on active nanoparticles, referring to the use of plasmonic NPs directly as photosensitizers.[50] In this group we can find TiO2, one of the most well-known NPs.[55-56] While its capacity to cause photo-oxidation is well-known, its potential in medical applications is greatly limited by its absorption which is mainly in the UV electromagnetic spectrum.[57] Due to the health risks associated with the use of an UV lamp, research has focused in shifting its absorbance spectrum towards the visible region.[58]

Fullerenes are another promising NP which can serve as PS. Fullerenes partly absorb in the UV spectrum, but also absorb light in the visible spectrum which makes them very interesting for clinical applications.[59] As first shown by Tegos et al[60], fullerenes were able to achieve a reduction in cell viability of 4 to 6 logs after a 10 minute irradiation for a wide range of Gram positive and Gram negative bacteria, improving the results shown by Toluidine Blue O, a

widely used PS against which they compared their results.[60] In another study by the same group,[61] they confirmed the clinical potential of fullerenes by proving that the antimicrobial effect could be repeated *in vivo* in a study done with mice. All in all, fullerenes represent a very promising, highly effective option in future aPDT applications. However, they still present several drawbacks, such as an absorption spectrum heavily biased towards lower wavelengths, which greatly limits its penetration in tissue, or its high hydrophobicity, which even after functionalization with water soluble polar groups has still not been entirely solved.[59, 62]

Still, the field of nanotechnology offers many different options, with new studies being carried out every day. A recent study done by Solorzano et al[63] showcased the promising properties of Copper (II) sulfide nanoparticles (CuS NPs), which could have many potential biosanitary applications. A study by Dai et al[64] using similar CuS NPs explored their applications in aPDT reporting a substantial antimicrobial effect.

3.1 Copper Sulfide Nanoparticles

CuS NPs are semiconductor chalcogenides that display interesting electrical, optical and catalytic properties.[63-65] Beyond their use in PDT and PTT, they have potential applications in the photodegrading of pollutants, biological labeling or DNA detection among others.[65] As plasmonic materials, CuS NPs absorb light in the NIR region of the electromagnetic spectrum, activating photodynamic and photothermal reactions thanks to the excitation of direct (band-to-band) transitions, indirect transitions and plasmonic photoexcitation. As previously mentioned, NIR absorption greatly increases the penetration of light in the skin, allowing for the treatment of deeper infections. [63-64]

In biomedical applications, we should always try to achieve a complete physiological biodegradation after the administration of a nanoparticulated material. A common worry in the field of nanomedicine is the biopersistence of NPs. This is precisely one of the main setbacks other plasmonic NPs. In an *in vivo* study on mice, Guo et al[65] demonstrated that after one month 96% of hollow gold NPs administered still remained in their bodies, and other studies done on carbon-based NPs like carbon nanotubes or graphene oxide have also shown a large physiological persistence. This is not the case for CuS NPs, as shown in the *in vivo* study carried out by and Guo et al[65], after a month 90% of the injected CuS NPs had

been degraded and subsequently excreted via the hepatobiliary route. Its subproducts are water-soluble and are easily excreted via urine and bile. [63, 65] An additional study carried out by Solorzano et al[63] on the biodegradation of CuS reported similar results, showing that under simulated physiological conditions CuS NPs degrade into soluble copper sulfates, which can be easily excreted.

The main advantages of CuS are thusly its capacity to absorb in the NIR spectrum and its high biodegradability. Other advantages of CuS are the low cost of its fabrication materials, its low cytotoxicity in the absence of light, and the ease of synthetizing functional NPs.[63-65] Figures 5 and 6 show a TEM images of the CuS NPs used in this work.

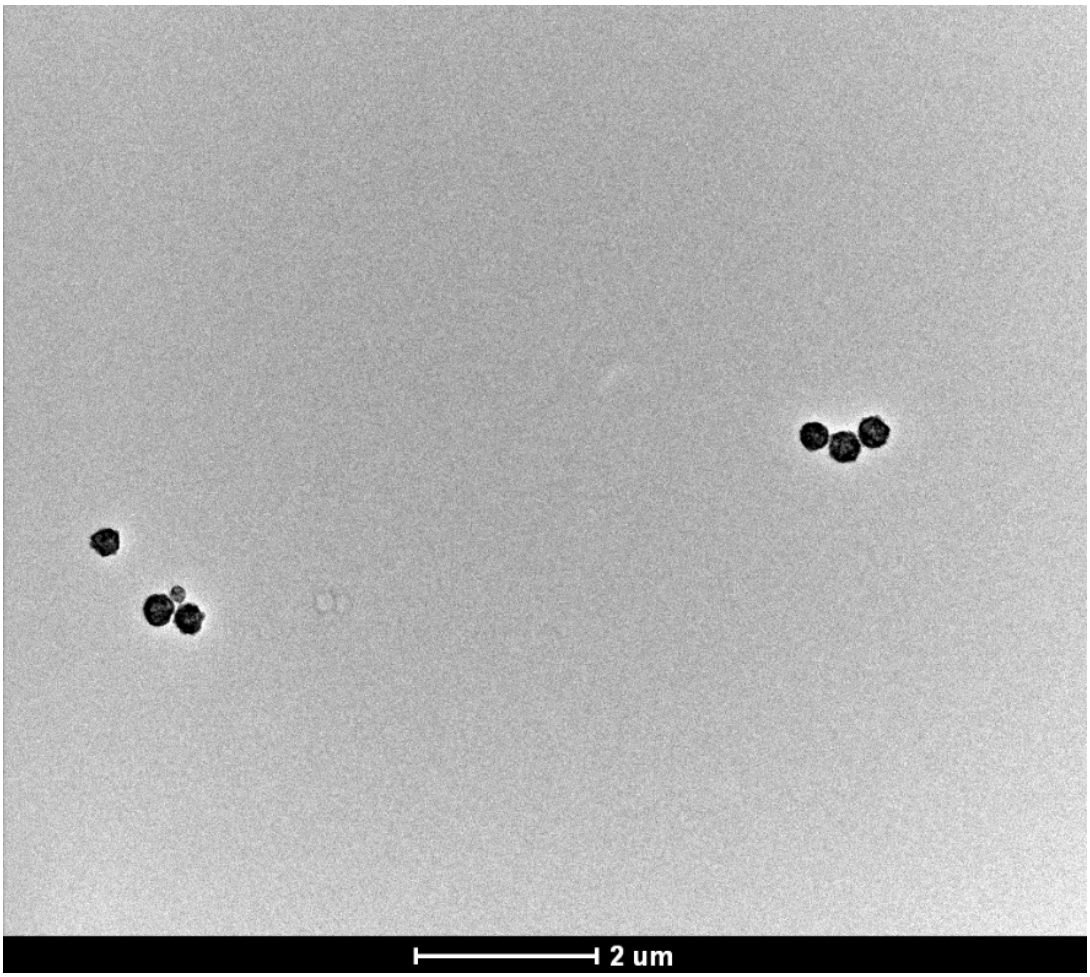

Figure 5 Figure 5 TEM image of CuS NPs.

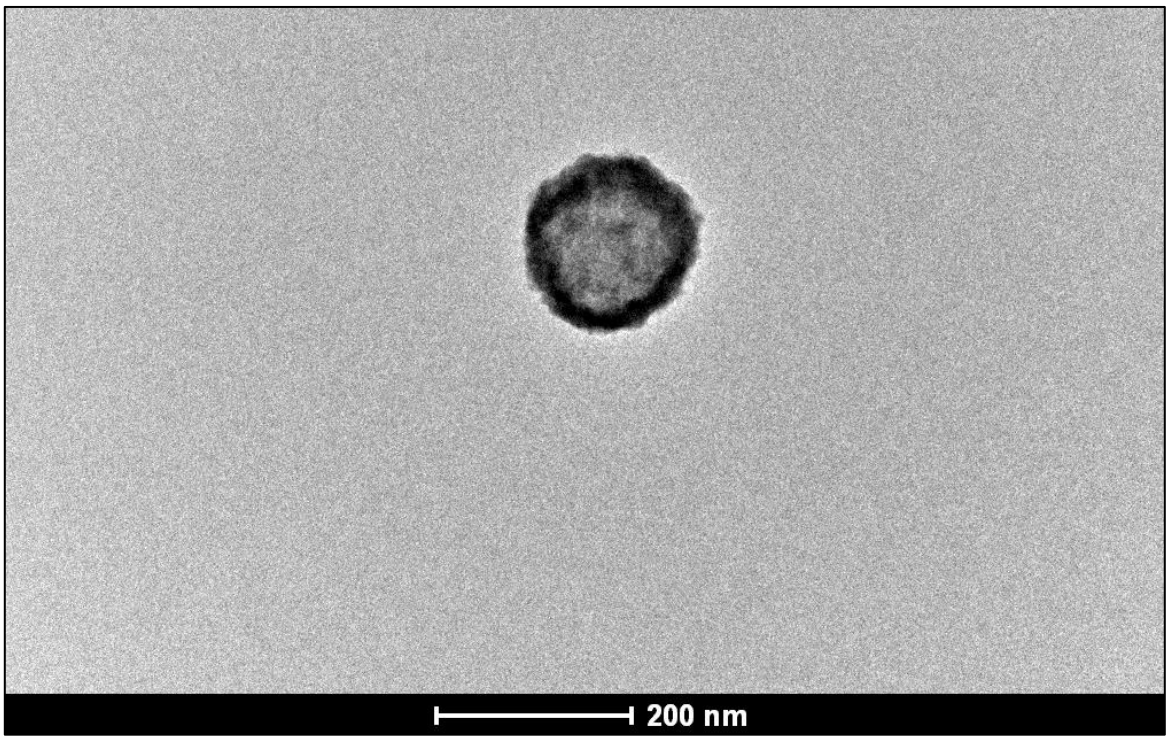

Figure 6 TEM image of CuS NPs.

4. Objectives

The importance of developing new solutions for the crisis that AMR represents cannot be overstated. aPDT has presented itself as a promising new option, proven to be effective against resistant microbes and so far, not known to cause any resistance thanks to its multitarget mechanism of action. Despite this, aPDT suffers from several shortcomings that might be hindering its spread in clinical settings. In this study to aim solve some of its problems, such as the susceptibility of PSs to photobleaching or the limited application in non-superficial skin infections. As much as possible we want to present a treatment capable of dealing with resistant strains of both Gram-positive and Gram-negative bacteria.

First, we studied the effectivity of ICG, a relatively unknown PS which absorbs in the NIR spectrum. A few studies exist backing up its use in aPDT, and its deeper penetration could be an advantage over other PS. Then, we studied the effectivity of our CuS NPs, whose higher resistance to photobleaching, high penetration and high biodegradability for a NP make a very interesting alternative to traditional PS. The possibility of using both PS in combination

was also explored as both absorb in the same wavelength. We hope this work serves as a first step towards the clinical application of new PS adding more versatility to aPDT treatments.

5. Material and Methods

Due to the space limitations, material and methods can be found in Annex 1.

6. Experimental overview

As mentioned, the properties of CuS NPs make them a very interesting choice for new aPDT experiments. If proven effective it could be a very valuable tool in the treatment of resistant bacteria. In order to show its effectivity, we selected two representative species of bacteria, one Gram + and one Gram -, and studied the effectivity of CuS NPs as a photosensitizer at different concentrations of CuS NPs and using different fluences of irradiation. Additionally, as in photodynamic reactions part of the energy is released as heat, we checked the maximum temperature achieved at different concentrations to check if part of the bactericide effect could be due to photothermal effect. We wanted to decouple the photothermal from the photodynamic effect. g

All experiments were also carried out using a different PS, in order to contrast the effectivity of CuS NPs. We choose indocyanine green (ICG) as it absorbs light in the NIR spectrum, in a range quite similar to our CuS NPs. In recent years, a few studies have been carried out on its possible application in aPDT and its antimicrobial effect[34, 36-38,41], while not quite as strong as other traditional PSs, has been proven. Nevertheless, given the novelty of this application for ICG, an additional study on its bactericidal effect is, in itself, interesting.

To study the possible synergistic effect of CuS NPs and ICG, experiments were carried out using both of them in combination to see if there were any synergistic or improved bactericide effect.

The photobleaching of both CuS NPs and ICG was determined by its absorbance spectra analysis before and after being irradiated by an 808 nm laser.

6.1 Selection of bacteria

The effectivity of aPDT tends to differ between Gram + and Gram – bacteria, with the later often being more resistant. This is thought to be related to the more complex double membrane of Gram – bacteria which hinders the passage of the PS into the bacteria.

As the aim of this work is to help alleviate the burden of AMR, we focused on bacteria with known resistance. Both selected bacteria are part of ESKAPE a group of six bacterial pathogens usually associated with antimicrobial resistance. As our Gram + bacteria, we chose *Staphylococcus aureus*, considered part of normal human flora of the skin and the mouth. Despite this, it is capable of causing skin infections, pneumonia, meningitis or even sepsis, which has made it one of the most common targets of aPDT studies.

For Gram -, we chose *Pseudomonas aeruginosa*, another common target of aPDT studies, it is interesting for its ability to form biofilms. In most studies, when targeting this pathogen, the fluence and dose must be increased in order to obtain the same bactericide effect as for *S. aureus*. [66]

In figure 7 we can see *S. aureus* (right) and *P. aeruginosa* (left) cultivated in a blood agar (BA) petri dish after a 24-hour incubation at 37ºC

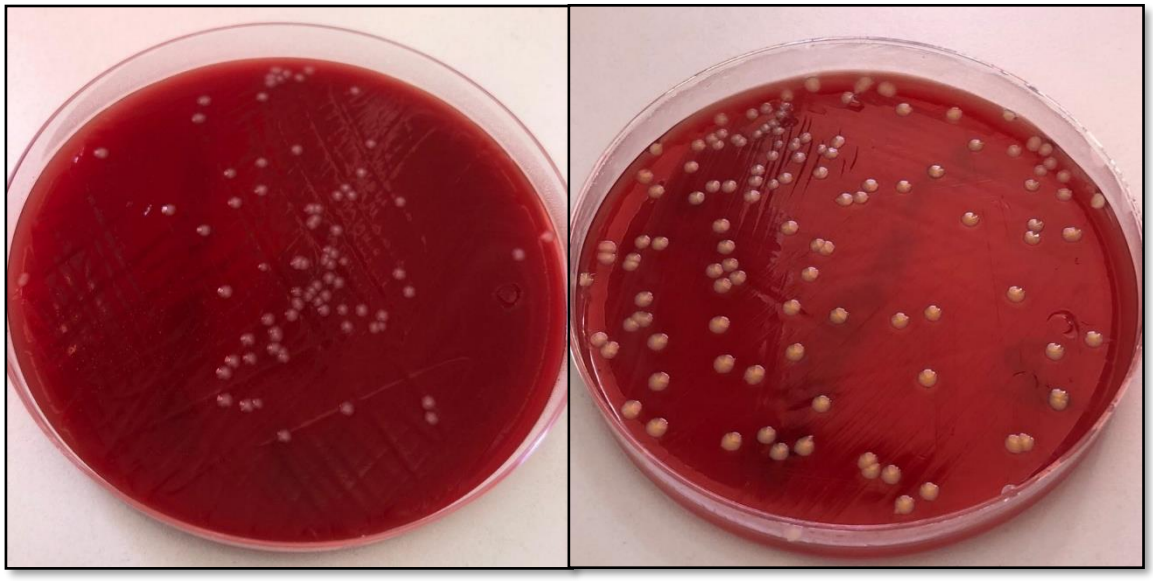

Figure 7 Left, photo of Pseudomonas aeruginosa grown on BA medium after 24 hours incubation at 37ºC. Right, photo of Staphylococcus grown on BA medium after 24 hours incubation at 37ºC.

7.Results

7.1 Heating curve

The photothermal conversion of PSs can be a detrimental side effect in aPDT. Temperatures above 42ºC can result in cellular damage.[67] This should be avoided as much as possible as high temperatures could not only result in discomfort or even burns in patients, it would also be impossible to determine whether the antimicrobial effect was caused due to the production of ROS by the PS or by the heat.

To ensure that our experiments would stay within physiological levels, calibration heating curves for both ICG and CuS were carried out using the maximum concentrations we had available of our PSs, as seen in figure 8. The maximum temperature for both (38.48ºC for ICG and 33.29ºC for CuS NPs) never went above 40ºC during the 6 minutes we recorded, showing that the bactericidal effect would not be caused by heat, and that should not cause any discomfort for potential patients.

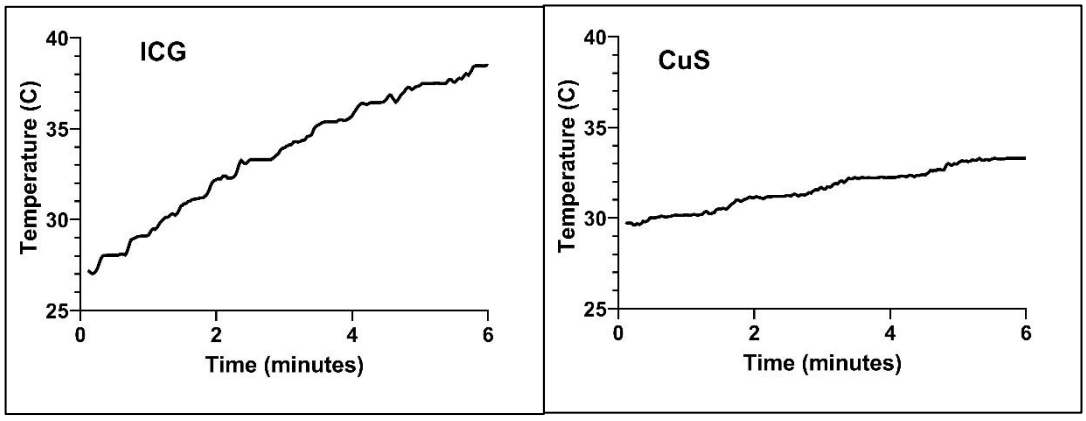

Figure 8 Heating curve of ICG 40 ul/mL and CuS (160 ul/mL) during 6 minutes of irradiation by an 808 nm laser set at 1 W/cm².

7.2 Antimicrobial photoinactivation therapy

Before studying the antimicrobial effect of ICG and CuS, first we had to determine the optimal parameters for our experiments. Using the heating curves as reference, we tried to find a range of fluences and concentrations that best showed the antimicrobial effect of both PSs, while keeping the temperature low. We also had to consider other limitations, such as the limited solubility of ICG, which tended to form aggregates at higher concentrations, and for CuS, higher concentrations proved problematic as the high density of the solution

refracted the light, thus lowering the penetration and effectivity of the treatment. Lower concentrations were also considered but the results obtained at the lowest concentration shown for both ICG and CuS already show a very slight antimicrobial effect, and so were not included. As for the fluences, a few repetitions were initially performed under low fluences, showing a much lower effectivity in comparison to the selected fluences, and so were discarded in the end. High fluences were not considered as the increase in exposure time and temperature were considered more disadvantageous. Lastly, for the experiments carried out using a combination of both PSs, due to time limitations we could only choose a maximum of six concentrations, which we tried to select to best showcase the potential of this combination. We compared the effect of both PS at high and low concentrations, then the effect of a high concentration of one combined with a low concentration of the other, and then intermediate concentrations for both.

The effectivity of the PSs was classified following the standards set by the Clinical and Laboratory Standards (CLSI) for *in vitro* antimicrobial tests,[68] which divided the antimicrobial agents between bacteriostatic and bactericidal depending on the reduction in viable bacterial density. Starting from an inoculum with a bacterial density of at least $\geqslant 5 \times 10^5$ cfu/mL, a reduction of 90 to 99%, or 1 to 2 logs, is considered to be a bacteriostatic effect, meaning that it does not kill bacteria, only prevents its growth, while on the other hand, a reduction of $\geqslant$99.9% or $\geqslant$3 logs, is classified as bactericidal effect, which in this case means that it is able to kill bacteria. The lowest concentration of an antimicrobial agent to achieve a bactericidal effect is called the Minimum bactericidal effect (MBC), and is often used as a measure of the effectivity of antimicrobial drugs. [68]

Results regarding control samples that were incubated in the dark to study the toxicity of the photosensitizers are shown in annex II. In all cases, they did not show a statistical difference with the control samples, irradiated without the photosensitizers, and so, for the sake of clarity they have been omitted in this section.

7.2.1 Photoinactivation of *S. aureus* by ICG

Figure 9 shows the *in vitro* aPDT results performed on *S. aureus* using different concentrations of ICG at two different fluences.

The highest antimicrobial effect recorded, at 6' or 180 J/cm^2 and a concentration of 40 µg/mL, was a reduction of 2.9 logs, close to the bactericidal threshold, but stayed within the limits of the bacteriostatic effect.

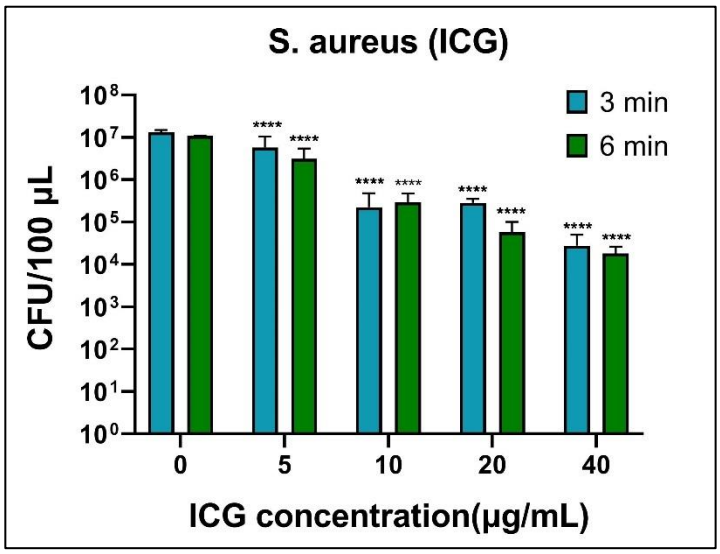

Figure 9 Antimicrobial effect of ICG for S. aureus ATCC 29213 in water suspensions with ICG concentrations of 0, 5, 10, 20 and 40 µg/mL , irradiated with an 808 nm laser set at 1 W/cm2 for 3' and 6'. Bars show cell viability measured in CFU/100 µL counted after a 24 hours incubation at 37oC. Each column is the mean ± standard deviation(n=3)

7.2.1 Photoinactivation of *P. aeruginosa* by ICG

Figure 10 shows the *in vitro* aPDT results obtained for *P. aeruginosa* using different concentrations of ICG at two different fluences.

MBC was only recorded at 6' or 180 J/cm^2 and a concentration of 40 µg/mL of ICG, with a reduction of 3.1 logs

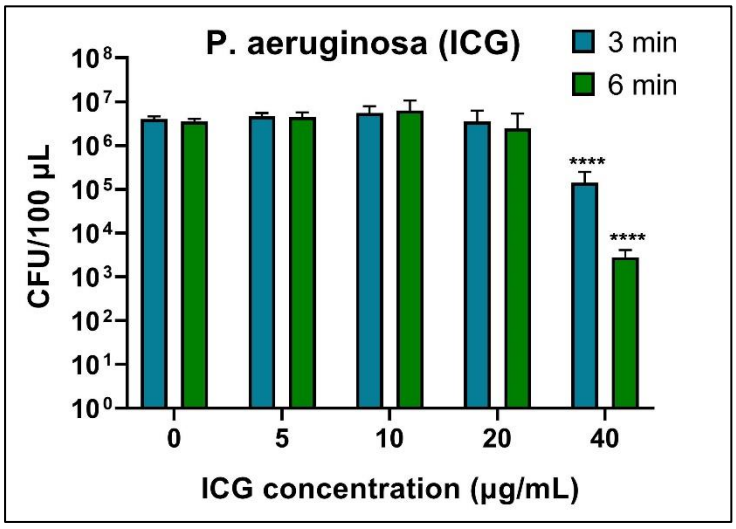

Figure 10 Bactericidal effect of ICG for P. aeruginosa ATCC 27853 in water suspensions with ICG concentrations of 0, 5, 10, 20 and 40 µg/mL, irradiated with an 808 nm laser set at 1 W/cm2 for 3' and 6'. Bars show cell viability measured in CFU/100 µL counted after a 24 hours incubation at 37°C. Each column is the mean ± standard deviation(n=3).

7.2.3 Photoinactivation of *S. aureus* by CuS NPs

Figure 11 shows the *in vitro* aPDT results obtained for *S. aureus* using different concentrations of CuS at two different fluences

The highest antimicrobial effect, recorded at 6' or 180 J/cm^2 and a concentration of 160 µg/mL, was a reduction of 2.7 logs, showing only a bacteriostatic effect.

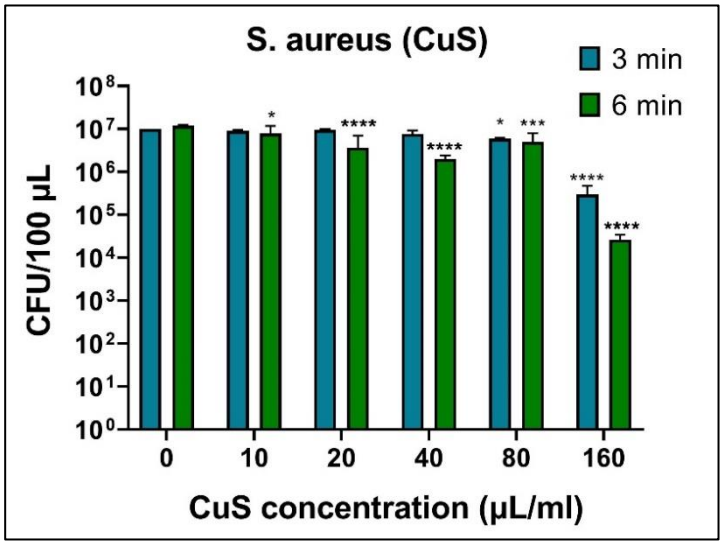

Figure 11 Bactericidal effect of CuS NPs for S. aureus ATCC 29213 in water suspensions with CuS concentrations 0, 10, 20 and 40, 80 and 160 µg/mL , irradiated with an 808 nm laser set at 1 W/cm2 for 3' and 6'. Bars show cell viability measured in CFU/100 µL counted after a 24 hours incubation at 37ºC. Each column is the mean ± standard deviation(n=3)

7.2.1 Photoinactivation of *P. aeruginosa* by CuS NPs

Figure 12 shows the *in vitro* aPDT results obtained for *P. aeruginosa* using different concentrations of CuS at two different fluences.

MBC was only recorded at 6' or 180 J/cm^2 and a concentration of 160 µg/mL, with a reduction of 3.2 logs.

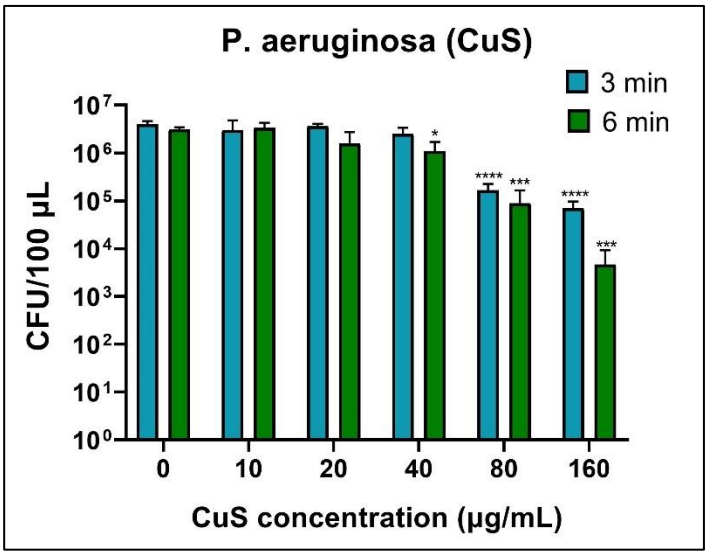

Figure 12 Bactericidal effect of CuS for P. aeruginosa ATCC 27853 in water suspensions with CuS concentrations 0, 10, 20 and 40, 80 and 160 µg/mL , irradiated with an 808 nm laser set at 1 W/cm2 for 3' and 6'. Bars show cell viability measured in CFU/100 µL counted after a 24 hours incubation at 37ºC. Each column is the mean ± standard deviation(n=3)

7.2.1 Photoinactivation of *S. aureus* by a combination of ICG and CuS

Figure 13 shows the *in vitro* aPDT results obtained for *S. aureus* using different concentrations of a combination of ICG and CuS at two different fluences.

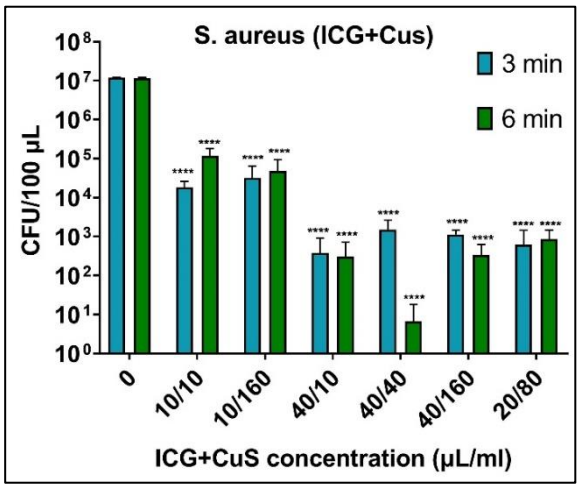

Figure 13 Bactericidal effect of ICG+CuS for S. aureus ATCC 29213 in water suspensions with CuS concentrations 0, 10+10, 10+160, 40+10, 40+40, 40+160, 20+80 µg/mL , irradiated with an 808 nm laser set at 1 W/cm2 for 3' and 6'. Bars show cell viability measured in CFU/100 µL counted after a 24 hours incubation at 37ºC. Each column is the mean ± standard deviation(n=3)

As the combination of both PS is not equal, it is not possible to properly speak of MBC, but we can observe a bactericidal effect with an irradiation of 6' for 40(ICG)+10(CuS), 40(ICG)+40 (CuS), 40(ICG) + 160(CuS), and 20(ICG) + 80(CuS) µg/mL, and with an irradiation of 3' for 10(ICG)+10(CuS), 40(ICG)+40(CuS), 40(ICG) +160(CuS), and 20(ICG)+ 80(CuS) µg/mL. The highest bactericide effect was recorded at 6' 40(ICG)+ 40(CuS) µg/mL, with a reduction of 6.5 logs, or 99.9999% of the present bacteria.

7.2.1 Photoinactivation of *P. aeruginosa* by a combination of ICG and CuS

Figure 14 shows the *in vitro* aPDT results obtained for *P. aeruginosa* using different concentrations of a combination of ICG and CuS at two different fluences.

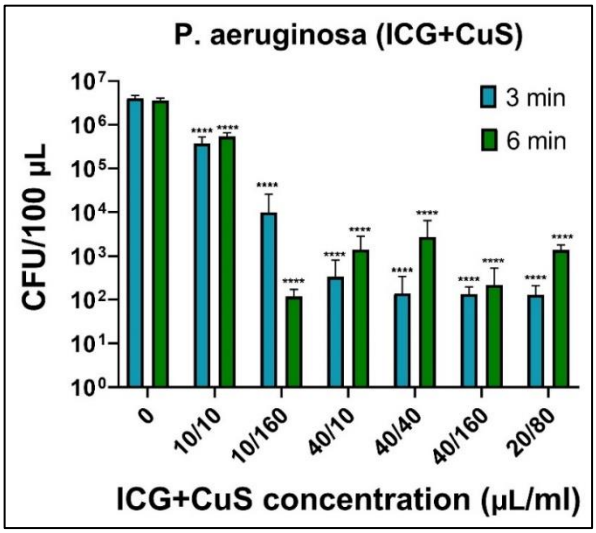

Figure 14 Bactericidal effect of ICG+CuS for P. aeruginosa ATCC 27853 in water suspensions with CuS concentrations 0, 10+10, 10+160, 40+10, 40+40, 40+160, 20+80 µg/mL, irradiated with an 808 nm laser set at 1 W/cm2 for 3' and 6' Bars show cell viability measured in CFU/100 µL counted after a 24 hours incubation at 37ºC. Each column is the mean ± standard deviation(n=3)

Again, it is not possible to speak of MBC, but we could observe a bactericidal effect at both 3' and 6' for concentrations of 10(ICG)+ 160(CuS), 40(ICG)+10(CuS), 40 (ICG)+40(CuS), 40(ICG)+160 (CuS) , and 20(ICG) + 80(CuS) µg/mL. The highest bactericidal effect, recorded at 6' or 180 J/cm^2 and a concentration of 10 µg/mL of

ICG and 160 µg/mL of CuS NPs, was a reduction of 4.5 logs, a reduction of over 99.99% of the present bacteria.

7.3 Photobleaching

The bactericidal effect of aPDT is limited to the time the PS is irradiated by its corresponding source of light. However, as we increase the irradiation time, the PS will slowly start to photobleach, losing its functionality until it can no longer absorb light, and so the generation of ROS and its antimicrobial effect will stop.[47] While higher concentrations of PS take longer to photobleach, the solubility of many PS is quite low (ICG among them), which limits the potential effectivity of these PS.[48] In our experiments, after irradiating ICG with an 808 nm laser for 3 minutes, a clear change of color from green to brown was observed as seen in figure 15.

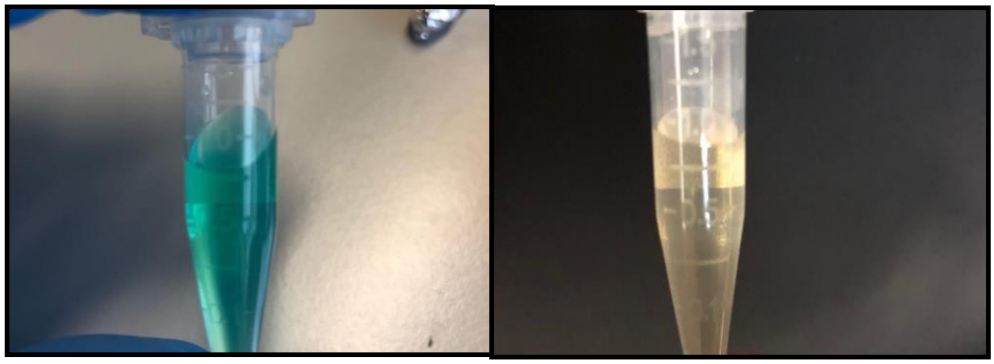

Figure 15 Left picture shows an Eppendorf with a solution of 400 ug/mL of ICG before irradiation. Right picture shows the same solution after a 3' irradiation with an 808 nm laser with an irradiance of 1 W/cm^2.

Photobleaching was corroborated comparing the absorption spectrum of the samples before and after irradiation. As we can see in Figure 16, this diluted sample of ICG practically lost all its absorption in the NIR range after 3’ minutes of irradiation with an 808 nm set to an irradiance of 1 W/cm^2.

And as seen in Figure 17, absorption spectra of CuS NPs were taken with the same conditions as ICG. In this case, both absorption spectra are identical. However, this does not mean that CuS NPs are immune to photobleaching, as it has been reported that under much higher fluences and irradiation times, they end up degrading and losing their absorption band.[63]

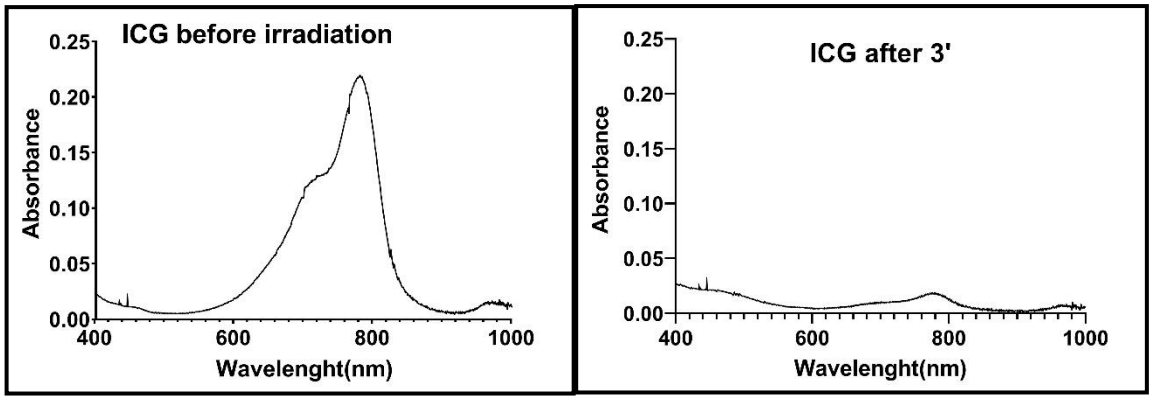

Figure 16 Left graph shows the absorption spectrum of CuS NPs before being irradiated. Right graph shows the absorption spectrum of the same solution after being irradiated for 3' with an 808 nm laser with an irradiance of 1 W/cm2.

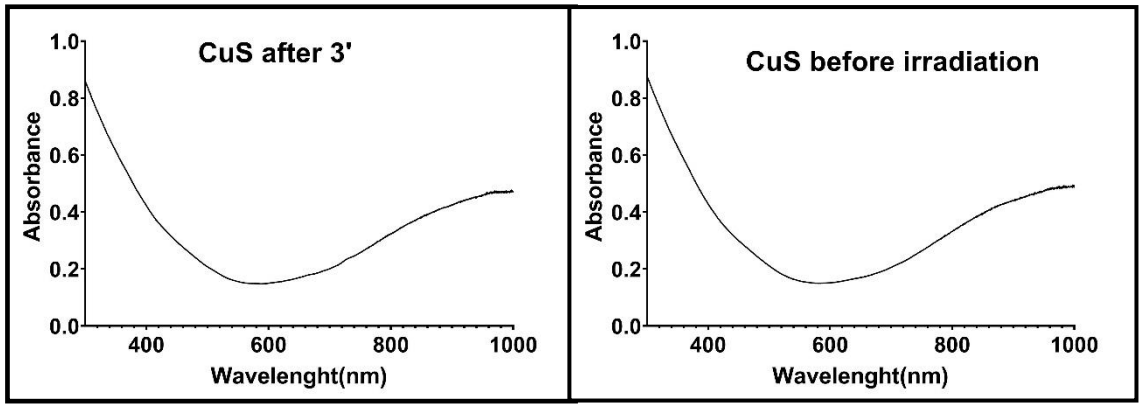

Figure 17 Left graph shows the absorption spectrum of CuS NPs before being irradiated. Right graph shows the absorption spectrum of the same solution after being irradiated for 3' with an 808 nm laser with an irradiance of 1 W/cm2.

8. Discussion

According to the results, both ICG and CuS seemed to have promising antimicrobial effects, however, before moving to further discussions on this topic, it is important to contextualize and compare our results to other similar studies done on this matter.

For ICG, the most relevant studies were the works of Omar et al[41] and Topaloglu et al,[34] which studied the antimicrobial effect of ICG against *S. aureus* and *P. aeruginosa*. Of the two, the study done by Omar et al[41] had a matching experimental setup with similar parameters.

Omar et al[41] studied a wide range of ICG concentrations, up to 200 μg/mL with fluences up to 411 J/cm^2, and while he obtained a complete inactivation of the treated bacteria, this antimicrobial effect was theorized to be partly due to the photothermal effect of ICG, as they

recorded temperatures close to 50ºC. The closest parameters we can find are 50 μg/mL of ICG (20% higher than us) irradiated by an 808 laser set at 1.27 W/cm^2 for 5', which corresponds to a light dose of 411 J/cm2 (around 15% higher than the one we used). With those parameters in *S. aureus* they recorded a reduction of 4 logs, which, given that both the fluence and the concentration of ICG were higher seems plausible in relation to the reduction of 2.9 logs we recorded.

In the case of *P. aeruginosa*, with those same parameters the effect was seemingly null, only obtaining a small reduction with a concentration of 200 ul/mL. On the other hand, in the results obtained by Topaloglu et al[34], while the experimental parameters differed more, with a concentration of ICG of 125 μg /mL and a fluence of 252 J/cm^2 they obtained a total reduction of all bacteria (around 8 logs), which seems more in line with our results, where a lower concentration of ICG (40 μg /mL) and higher fluence (360 J/cm^2) obtained a reduction of 3.1 logs. The reason for this difference could be in the strain of the bacteria used, while both Topaluglu et al[34], and us used *P. aeruginosa* ATCC 27853, Omar et al[41] used *P. aeruginosa* PA01.

It is also important to see how ICG outperforms other more traditional PS. Methylene blue (MB) served as a perfect example as we could find several studies, such as the ones performed by Laguna et al[30], which used a very similar experimental design. Moreover, while MB still absorbs in the visible light spectrum, its maximum absorption λ is 665nm which is rather high for an organic PS. In the study done by Laguna et al[30] regarding *S. aureus*, they were able to reduce the population of bacteria by 6 logs with only 0.62 μg /mL of MB and fluence of just 18 J/cm^2. Despite these outstanding results, the use of MB as PS has one major drawback, the lower intensity of the required sources of light increases treatment time exponentially.[30] In this study, in order to obtain a fluence of 18 J/cm^2 the samples had to be irradiated for 43', which limits the potential clinical applications of MB.[30]

Across the three experiments, ICG showed to be quite effective against Gram + bacteria such as *S. aureus*. However, the effectivity of ICG on Gram - bacteria such as *P. aeruginosa* seems more divisive. In general, as we mentioned before, Gram – bacteria tend to be more resistant to most antibacterial treatments, probably due to the more complicated structure of their bacterial cell wall. Despite mixed results, given the lack of new options for the treatment of

Gram – bacteria, we believe that it would be worth to further study the effectivity of ICG for the treatment of Gram – bacteria.

Comparing the effectivity of CuS is harder, as, to the best of our knowledge, there is not much literature available on this subject. We were able to find one study by Dai et al[64], which studied the aPDT capabilities of functionalized CuS nanoclusters against *S. aureus* and *P. aeruginosa*. Experimental setup also differed as they used a 980 nm laser with a higher fluence of 450 J/cm^2. The highest concentration of CuS they studied were 44 μg/mL, at which they obtained a cell viability of 20% for both *S. aureus* and *P. aeruginosa*. Compared to the closest parameters in out experiments at a fluence of 360 J/cm^2 and a concentration of CuS of 40 μg/mL we recorded a similar cell viability of 18% for *S. aureus* and 17.75% for *P. aeruginosa*. At these low concentrations, we can observe an antimicrobial effect, but it is not enough in any of these cases to be classified even as bacteriostatic.

Next, we compared the effectivity of CuS NPs with other aPDT studies done with NPs, such as TiO2 NPs and fullerenes. The antimicrobial efficacy of Photocatalytic TiO_2 NPs, and its ability to generate ROS are well-known.[56] TiO_2 absorbs in the UV range and so, irradiance is much lower, and the irradiation times used tend to be much higher. While the risks associated with a UV irradiation have made it so TiO_2 is mostly used for the sterilization of water, food, surfaces, et, Asahara et al[57] realized an *in vitro* study on its effectivity against *S. aureus* with the idea of studying future clinical applications. The highest antimicrobial effect was achieved with 19 μg /mL of TiO_2 irradiated for 60 minutes with an UV light with an irradiance of 1.82 mW/cm^2, for a total of fluence of 6.5 J/cm^2. Although part of the effect is due to the intrinsic bactericide effect of UV light, which after 60 minutes has reduced the survival ratio around a 35%, with TiO_2 the reduction increases to 99.6%. A major drawback for TiO_2 which can limit its potential clinical applications is the negative effect of UV light on the human body, because it damages nuclear DNA and so, present efforts focus on the modification of the TiO_2 spectra so that it absorbs in the visible light spectrum.[59] Compared to this, we were able to obtain a very similar bactericidal effect in a tenth of the irradiation time, and without the risks of using UV-light. Unfortunately, while the capacity of TiO_2 to inactivate *P. aeruginosa* is well-documented,[56] we were unable to find any similar study that contemplated its clinical application.

The application of Fullerenes as PS for aPDT is also well-documented both *in vivo* and *in vitro*.[60-61] Tegos et al[60] carried out an in vitro study on the antimicrobial effect of fullerenes for both *S. aureus* and *P. aeruginosa* among others. For S. aureus, a fluence of 2 j/cm2 and a concentration of 100 μg/mL were enough to cause a reduction of 4-5 logs. *P. aeruginosa* proved to be a bit more resistant but with a concentration of 100 μg/mL and a fluence of 16 μg/mL, they obtained a reduction of 3-5 logs. The low light doses meant that irradiation could be achieved in around 10 minutes. Compared to our results, fullerenes proved more effective, under lower light doses and concentrations, and if further researched they could be a very valuable tool in the treatment of very superficial skin infections.

Reviewing the antimicrobial effect of CuS and ICG at their highest concentration and fluences, both barely stayed under the bactericidal threshold for *S. aureus*, with ICG showing slightly better results, and for *P. aeruginosa* both had a bactericidal effect, in this case with CuS NPs showing a slightly higher antimicrobial effect. It is worth noting that in order to reach the same effectivity as ICG at 40 μg/mL, a concentration 4 times higher of CuS NPs was necessary.

All in all, the antimicrobial effect obtained for CuS is promising. A reduction of around 99.9% of the bacteria is considered to be bactericidal. However, we must remember that the bactericidal effect is only present during the short irradiation, after which, if enough bacteria are left, the infection will reappear.

For both PS, a higher bactericidal effect might have been achieved by increasing the concentration or the fluence, but this presented additional problems. The solubility of ICG is quite low, and it quickly formed aggregates when we tried to increase the concentration, and the high photothermal efficiency of ICG means that, in any case, higher fluences and concentrations could result in very high temperatures, as shown by Omar et al.[41] As aPDT is meant to be used on the skin of the patients, we must avoid high temperatures as we could risk damaging their skin, plus it could be quite painful for them. Moreover, even if we are able to keep the temperature within physiological levels, an increase in the light dose might not necessarily result in a higher antimicrobial effect due to its sensibility to photobleaching. In the case of CuS NPs, it might be worth studying the effectivity of an increased fluence in future studies, but a higher concentration would soon show a decreased effectivity. The high density of the CuS NPs at high concentrations results in a dispersion of the light which lowers

the overall effectivity of the therapy. Moreover, we also considered the fears regarding the possible side-effects of NPs in the long run. CuS NPs have shown to be highly biodegradable but public opinion on NPs biopersistence is still quite negative.

In the end, we decided that the most interesting option to increase the bactericidal effect of CuS NPs was to try and study their effect in combination ICG.

On *S. aureus* the results were very promising. Even at the lowest concentration tried, 10 μg/mL of ICG and 10 μg /mL of CuS NPs, we obtained a reduction of 3 logs or a 99.9 reduction with a fluence of 180 J/cm^2 or 3 minutes of irradiation. A concentration of 10 μg /mL of ICG by itself only achieved a reduction of 0.8 logs and 10 μg /mL of CuS NPs did not have any significant effect. This represents an increase of 2.2 logs over the additive effect of both PSs by themselves. The highest bactericidal effect was not achieved at the highest concentrations, quite possibly due to the aforementioned dispersion of the light that such high concentrations could induce. The best results were obtained with 40 μg /mL of ICG and 40 μg/mL of CuS NPs, which showed a reduction of 6.5 logs or over 99.9999%. Again, a concentration of 40 μg/mL of ICG showed a reduction of 2.9 logs and 40 μg /mL of CuS had a reduction of 0.7 logs. In this case, this meant an increase of 2.9 log over the additive effect of both PS in separate.

On *P. aeruginosa*, we obtained similar results. While the lowest concentrations did not have such a high effect, we saw reductions of at least 3 logs for all the other concentrations. The highest effect was obtained with 10 μg /mL of ICG and 160 μg /mL of CuS NPs, which showed a reduction of 4.5 logs. A concentration of 10 ug/mL of ICG did not show any statistically significant effect and 160μg/mL of CuS had a reduction of 3.2 logs. Representing an increase of 1.3 logs over the additive effect of both PS separately.

While further investigation on this matter is necessary, these results point to the existence of a synergistic between these two PS. After studying the mechanism of action of these, we believe that one possible explanation for this effect resides in the differences between the mechanisms of photodynamic reaction that each of these PS follows. ICG has been known to favor Type II reactions, with a high production of singlet oxygens, [39] while on the other hand, CuS NPs seem to follow Type I reactions, with a higher production of peroxide oxygens. [69]

As the targets for each of the ROS is different, the combination of both routes might explain the observed synergistic effect. In the future, the next step would be to do a Fractional Inhibitory Concentration Index (FICI), used to determine if an effect is truly synergistic or not.

Another important point is that we were able to obtain similar bactericidal effects with the same concentrations of PS for both *S. aureus* and *P. aeruginosa*. Skin infections are very rarely caused by one single type of bacteria and so, aPDT treatments should be effective against both Gram + and Gram – bacteria. [70]

9. Conclusions and future work

After reviewing our results, we believe that CuS NPs have a potential application as a new PS in aPDT. It has shown to have an antimicrobial effect using subcytotoxic concentrations, while also keeping the temperature of the solutions low. It is of special interest their ability to produce similar results in both Gram positive and Gram negative bacteria. By itself, while its antimicrobial effect is on the low side compared to other PS its deeper penetration could make it a very interesting option for deeper infections, and its much shorter irradiation time could make it an attractive choice for patients. In combination with ICG, they showed a strong synergistic effect, achieving much higher antimicrobial effects using lower concentrations, and it is our belief that these results merit further investigation on this matter.

Another possibility that could be interesting in the future is to study their effect in combination with topical antibiotics, as these strategies have proven to be quite effective for other PS. [29-30]

In the future we would like to study the effectivity of this treatment with other resistant microorganisms, not only limited to bacteria. Fungi have proven to be traditionally harder to kill[13] and are also often also present in skin infections, so this would the logical next step.

All in all, we hope these results help pave the way for future research on CuS as we hope they might help enhance the versatility and effectivity of aPDT treatments in the not so distant future.

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Annex 1. MATERIAL AND METHODS

1.1 Microorganisms

Staphylococcus aureus ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were obtained from the American Type culture collection (ATCC;Rockville, MD). Columbia Blood agar (BA) was purchased from Oxoid.

1.2 Photosensitizers

1.2.1 Copper Sulfide Nanoparticles

All the chemicals used in the synthesis (polyvinylpyrrolidone, 10.000 Da Mw; polyvynilpyrrolidone 30 (PVP K30), 40 000 DaMw; polyvinylpyrrolidone, 50.000 Da Mw; copper(II) chloridedihydrate, ACS reagent ≥99.0%; sodium sulfide nonahydrate, ACS reagent ≥98,0%; hydrazine, 35 wt. % in water; sodium hydroxide, ACS reagent ≥97%,) were purchased from Sigma-Aldrich and used without further purification.

The synthesis of CuS NPs using a batch reactor was performed following the work of Ramadan et al[71] with some modifications. Compared to the original work, the reaction was scaled up five times, and due to the inherent variability of this synthesis method, the synthesis was performed in parallel in two open flasks. The synthesis was performed at 70ºC. In the open flasks, 240 mg of PVP K30 was dissolved in 25 mL of DDI water with 100 µL of a 0.5 M solution of $CuCl_2$ and 25 mL of water with its pH adjusted to 9. Next, 6,4 mL of hydrazine solution was added under stirring, leading to the formation of CuS seeds; finally, 200 µL of Na_2S (320 mg/mL) were added to the previous dispersion, after which it was kept under heating at 70ºC for two hours. All the solutions were added consecutively without dwell times. In figure 18 we can see the setup of the synthesis and observe the characteristic yellow color we obtain after adding the hydrazine (right flask) and how it turns a deep brown after adding the Na_2S (left flask). As seen in figure 19, after the two-hour synthesis the solution has turned deep brown-greenish, after which the resulting CuS NPs were thoroughly washed in DDI water by successive cycles of centrifugation.

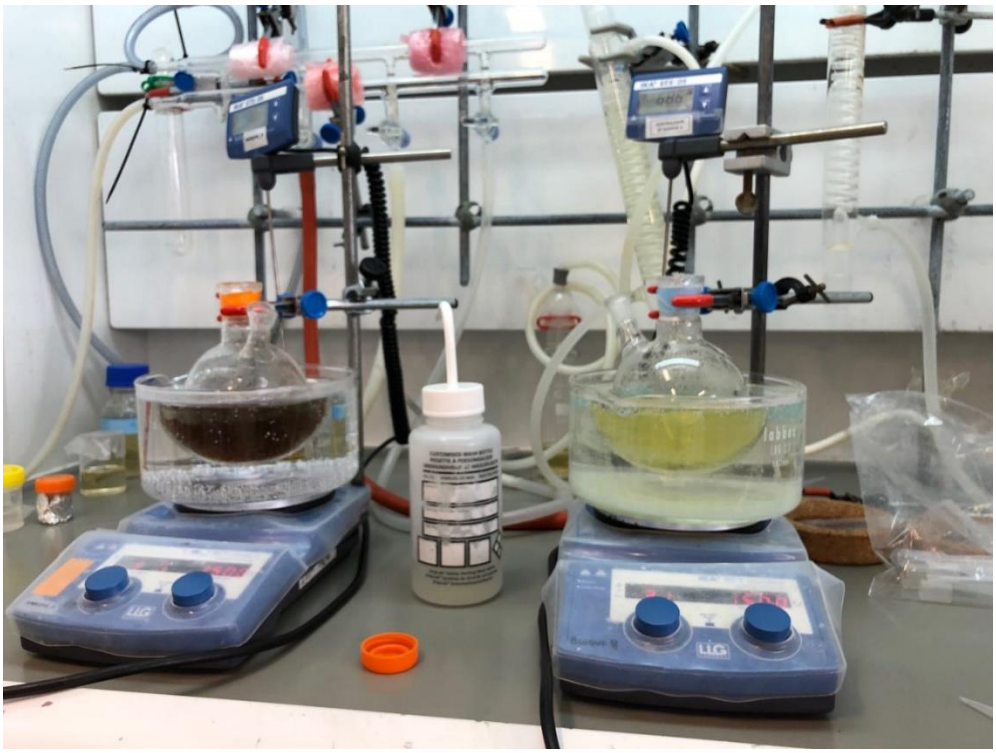

Figure 18 Picture of the experimental setup. On the right flask, we can see the characteristic yellow color obtained after adding the hydrazine. On the right we can already see the dark brown color obtained in the next step, after adding Na_2S.

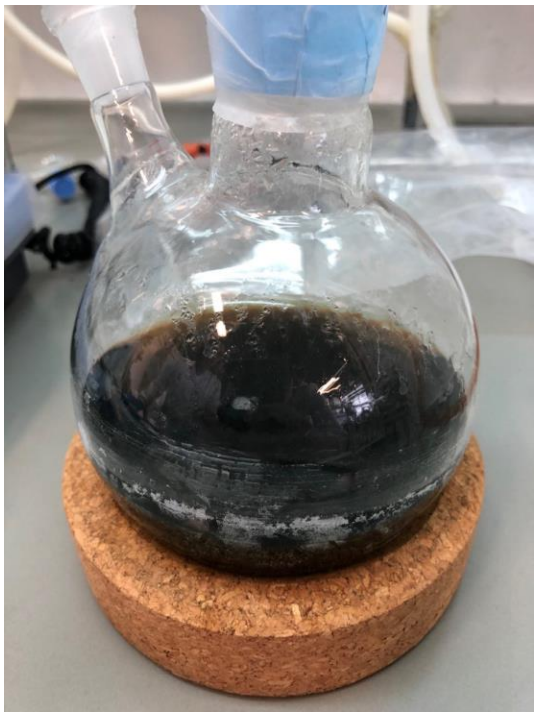

Figure 19 Picture of the dispersion after two hours at 70 oC. The color has turned brown-greenish, and the nanoparticles have been formed.

1.2.2Indocyanine Green

Indocyanine green I2633 25 mg was purchased from Sigma-Aldrich. ICG was diluted in distilled water and covered in aluminum foil to prevent photobleaching.

1.3 Characterization

The optoelectronic properties of copper sulfide can vary depending on its stoichiometry from covellite (CuS) to djurleite (Cu1.97S), digenite (Cu1.8S), anilite, (Cu1.4S), and chalcocite (Cu2S).[63] The maximum in the extinction spectrum in the NIR region at 1050 nm is reached for covellite. With other crystalline phases showing minimal absorption at those wavelengths. As the aim of the synthetized NPs was aPDT, the characterization of its optoelectronic properties was necessary. UV−vis absorption spectra of both ICG and CuS NPs were evaluated via a UV−vis−NIR spectrophotometer (Jasco V670, Tokyo, Japan)

The absorption spectrum of both CuS and ICG can be seen in Figure 16 and 15, respectively.

1.4 Experimental design

Selected bacteria were seeded on BA and cultured overnight. Stock inoculum suspensions were prepared in bidistilled water and adjusted to optical densities corresponding to 0.5 McFarland which for these microorganisms corresponds approximately to 10^8 cell/mL. McFarland scale is recommended for performance of susceptibility testing by CLSI [72] and EUCAST.[73]

Then, the 808 nm wavelength laser diode (6 × 8 mm^2 spot size; Optilas model MDL-III-808-2W, Changchun New Industries Optoelectronics Technology Co., Ltd., Changchun, China) was adjusted to an irradiance of 1 W/cm^2 using a power controller (Model PD300-3W, Ophir Laser Measurement Group, Logan, UT, USA). After that, 90 μL of the prepared inoculums were added to several 0.5 mL Eppendorf tubes. Then, 10 μL of varying concentrations of the photosensitizer (ICG, CuS NPs or both in which case 20 μL would be added) were added, so that the final volume in the Eppendorf tube was 100 μL. Irradiation was then carried out at point-blank range with no preincubation period; the suspensions were immediately subjected to irradiation with fluences of either 180 J/cm^2 (3 minutes) or 360 J/cm^2 (6 minutes). As control samples, first we exposed samples without photosensitizer to the same fluences, to check if the laser could have any bactericide effect by itself, and then, in parallel to the

irradiated sample, another identical sample was kept in the dark to check the toxicity of the PS. An image of the experimental setup in Miguel Servet hospital can be seen in figure 20.

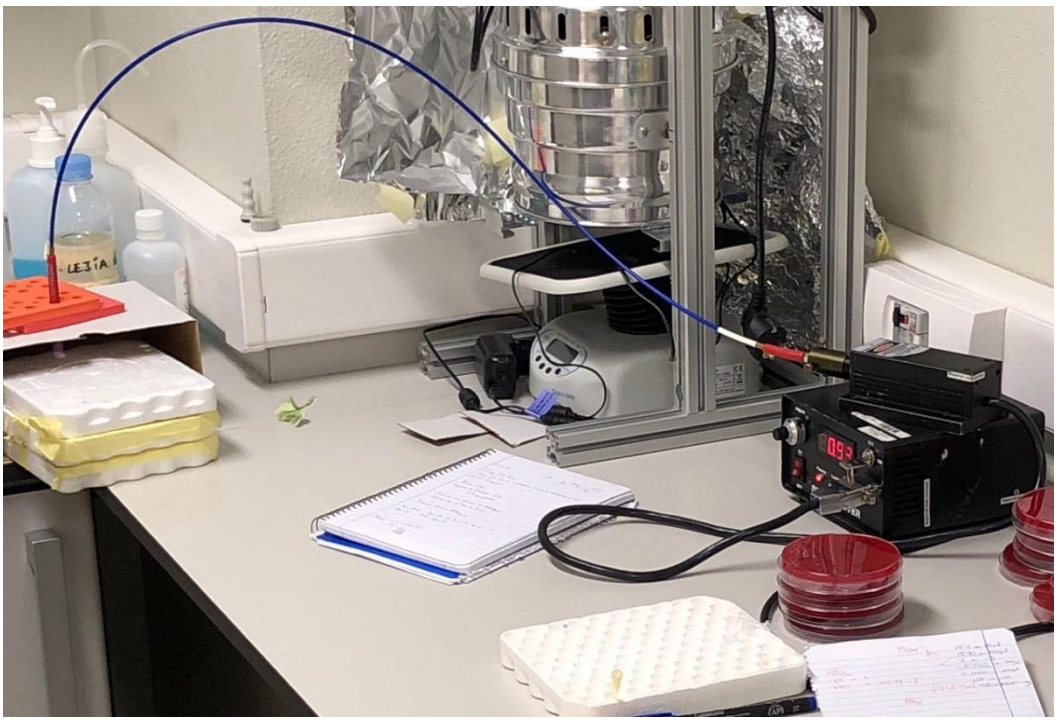

Figure 20 Picture taken of the experimental setup for the irradiation in the laboratory of Microbiology in Hospital Miguel Servet.

After completing the aPDT protocol, samples and controls were cultured on BA and incubated overnight at 37ºC. The effectiveness of the aPDT treatment was assessed by counting the number of CFU/100µl using a Flash and Go automatic colony counter (IUL, S.A, Spain) and comparing the results with controls. All experiments were carried out at least 3 times.

1.5 Data analysis

All data was analyzed with Graphpad 8.0.2, using Two-way anova on the data to determine the statistical significance of the results. The asterisks in the graphs should be interpreted as:

* Represents a P-value from 0.01 to 0.05 and the difference is considered significant.

** Represents a P-value from 0.001 to 0.0005 and the difference is considered very significant.

*** Represents a P-value from 0.01 to 0.05 and the difference is considered extremely significant.

**** Represents a P-value below 0.01 and the difference is also considered extremely significant.

Annex 2. aPDT controls

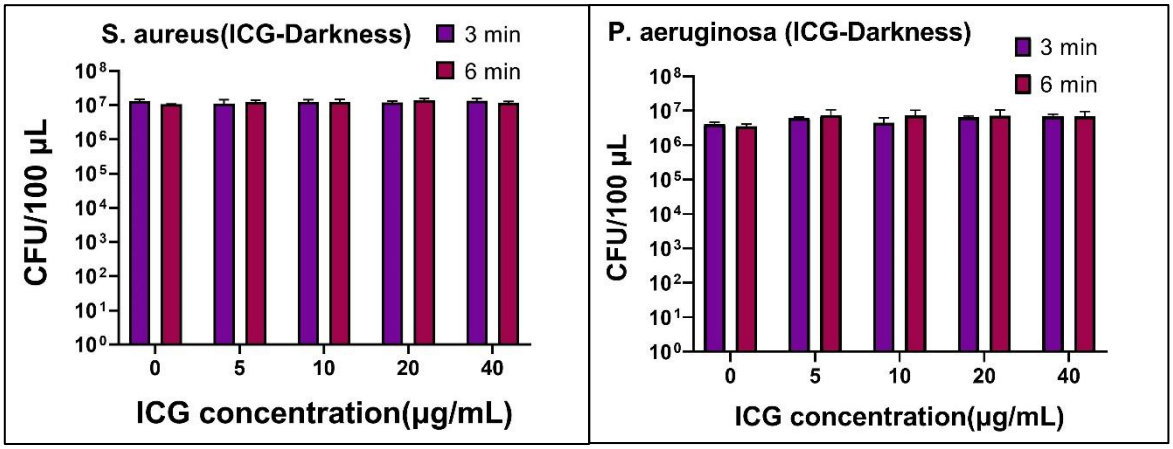

Figure 21 Bactericidal effect of ICG for S. aureus ATCC 29213(left) and P. aeruginosa ATCC 27853 (right) in water suspensions with ICG concentrations of 0, 5, 10, 20 and 40 µg /mL , incuvated in the darkness for 3' and 6'. Bars show cell viability measured in CFU/100 µL counted after a 24 hours incubation at 37ºC. Each column is the mean ± standard deviation(n=3)

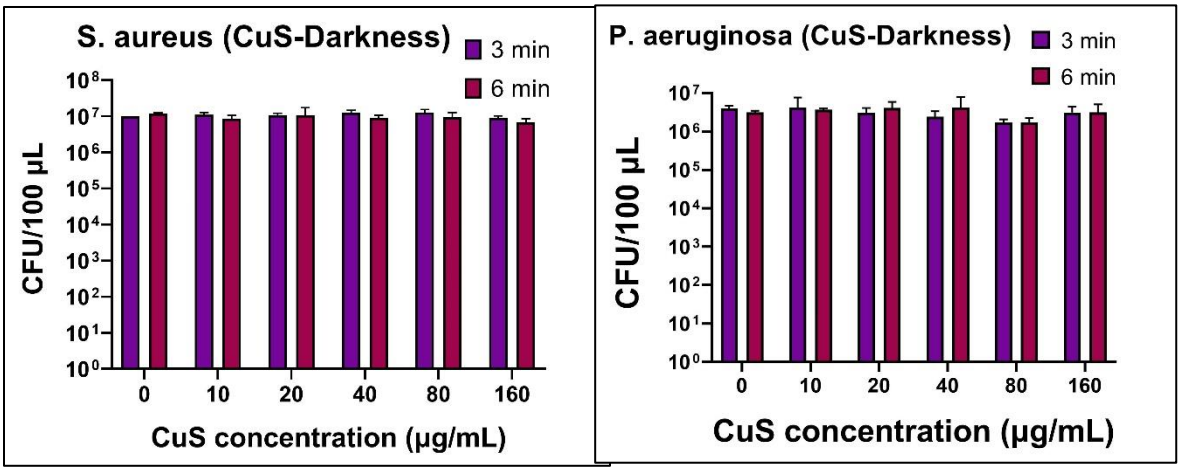

Figure 22 Bactericidal effect of CuS for S. aureus ATCC 29213(left) and P. aeruginosa ATCC 27853(right) in water suspensions with ICG concentrations of 0, 10, 20, 40, 80 and 160 µg /mL , incuvated in the darkness for 3' and 6'. Bars show cell viability measured in CFU/100 µL counted after a 24 hours incubation at 37oC. Each column is the mean ± standard deviation(n=3)

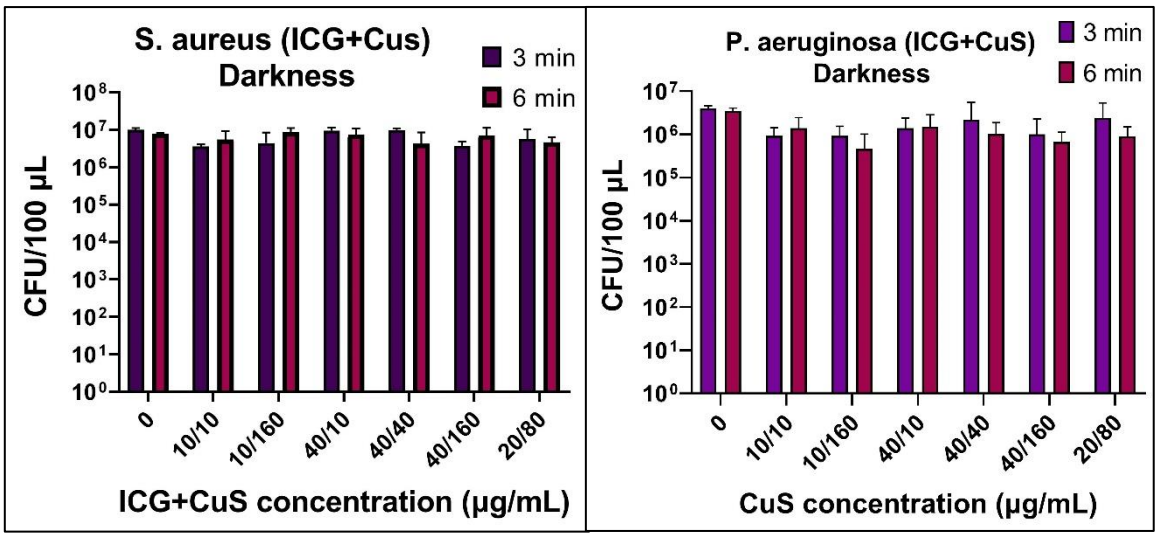

Figure 23 Bactericidal effect of ICG+CuS for S. aureus ATCC 29213(left) and P. aeruginosa ATCC 27853(right) in water suspensions with ICG concentrations of 0, 10+10, 10+160, 40+10, 40+40, 40+160, 20+80 µg /mL, incuvated in the darkness for 3' and 6'. Bars show cell viability measured in CFU/100 µL counted after a 24 hours incubation at 37oC. Each column is the mean ± standard deviation(n=3)