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The role of the light source in antimicrobial photodynamic therapy

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Antimicrobial photodynamic therapy (APDT) is a promising approach to fight the growing problem of antimicrobial resistance that threatens health care, food security and agriculture. APDT uses light to excite a light-activated chemical (photosensitiser), leading to the generation of reactive oxygen species (ROS). Many APDT studies confirm its efficacy *in vitro* and *in vivo* against bacteria, fungi, viruses and parasites. However, the development of the field is focused on exploring potential targets and developing new photosensitisers. The role of light, a crucial element for ROS production, has been neglected. What are the main parameters essential for effective photosensitiser activation? Does an optimal light radiant exposure exist? And finally, which light source is best? Many reports have described the promising antibacterial effects of APDT *in vitro*, however, its application *in vivo*, especially in clinical settings remains very limited. The restricted availability may partially be due to a lack of standard conditions or protocols, arising from the diversity of selected photosensitising agents (PS), variable testing conditions including light sources used for PS activation and methods of measuring anti-bacterial activity and their effectiveness in treating bacterial infections. We thus sought to systematically review and examine the evidence from existing studies on APDT associated with the light source used. We show how the reduction of pathogens depends on the light source applied, radiant exposure and irradiance of light used, and type of pathogen, and so critically appraise the current state of development of APDT and areas to be addressed in future studies. We anticipate that further standardisation of the experimental conditions will help the field advance, and suggest key optical and biological parameters that should be reported in all APDT studies. More *in vivo* and clinical studies are needed and are expected to be facilitated by advances in light sources, leading to APDT becoming a sustainable, alternative therapeutic option for bacterial and other microbial infections in the future.

1. Introduction

Due to the widespread use of antibiotics in various areas of our life, from medicine to farming, we are currently facing an ever-increasing threat from antimicrobial resistance (AMR)[1–6] which, is so severe that it has been called the "post-antibiotic apocalypse".[7,8] We need to explore alternative ways to kill infectious bacteria, as otherwise many diseases, routine surgery and injuries could lead to serious infections, sepsis and death. The problem applies not only to bacterial infections, but also those caused by fungi and parasites. The dynamic expansion of

AMR has reached even the most remote and uninhabited areas of the planet, such as High Arctic soils where in 2019 antibiotic resistance genes were discovered.[9] It has been shown that antibiotic resistance can develop very quickly – in one study wild type *Escherichia coli* took just eleven days to develop mutations allowing them to survive in an antibiotic at 1000 times the concentration that was initially lethal.[10] A serious effect of this worldwide crisis is increasing mortality among patients infected by drug resistant pathogens.[11–15] Importantly, AMR can also lead to amputation in some cases of incurable bacterial infections.[16,17]

Photodynamic therapy (PDT) provides an alternative way of killing bacteria.[18] It uses light in combination with a light-activated chemical (photosensitiser) in the presence of oxygen to generate reactive oxygen species (ROS) that kill nearby cells. It is commonly used to treat many skin cancers, and is also used for the treatment and palliative care of internal cancers.[19–21] There is now great interest in the ability of PDT to kill bacteria and other microbes. Antimicrobial Photodynamic Therapy (APDT) is known

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by many names such as Antimicrobial Photodynamic Inactivation (aPDI/APDI)[22–26] Antimicrobial Photoinactivation,[27–29] Antimicrobial Photodynamic Therapy (aPDT/APDT),[30–37] lethal photosensitization,[38–40] photoactivated disinfection (PAD)[41–43] (in the dental field), Photodynamic Antimicrobial Chemotherapy (PACT)[44,45] and Photodynamic Inactivation of Bacteria (PIB).[46–48] All these therapies differ only in the name as they all work in the same way *i.e.* by using light to activate a photosensitiser that generates ROS that kill the pathogen. In this review, the term APDT will be used for the rest of the article, regardless of whether the studies were conducted *in vivo* or *in vitro*.

Photosensitisers can undergo two types of photochemical reactions, both using triplet oxygen ($^3O_2{}^3\Sigma_g{}^-$) as a reagent. Type I, is based on the production of superoxide anions ($O_2{}^-$), leading to the formation of various free radicals (including hydroxyl radicals $HO^\bullet$, peroxyl radicals $ROO^\bullet$ and alkoxyl radicals $RO^\bullet$) and radical ions (radical cation of thymine or guanine). Type II reactions involve the production of singlet oxygen ($^1O_2{}^1\Delta_g$), which is highly reactive.[28,29,49,50] Inactivation of pathogens *via* photodynamic processes is classified as a type of Photodynamic Therapy (PDT) and poses an attractive and most importantly effective alternative treatment against viruses,[51–56] fungi,[57–63] parasites[64–68] and bacteria.[23,27,37,69–71]

Over the last 20 years most of the work towards APDT has been focused on the properties of photosensitisers. The biological diversity of microorganisms is a challenge to the efficacy of this therapy. One of the main problems is the composition of pathogen cell walls. Gram-positive bacteria have a thicker peptidoglycan layer (20–80 nm) with polysaccharides, peptidoglycolipids and teichoic acids attached covalently, whereas Gram-negative bacteria have a thinner peptidoglycan layer (5–10 nm) with Braun's lipoprotein, in the outer membrane.[49] Therefore, a great effort has been undertaken to optimise the properties of photosensitisers, which would allow the

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treatment of both a wide range of infections, and specific targeted infections. The short lifetime of singlet oxygen (3 μs in a metabolically-functioning single cell[72] or H_2O[73]) is the reason why the immediate proximity of the photosensitiser to the microbial cell is needed for the best results.[28]

As PDT involves the interaction of light with photosensitisers, the light source is just as important as the photosensitiser. In fact, the development of the field has been largely shaped by the availability of light sources. As photosensitisers have been recently reviewed,[74,75] our focus is on the role of light and light sources in APDT. It is timely because advances in optoelectronics, including lasers, LEDs and organic LEDs have transformed lighting and are poised to transform photodynamic therapy. In order to keep the review a manageable size, we focus on the simple, long-established, widely available photosensitiser methylene blue (MB) that is approved by the Federal Drugs Administration for the treatment of methemoglobinemia and is widely used off-label for PDT.[76] For the first time we bring together and analyse data for all published APDT studies using MB in the period 2000–2022. We explore whether parameters such as light source, irradiance (incident light intensity per unit area, usually measured in W cm^{-2}) and radiant exposure (incident light energy per unit area, usually measured in J cm^{-2}) influence the effectiveness of APDT. We then discuss the implications for the future of the field.

2. The meaning of light in antimicrobial photodynamic therapy

Light is an essential element for APDT, and the characteristics of the light required, in turn, define the most suitable light sources. In this section we consider the role of the wavelength of light and light-tissue interactions in APDT.

2.1 The optimal treatment wavelength

APDT studies *in vitro* have shown that this method can be effective for killing a wide range of pathogens, both prokaryotes and eukaryotes.[28,77,78] There are more constraints to consider when performing APDT *in vivo*. The wavelength of the photoactivation light, crucial for ROS production, has to be safe for host (human) cells. Consequently, ultraviolet light (<400 nm) should be avoided in APDT treatment, due to its high potential for DNA mutagenesis leading to oncogenesis and forming toxic products of tryptophan, tyrosine and riboflavin.[79–82] UV light exposure (7–25 J cm^{-2}) during *in vivo* studies resulted in drastic changes in the dermis and epidermis and promoted oxidative stress. *In vitro*, it led to the apoptosis of fibroblasts, the formation of the tumour suppressor p53 and thymine dimers, the occurrence of sunburn cells and skin pigmentation increase.[83,84] However, UV-B sunlight is crucial for humans to produce cholecalciferol (vitamin D_3), the production of which is required for calcium and phosphate homeostasis.[85] Moreover, PUVA (psoralen and ultraviolet A) and narrowband UVB (NB-UVB) therapy are based on UV light and are widely used to treat psoriasis. Nevertheless, prolonged use of the above treatments has been reported to have carcinogenic effects, including non-melanoma skin cancers (NMSC) squamous cell carcinomas (SCC) and basal cell carcinomas (BCC).[86–88]

The possible toxicity of blue light has been explored in studies on cell lines. In 2010 Liebmann *et al.* investigated the influence of solar irradiation at longer wavelengths on human skin cells.[89] Their research demonstrated the cytotoxic effect of blue light at high fluences (412, 419, 426 nm: 66–100 J cm^{-2} and 453 nm: >500 J cm^{-2}) on keratinocytes, endothelial cells and proliferating T lymphocytes (proliferation reduction). The significant proliferation decrease caused by blue light irradiation is the basis of hyperproliferation treatment, for example against psoriasis as an alternative to PUVA therapy. Opländer

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et al., also studied the effect of blue light on skin. They showed the dependence of cytotoxicity on the applied wavelength and defined the range of blue light (400–460 nm) characterised by antiproliferative properties.[90] Masson-Meyers *et al.*, in turn, have suggested that light of a wavelength of 470 nm (at 5 J cm^{-2} fluence only) does not diminish wound healing (*in vitro*) and might even stimulate the tissue recovery process.[91] A similar effect of a low-dose blue light was observed by Mignon *et al.* in 2016, and was attributed to enhancement of collagen production, but further research (Mignon *et al.* 2017) with several light-exposures applied (more than one irradiation) showed a toxic impact of light with wavelength between 450 and 590 nm on cellular metabolic activity.[92,93] Blue light has been reported to be harmful to human retinal pigment epithelial (RPE) cells, causing so-called blue light-induced apoptosis (BLIA).[94–97] Blue-green light is also considered to be damaging to the retina cells,[98,99] however, Arnault *et al.* showed that green light with wavelength of 550 nm does not exhibit toxic effects on RPE.[100] There is no evidence of green light being cytotoxic to skin, and it may be applied in skin lesion treatment.[100] However, overall the effect of blue light remains a matter of debate as studies on patients have not shown toxicity of blue light, and according to the best available clinical research evidence, blue filters are not needed to protect eyes.[101]

Interestingly, in the case of red light (632, 648 nm) and NIR light (850, 940 nm) no negative effects are observed.[89] In the last 50 years red light therapies have been extensively investigated and successfully applied in the treatment of various conditions such as wound healing, skin diseases, traumatic brain injuries, Alzheimer's and Parkinson's disease, depression and inflammatory diseases.[92,102–106] Wavelengths in the red and NIR are preferred for photodynamic therapy (whether antimicrobial or cancer) for several reasons. Firstly, there are well-known photosensitizers, such as derivatives of chlorins, bacteriochlorins, phthalocyanines, and phenothiazine-based dyes, absorbing this range of light very efficiently.[107–109] Secondly, red and NIR light penetrate skin significantly deeper than shorter wavelengths, and finally, so far there is no evidence that they are toxic to cells.

2.2 Light propagation in human tissue

The propagation of light in tissue is a key step in PDT as light must reach the photosensitiser in the target tissue. Light can be reflected from the surface of tissue, and once inside it can be strongly scattered and absorbed. In fact, tissue (including skin) scatters light so strongly that each ray (or photon) can be scattered many times before it is absorbed, making light propagation in tissue a complex problem to study. The regular reflectance of skin illuminated by light at normal incidence is around 4–7%, and the rest of incident light is either scattered or absorbed in the tissue.[110] Therefore, light absorption and light scattering are the dominant processes to understand in light-tissue interaction.

The absorption of light in tissue results from different components including water, oxyhaemoglobin and haemoglobin in the epidermis, melanosome, vessel wall, and whole blood.[111] It depends on the wavelength, and can be characterized by absorption coefficients $\mu_a(\lambda)$ as shown in Fig. 1.
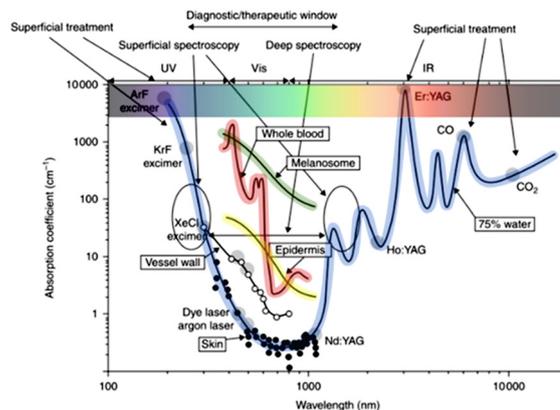

Fig. 1 Primary absorption spectra of biological tissues and wavelengths of lasers commonly used in medicine. Reprinted with permission from Altschuler and Tuchin.[111]

Light scattering is a photon-matter interaction which alters the direction of propagation of the photon. It occurs at the interface of different tissue components due to the differences in refractive index. Structures much smaller than a wavelength of light lead to Rayleigh scattering, whilst structures comparable to or larger than the wavelength of light lead to Mie scattering.[112] The scattering coefficient is also wavelength-dependent and can be represented as $\mu_s(\lambda)$. In modelling, the reduced scattering coefficient $\mu'_s(\lambda)$ is often used, and is given by $\mu'_s(\lambda) = \mu_s(1 - g)$, where the anisotropy parameter g is the average of cosine of the scattering angle. Fig. 2 shows the wavelength-dependent reduced scattering coefficient for different scattering types.[113] Different simulations have been used for calculating the light propagation in tissue for sensing or calculation of absorbed dose of light.[114–116]
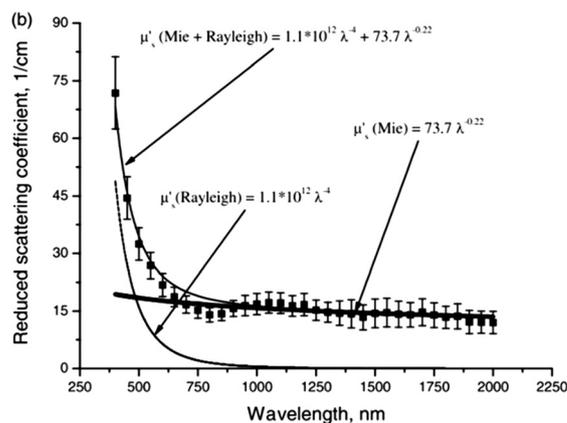

Fig. 2 Reduced scattering coefficient of human skin as a function of wavelength. The symbols show the averaged experimental data and standard deviation represented by vertical lines. The contribution of Mie and Rayleigh scattering, and their combination are also presented. Reprinted with permission from A. Bashkatov.[113]

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Monte Carlo simulations are the most widely used method for simulating light transport in tissue. Photon packets are generated, and their trajectory through the tissue is recorded.[117] Once launched, a photon will travel a certain distance and come across different events (absorption, scattering, reflection or transmission). The photon changes direction many times until it is terminated by escaping from the tissue or absorption in the tissue. Reflection or transmission is recorded when the photon escapes from the tissue, and the position of the photon is recorded when it is absorbed by the tissue. The recorded ray history is then analysed after the simulation is completed. As the number of photon trajectories studied increases, the simulation becomes more accurate. In combination with measured values of the optical properties of tissue, Monte Carlo simulations provide a detailed understanding of light propagation in tissue.

The penetration depth of light is a key parameter in evaluating the absorbed dose of light in photodynamic therapy. It is normally defined as the depth at which the incident intensity (irradiance) decreases to 1/e of its initial value. The light penetration depth can be calculated using the absorption and reduced scattering coefficients,[113] giving a relationship between wavelength and light penetration depth into the skin as shown in Fig. 3. For photodynamic therapy, a commonly used wavelength range is from 600–900 nm because these wavelengths are safe and can propagate further into tissue than shorter wavelengths.[110,113] We can see that the calculated penetration depth at 600, 700, 800 and 900 nm are 1.5, 2.0, 2.3 and 2.5 mm respectively.[113] Also, Monte Carlo simulations have shown that beam width can affect the penetration depth. An increase in beam width from 1 to 5 mm can significantly increase the penetration depth, but a further increase in beam width has little effect on it.[112]

3. Light sources and their properties

Light is crucial for PDT as it excites the photosensitiser, which then leads to the generation of reactive oxygen species. Hence the light source is very important and needs to fulfil several
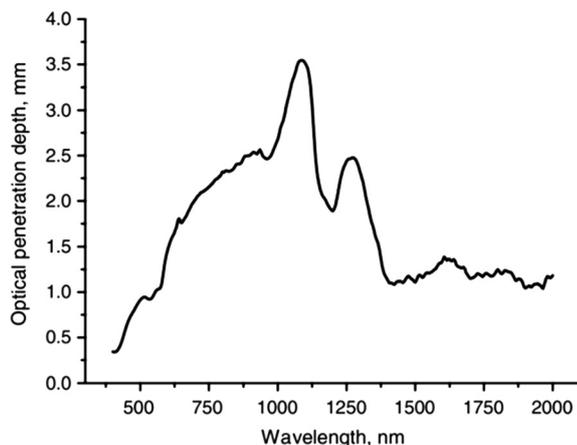

Fig. 3 Optical penetration depth of light into skin at wavelengths from 400 to 2000 nm. Reprinted with permission from A. Bashkatov.[113]

requirements to achieve effective PDT. First, the wavelength(s) of the light source needs to be absorbed by the photosensitiser, and so needs to be in the region of the absorption spectrum of the photosensitiser. Second, as explained above, the wavelength must be able to propagate far enough in tissue for the desired treatment. Third, the light source needs to deliver sufficient irradiance of light to perform PDT, but should not be so high as to cause unnecessary pain due to heat generation.[118] In addition to irradiance and spectrum, there are many other parameters such as light uniformity, equipment size, cost and safety issues which should also be considered. In this section, the advantages and disadvantages of different light sources that are used in antimicrobial photodynamic therapy will be discussed.

3.1 Lasers

LASER is an acronym for Light Amplification by Stimulated Emission of Radiation and is the most widely reported light source for APDT. Lasers generate monochromatic, coherent and collimated light of high irradiance. There are several popular choices of lasers for APDT such as argon ion lasers, metal vapour-pumped dye lasers, Nd: YAG lasers and diode lasers.[119,120] They share the benefit of being able to deliver high irradiance from hundreds of mW cm^{-2} to several W cm^{-2}. These irradiances are higher than for other light sources, but limited to a small area of irradiation. Their narrow emission bandwidth can specifically target the absorption peak of photosensitisers. However, the available wavelengths are limited, hence, the use of dye lasers which use organic dye molecules as the gain medium enabling a wide range of wavelengths to be generated to match the absorption of various photosensitisers. Although lasers are widely installed in clinics, they have some limitations including being expensive, cumbersome, the emitted beam usually being rather small and potential eye safety problems. Sometimes lasers are coupled into fibre bundles, which can be more convenient for delivering light to a patient. The unique feature of laser light, namely its coherence is not needed for PDT and so a range of other light sources is also suitable for PDT.

3.2 Lamps

Lamps have a very long history in light therapy, dating back to the late nineteenth century when the "Finsen lamp" was first used to treat *lupus vulgaris*.[121] Today there are several lamps in use for APDT including tungsten filament, Xenon arc, metal halide, sodium and fluorescent lamps.[140] Lamps are normally less expensive and easier to handle than lasers and they deliver light irradiance from several to hundreds of mW cm^{-2}. Unlike lasers, lamps such as sodium lamps and fluorescent lamps can be used for large-area treatment without coupling to fibres.[120] Furthermore, the broadband lamps emit a wide range of wavelengths which can cover the whole visible spectrum including the absorption of many commonly used photosensitisers. However, adverse effects such as heat generation by infrared light and tissue damage by ultraviolet light can cause problems.[138] For this reason, spectral filters are usually applied to cut off wavelengths not matching the absorption of the photosensitiser.

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The spectra of filtered lamps for APDT will finally depend on the filters applied, the bandwidth typically ranges from 10 to 100 nm. There are a few limitations of filtered lamps such as large equipment size, expensive spectral filters, and low efficiency due to a combination of the limited efficiency of the lamp itself and to a significant fraction of the light output being blocked by the filters.

3.3 Light-emitting diodes (LEDs)

LEDs are a common light source in which a voltage is applied to a semiconductor, leading to injection of charges and emission of light. The semiconductor can be an inorganic material (such as GaAs or GaN), or an organic semiconductor. However, in practice the term "LED" is used for inorganic semiconductor LEDs, and organic LEDs are called "OLEDs". LEDs are widely used in lighting and displays, and are also used as light sources for medical treatment. Commonly used materials for LEDs are InGaN, AlGaInP, AlGaAs and GaP. The emission wavelength is determined by the band gap of the semiconductor and can range from the UV to infrared regions of the spectrum, depending on the material. Many LEDs (especially visible and near infra-red) are low-cost in comparison to other light sources, and they can deliver high irradiances up to hundreds of mW cm^{-2} in an energy-efficient way. Like lamps, LEDs are neither monochromatic nor coherent, but their emission spectrum is much narrower – typically 20–40 nm wide. The available wavelengths of emission of LEDs cover most photosensitisers, and so LEDs can be selected to match the absorption of a photosensitiser, and used without a filter.

LEDs can be mounted as arrays for large-area treatment. Currently, PDT devices based on LEDs are used in hospitals and clinics, but they are still large and cumbersome. However, as individual LEDs are small, arrays of them can be made into smaller, wearable devices.[122] An important consideration in all LED based devices is how to achieve uniform illumination.[123] This is because LEDs are effectively point sources and so an array of them will naturally be brighter at each LED than in between. For the large hospital-based LED light sources this problem is overcome by placing the array at a distance ($\sim$10 cm) from the patient so that the light from neighbouring LEDs overlaps when it reaches the patient. For a wearable device the light needs to be spread by diffusers which lead to some loss, and also means the light source needs some thickness ($\sim$1 cm) to allow the light to spread out.

3.4 Organic light-emitting diodes (OLEDs)

As well as the inorganic semiconductors discussed above, there are organic semiconductors. These are carbon-based materials which are conjugated, leading to extensive electron delocalisation and semiconducting properties. In contrast to inorganic semiconductors which are rigid, brittle, crystalline materials grown epitaxially, organic semiconductors are often amorphous and flexible. They can readily be deposited from solution or by evaporation and their properties can be tuned by changing their chemical structure. This combination of simple fabrication, flexibility and scope to tune emission across the visible means that OLEDs are now widely used to make vivid and attractive mobile phone displays, and increasingly used for televisions.

OLEDs are lightweight, thin and easy to fabricate by evaporation or solution-based processes. They are intrinsically area light sources (in contrast to inorganic LEDs which are generally point light sources) and so are very suitable for illuminating an area uniformly, as desired in PDT. OLEDs can also be flexible, allowing them to conform to human skin. The available emission wavelengths range from near UV to NIR with a typical emission bandwidth of 60–100 nm. The wavelength can be tuned by changing the chemical structure of the emitter, and fine-tuned using microcavity structures.[124–129] The first studies of OLED mediated photodynamic therapy are encouraging and suggest that OLEDs could become the ideal light source for ambulatory PDT.[123,125] So far OLEDs give lower emission irradiance (around 5 mW cm^{-2}) than most other light sources but progress is being made towards higher light outputs. The lower irradiance requires a longer exposure time to achieve a given radiant exposure of light, but this is acceptable for an ambulatory device, and has the potential to reduce pain commonly experienced in PDT.

3.5 Daylight

Apart from artificial light sources, PDT using daylight has been promoted in recent years.[130–134] Daylight PDT makes use of sunlight which has a broad-spectral range from UV to IR region, so there are many available photosensitisers that can be used. It has some attractive features: it is free of cost, can be accessed without visiting clinics and can illuminate a very large area with high uniformity.[135] These features mean daylight PDT has been successfully used to treat actinic keratosis on the scalp.[136–139] However, as sunlight is very variable in irradiance, radiant exposure is poorly controlled in daylight PDT. As APDT is likely to be used in situations such as wound healing, and wounds can be on many areas of the body, daylight PDT is likely to be less suitable for APDT than for the treatment of actinic keratosis.

3.6 Other light sources

In addition to the light sources described above, some APDT research simply reports using "non-coherent" or "non-thermal" light sources without giving details of the actual light source used. Even if essential light parameters such as radiant exposure, light irradiance, power output, or wavelength are presented, this may leave questions about the type of light source, mode of operation, appearance of the device, or size of irradiation area. There are also occasional reports of other light sources such as endoscopy systems, photopolymerisers and supra-luminous diodes (SLD).

4. Light sources for antimicrobial photodynamic therapy – approaches to searching and comparison

4.1 Methylene blue as a reference photosensitiser

Singlet oxygen plays a very important role in photochemistry and PDT. One of the most commonly used reference

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compounds to appraise quantum yield of singlet oxygen generation $\varphi(^1O_2)$, which is the essential parameter of the photosensitiser, is methylene blue (MB) known also as methylthioninium chloride. For MB in water, $\varphi(^1O_2)$ is 0.52.[140] In aqueous solution methylene blue can form dimers, and there is an equilibrium between monomers and dimers that depends on factors such as, for instance, pH and temperature.[141] Methylene blue was synthesised for the first time in 1867 by Heinrich Caro with the main purpose of cotton staining. A breakthrough was Guttmann and Ehrlich's discovery of its antimalarial properties, thus methylene blue has become the first clinical synthetic antiseptic.[142,143] It has become a widely used diagnostic agent and drug to treat congenital and induced methemoglobinemia, vasoplegic syndrome and ifosfamide-induced encephalopathy.[144–146] MB is water soluble but *via* the uncharged and lipophilic intermediate called leucomethylene blue (LMB/MBH), passes through the cell membrane and promotes non-enzymatic reduction of Fe^{3+} methaemoglobin ions results in normal haemoglobin.[147] Inside the cell LMB is oxidized to MB, where high intracellular concentrations (20–100 μM) lead to the increase of phosphogluconate activity, oxygen consumption and glutathione oxidation.[148] Dihydronicotinamide-adenine dinucleotide phosphate (NADPH) is then involved in the reduction of oxidized MB (MB^+) to its active reduced form MBH, which causes activation of the glucose 6-phosphate dehydrogenase and therefore pentose phosphate pathway stimulation.[149]

MB is also involved in other biological processes, such as enhancing cell proliferation in HGPS cell lines and improving cellular respiration of astrocytes.[150,151] The regular clinical concentration of methylene blue solution is 10 mg mL^{-1} (1%) and the therapeutic dose is <2 mg kg^{-1} body weight.[143] MB is widely available and has been very broadly used in PDT, so has been chosen as the focus of this review, and the basis for comparing usefulness of various light sources.

4.2 The protocol of searching

This literature review includes over 330 publications and papers published from January 2000 to September 2022 collected from the platform Web of Science (formerly ISI Web of Knowledge) across all databases. The research was based on a topic search of *methylene* AND *blue* AND *photodynamic*, thus titles, abstracts and keywords were searched and 1892 articles and proceedings were identified for further consideration. From this search, original papers and proceedings about methylene blue mediated APDT (MB-APDT), with a water, phosphate-buffered saline (PBS) or physiological saline (0.9%NaCl w/v) solution of a photosensitiser, were analysed. Both *in vitro* and *in vivo* experiments (animal models and human studies) were included. Only articles in the English language were considered. In the end 503 published articles and proceedings were identified from which 330 have sufficient information to use for further analysis. The major aim was to identify the parameters of light used. Additional information collected includes photosensitiser concentration, APDT target, reduction in pathogen cells, and various experimental details

such as initial number of cells, the distance between sample and light source, pre-irradiation incubation time (preincubation), and method of cell survival determination.

5. Results

The results of the search on APDT using methylene blue are shown in Table 1. The table summarises the main types of light sources used, together with the key experimental conditions for APDT.

Data from each group was converted to the most frequent measurement units: watts (W) for power output, milliwatts per square centimetre (mW cm^{-2}) for illumination irradiance, joules per square centimetre (J cm^{-2}) for radiant exposure, nanometres (nm) for wavelength, and minutes (min) for exposure time. The leading unit for photosensitiser concentration is milligram per millilitre, however, due to a large range of data including very low concentrations, we have used micrograms per millilitre (μg mL^{-1}). For all parameters, the range of values used in the papers identified is presented. Where relevant, the mean is also shown. As there is a wide spread of many parameters, but with some tendency to concentrate around particular values, the mode (*i.e.* most frequently occurring value) was also calculated. Empty spaces in the table mean lack of literature data. In this review, no attempt was made to calculate any parameters due to the use of only data explicitly provided in the literature.

5.1 The targets of methylene blue-mediated antimicrobial photoinactivation

From the analysis of 330 published articles and proceedings, 84% are *in vitro* studies, 8.5% in animals and 7% on patients. For animal studies, the murine model is most common and used in approximately half of the studies. The targets of APDT were mainly bacteria (56% of all cases) followed by fungi (29% of cases), virus (8% of all cases), parasites (4.5% of cases), plants (1.5% of all cases) and animals (1% of all cases) (Fig. 4).

The calculations of the percentage distribution of MB-APDT targets were based on the biological diversity (variety of species) of the organisms studied over the years 2000–2022. The most frequently studied bacterium of bacteria targets was *Staphylococcus aureus* (23%), including antibiotic resistant varieties such as Methicillin-resistant *Staphylococcus aureus* (MRSA). The next most popular bacterial targets were *Escherichia coli* (12.5%), *Enterococcus faecalis* (10.5%), *Pseudomonas aeruginosa* (9%) and *Streptococcus mutans* (8%) (Fig. 5a). A possible reason for *S. aureus* being a more common target is that it is a widespread skin pathogen of major clinical concern that is often antibiotic resistant and therefore an attractive target for APDT.[169,239,397,452]

Gram-positive bacteria account for 55% of the bacterial species tested, while 45% are Gram-negative. Considering this division of bacterial targets, the % distribution of the most frequently studied species, *Staphylococcus aureus*, constitutes almost half (41%) of the Gram-positive bacteria studied (Fig. 5b),

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Table 1 The parameters of light sources used in studies of methylene blue mediated APDT published between 2000 and 2022

| Light source (according to the number of research) | Light parameters | | | | | | | |
	Power output [W] range **mode** mean	Irradiance [mW cm^{-2}] range **mode** mean	Radiant exposure [J cm^{-2}] range **mode** mean	Wavelength [nm]	Irradiation time [min] range **mode** median	PS concentration [μg mL^{-1}] range **mode** median	No. of papers	Ref.
LASER	0.021–7 **0.040** 0.25	0.35–2.1 × 10^6 **100** 26 789	0.24–6048 **30** 128	630–690	0.17–120 **5** 4	0.001–3.2 × 10^5 **100** 24.47	163	33, 57, 71 and 152–311
LED	0.1–0.69 **0.24** 0.27	0.15–1100 **100** 104	0.24–305 **10** 47	560–780	0.33 – 200 **10** 10	0.0003–5 × 10^4 **31.98** 17.59	108	59, 60, 64, 67, 68, 128, 194, 231, 233, 275, 301 and 311–407
Lamp	0.165–2.8 **n/d** 1222	1.6–7 × 10^3 **40** 306	0.0075–1010 **200** 55	350–IR	2 – 360 **10** 20	0.003–5 × 10^3 **3.2** 6.4	49	22, 70, 174, 181, 185, 193, 205, 217, 281, 316, 320, 401 and 408–444
Other in-/ non-coherent light	0.3–6.88 **0.70** 0.7	32.5–400 **100** 117	0.9–360 **40** 45	400–800	0.2–120 **4** 6.4	0.032–511.76 **6.4** 6.4	10	46 and 445–453
OLED organic light-emitting diode		1.5–9,44 **n/d** 6	2–129.6 **n/d** 36	570–750	47–180 n/d 104	1.25; 5	3	125, 128 and 129
Solar radiation		2.9–579.2 **n/d** 174		UV–IR	30; 540	0.05–63.97 11.7	3	454–456
OPO oscillator optical parametric		10; 250	30; 90	665	2; 3.3	16	2	157 and 457
Endoscopy system		150			5; 10; 15	20; 200; 400	1	458
HHP hand-held photopolymerizer		350–500	2	400–500	1; 3	0.032; 3.2	1	459
Non-termal light				664	24	1; 500	1	189
SLD supra-luminous diodes	5.1	7.8; 120	3; 12; 24; 40; 60	625	0.5; 2.3; 4.6; 7.8; 11.6	90	1	460

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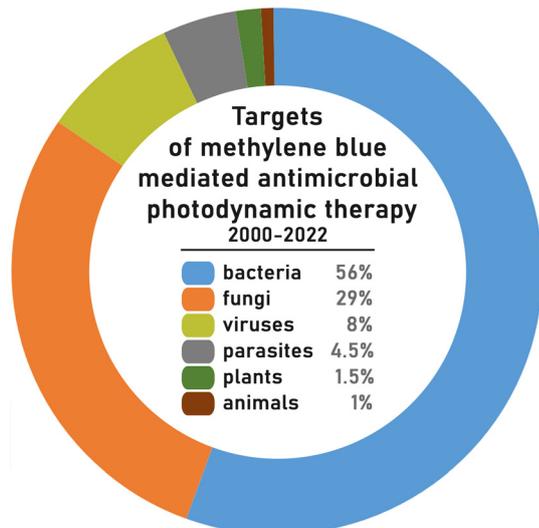This journal is © The Royal Society of Chemistry 2023

Fig. 4 Targets of methylene blue mediated antimicrobial photodynamic therapy in studies conducted between years 2000–2022 based on the species diversity.

whilst *Escherichia coli* is investigated in nearly 1/3 (27%) of the papers regarding APDT against Gram-negative bacteria (Fig. 5c). Amongst fungi, the most commonly investigated fungus genus was *Candida* spp. (*C. albicans*, *C. krusei*, *C. parapsilosis*). The leading species were *Candida albicans* (50%), *Candida krusei* (5%) and *Candida parapsilosis* (4%) (Fig. 5b). The tested microorganisms were mostly obtained from American Type Culture Collection but in some cases pathogens, especially *S. aureus*, were isolants or clinical strains (Table 1).[60,195,210,211,251,363,421,461] Several studies focused on APDT of oral microflora, in particular, bacteria found in dentin, usually isolated from patients with deep caries lesions[153,216,257,263,282,284,362,385]

Some studies also investigated MB-APDT targeted towards viruses, including vesicular stomatitis virus (VSV), bovine viral diarrhea virus (BVDV), dengue virus, West Nile virus (WNV), hepatitis C virus (HCV), herpes simplex virus type 1 (HSV-1) and SARS-CoV2.[215,297,299,300,311,314,321,331,403,408,409,411]

Anti-parasitic photodynamic therapy investigations were dominated by *Leishmania* spp. (*L. braziliensis*, *L. amazonensis*, *L. major*, *L. mazonensis*)[67,128,196,198,238,324,340,356,368] and only two publications studied MB mediated photoinactivation against an alternative parasite species, which were *Trichomonas vaginalis* and *Trypanosoma cruzi*.[64,286]

5.2 The concentration of methylene blue in APDT

The concentration of MB used in APDT of a range of pathogens is shown in Fig. 6. The photosensitiser concentration was reported in three different ways as molar concentration (mostly μM), mass concentration (mostly mg mL^{-1}) or a percentage (%). The reported molar concentrations ranged from 10 nM (3.2×10^{-6} g L^{-1}) to 1 M (319.84 g L^{-1}), the mass concentrations ranged between 0.001 μg mL^{-1} (3.12 nM) and 50 mg mL^{-1} (0.16 M) and the percentage concentrations ranged from 0.0001% to 2%. The highest concentration (319.84 g L^{-1} = 1 M)
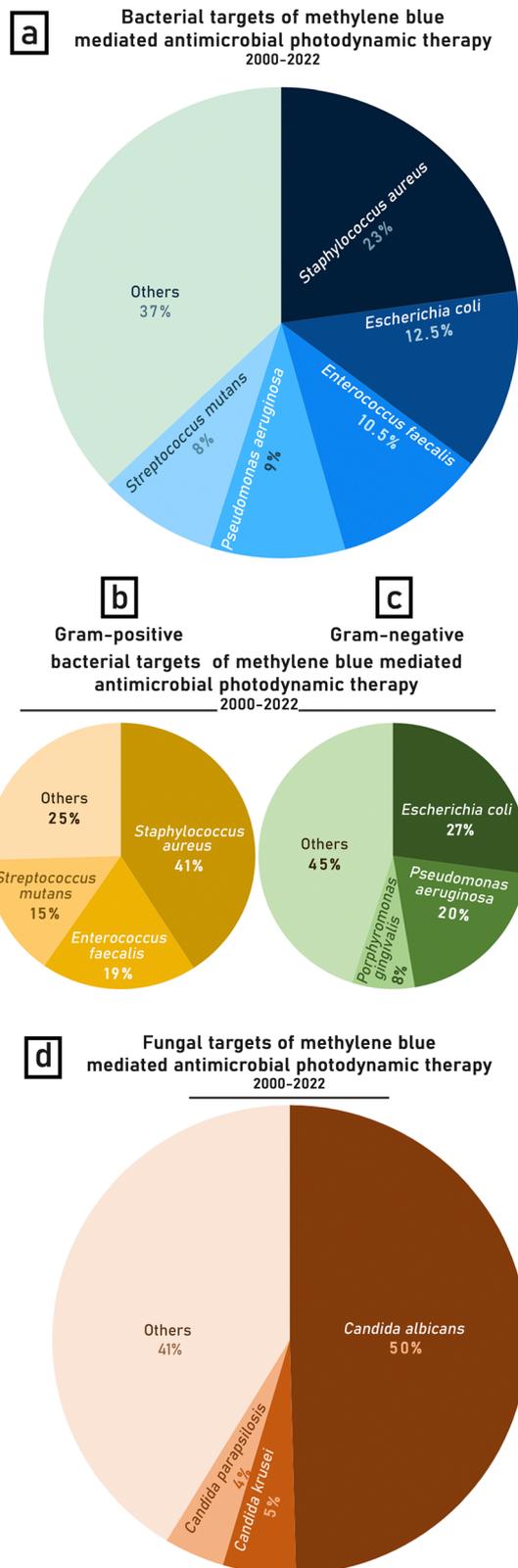

Fig. 5 Targets of MB-mediated antimicrobial photodynamic therapy in studies conducted between years 2000–2022 based on the number of publications. (a) all bacteria, (b) Gram-positive bacteria, (c) Gram-negative bacteria and (d) fungi.

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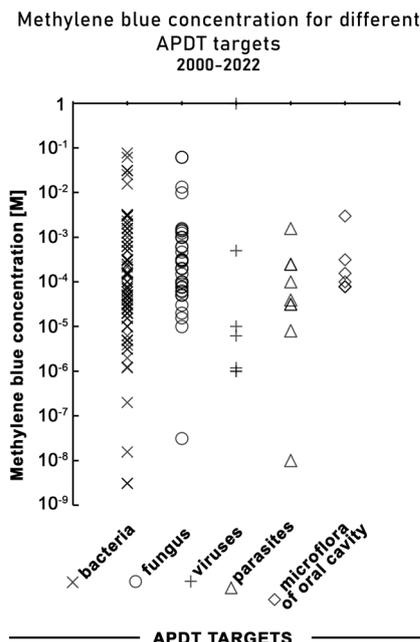*Chem. Soc. Rev.*, 2023, 52, 1697–1722 | 1705

Fig. 6 Methylene blue concentrations (on a log scale) used in MB-mediated antimicrobial photodynamic therapy in studies conducted between 2000–2022.

was tested on Herpes simplex viruses cultured in infected monolayers of cell lines, and the lowest on *Mycoplasma salivarium in vitro*.[332,353] We note that the highest concentrations reported seem unreasonable as the solubility of methylene blue in water is reported to be up to 0.13 M (43 g L^{-1}) thus concentrations of MB greater than its water solubility were rejected from further analysis.[462] For antifungal and antibacterial PDT the methylene blue concentration range is very broad but accumulation of values is observed between 0.31–0.031 mM (0.1–0.01 g L^{-1}) (Fig. 6). Based on all the presented concentration values, the mode for methylene blue concentration is 0.1 mM (0.032 g L^{-1}).

The APDT effect is also highly dependent on the concentration of photosensitiser thus the analysis of log reduction as a function of MB concentration was performed (Fig. 7). In general, no correlation between reduction and MB concentration is
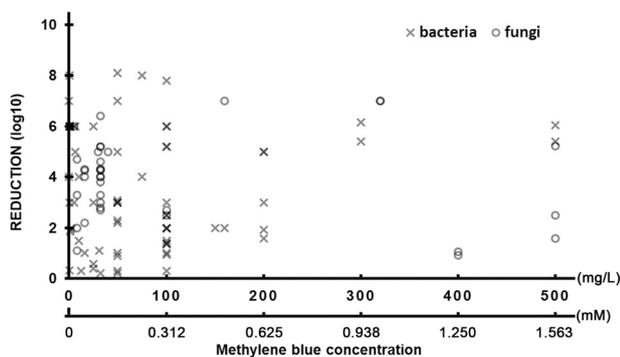

Fig. 7 The reduction of targeted pathogen number *via* antimicrobial photodynamic therapy as a function of applied methylene blue concentration in studies conducted between 2000–2022.
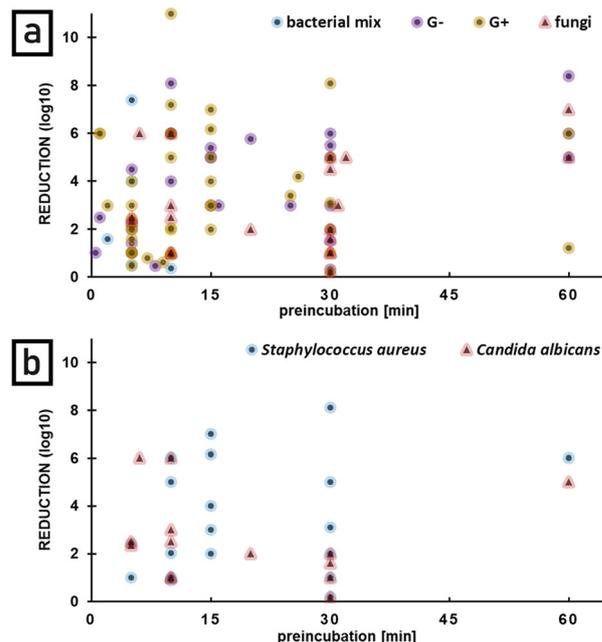

Fig. 8 Log reduction of targeted pathogen number as a function of preincubation time for bacteria (bacterial mix – mixture of bacteria or unidentified bacterial microflora; G(−) – Gram-negative bacteria; G(+) – Gram-positive bacteria) and fungi (a) and the two major pathogens *Staphylococcus aureus* and *Candida albicans* (b) investigated in studies concerning antimicrobial photodynamic therapy using methylene blue conducted between 2000 and 2022 based on species diversity.

observed either for fungi or bacteria. A reduction higher than 6 logs can be obtained in a broad range of photosensitizer concentration (32–320 µg mL^{-1}). For some bacterial targets significant reduction (8 log 10) is achievable with relatively low concentration <60 µg mL^{-1} (Fig. 7). Some studies show evidence of improved reduction by increasing photosensitizer concentrations, but this effect depends on the pathogen species and is not linear.[290]

Since methylene blue toxicity in the dark is observed in case of many pathogens, preincubation time, defined as a time of pathogen incubation with photosensitizer without light, appears to play important role in APDT effect. The longer preincubation time the higher log 10 reduction in case of bacteria is observed (Fig. 8a).

There is a correlation observed especially in case of *Staphylococcus aureus* which analysis is based on the biggest number of data collected for one bacterial species. For *Candida albicans* no correlation was observed and longer preincubation (up to 30 min) reduces the final reduction. The same trend was observed in case of other fungal species. No data are available for preincubation between 35 and 60 minutes (Fig. 8b).

5.3 The light sources used for methylene blue mediated APDT

The types of light sources used are shown in Fig. 9. The most common light sources reported for MB-mediated APDT were lasers (48.5%), LEDs (32%), and lamps (13%). The other light sources category (6.5% in total) includes so-called *non-coherent*

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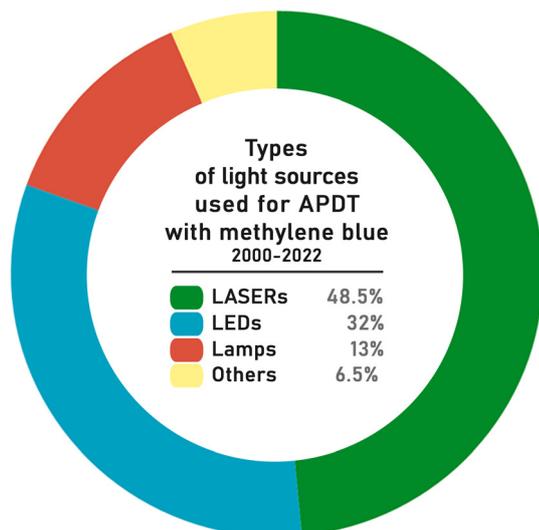This journal is © The Royal Society of Chemistry 2023

Fig. 9 Diversity of applied light sources for MB-mediated antimicrobial photodynamic therapy in studies conducted between years 2000–2022 based on the number of publications.
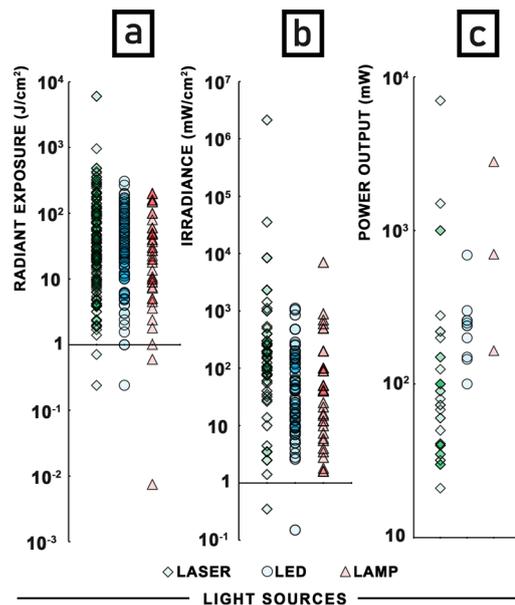

Fig. 10 The irradiation parameters in MB-mediated antimicrobial photodynamic therapy in studies conducted between years 2000–2022. The graphs show the power output (a), irradiance (b) and radiant exposure (c) for lasers, LEDs and lamps, which are the three most common light sources in MB-mediated APDT. All values are in log scale due to their broad ranges. The overlapping of the markers on the graphs shows the most common values.

light sources (name taken from publication methodologies), solar radiation,[454–456] optical parametric oscillator (OPO),[457] hand-held photopolymerizer (HHP),[459] endoscopy system,[458] superluminescent diodes (SLED or SLD)[460] and organic light-emitting diodes OLEDs[125,128,129] (Fig. 9).

The key irradiation parameters for APDT are shown in Fig. 10. The issue of power output is quite problematic. Many publications, especially in the case of lamps, do not specify if the reported power is the optical output power or the electrical input power. For preparing Fig. 10a only values of power output were used, limiting us to only three reports of lamps. Consequently, the highest power output was observed for a laser (7 W).

Due to heat generation, lamps require a cooling element (water tank or ice), commonly assembled between the light source and irradiated sample.[408,409,413,442–444] Heating is much less of a problem for LEDs because of their much higher efficiency. Lasers, as the most common light source are characterized by the broadest range of power output values.

The most common power output (mode of the distribution) was 40 mW for lasers and 240 mW for LEDs (Fig. 10c). However, when arithmetic means are considered, the average power output of lasers was 257 mW and 274 mW for LEDs. In case of lamps, there are only three reports that explicitly mention power output. The irradiance was the second most often reported parameter (180 publications) ranging from 0.15 mW cm^{-2} (LED)[275] to 2123 W cm^{-2} (laser).[256] For lasers and LEDs the most frequent irradiance value was 100 mW cm^{-2}, for lamps 40 mW cm^{-2} (Fig. 10b). Unfortunately, in the case of many studies this parameter was disregarded, nevertheless, strong tendency is observed here. The radiant exposure, referring to the irradiated surface area as well as irradiance and directly depending on irradiation time, was the quantity most commonly reported (256 publications). The maximum radiant exposure found was directed against *Candida albicans* murine

models *in vivo* and was 6048 J cm^{-2} (laser).[264] The most common radiant exposure (mode of the distribution) was 30 J cm^{-2} for lasers, 10 J cm^{-2} for LEDs, and 200 J cm^{-2} for lamps for both *in vivo* and *in vitro* studies (Fig. 10a). Because of methylene blue, almost all research used red light for the photoactivation process. Studies using a laser with a wavelength exceeding the absorbance spectrum of the dye were excluded from further analysis. Lasers as monochromatic light were described by only one wavelength. For LEDs the maximum wavelength of emission was usually presented. In the case of lamps, investigators usually marked the presence of filters that allowed them to obtain light with a wavelength usually longer than 630 nm or the range of light wavelengths was presented (Table 1).

The distance between the light source and illuminated area during the photodynamic inactivation process should be reported to understand the geometry of the study. When the light source releases large amounts of energy in the form of heat, the temperature of the surface under direct light may drastically increase, leading to a thermal (not photodynamic) lethal effect. The largest distance between the sample and light source of 3 meters was used in APDT of a virus, and photoinactivation was still observed.[314] In the dental field optical fibres were usually introduced into the tooth, as close to the pathogen as possible, reducing the distance between the light source and the irradiated object to a minimum.[206,207,241] The range of distances between the irradiated area and the light source was in the range of 0.1–300 cm, with the most common value being 1 cm. It should be noted that only 20.9% of

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research reported the distance of the light source from the sample.

5.4 The effectiveness of different light sources in microbial load reduction

Many different light sources were applied in antimicrobial photodynamic therapy with a broad range of power output, emission properties, applicability, and availability. Therefore, a question arises: is there any difference in APDT effectiveness dependent on the applied light source and its properties. The reduction of microbial load shown as a logarithm is the commonest approach to present the antimicrobial effect of photoinactivation. For this reason, it was chosen as the basis for the comparison of the effectiveness of different light sources in APDT. There is no doubt that bacteria are the most frequent study target of photoinactivation thus the greatest amount of data is collected in the area of antibacterial action of APDT. Fig. 11 shows that although there is a broad range of reductions achieved, all light sources can achieve a large reduction, and that the reduction does not appear to depend strongly on the light source. The figure does not include parasites because (apart from one exception) the publications on this topic did not include all the information needed (reduction in microbial load, irradiance and radiant exposure).

For laser APDT, there is a high density of points around 1 log 10 reduction (90% mortality of bacteria), indicating this is the most common value. The average reduction is higher for LEDs and lamps.

Fig. 12 plots the effectiveness of APDT as a function of radiant exposure ($J cm^{-2}$) of light delivered. Fig. 12a shows results for bacteria, fungi and viruses, and it can be seen that
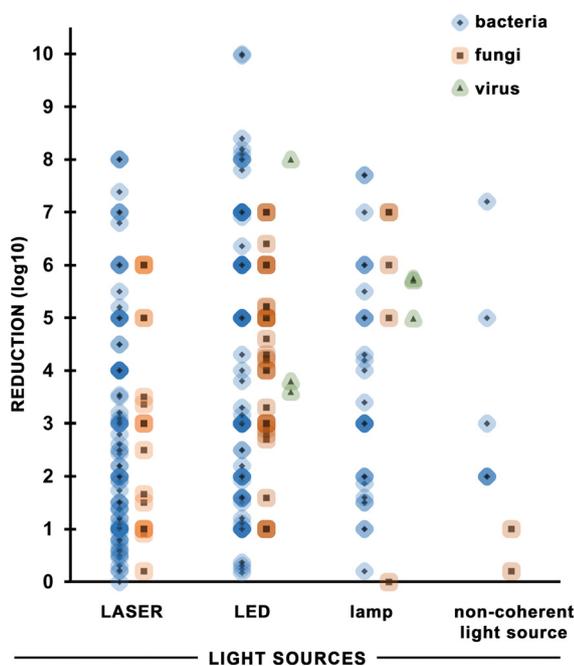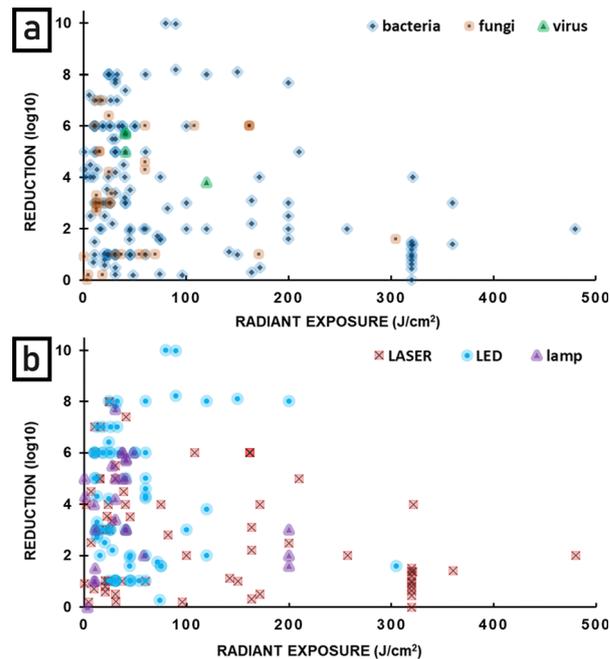

Fig. 12 The reduction of targeted pathogens number *via* antimicrobial photodynamic therapy as a function of applied radiant exposure and pathogen (a) and light source (b). The overlapping of the markers on the graphs shows the most common values.

APDT can be effective in greatly reducing the numbers of all these microbes. Fig. 12b shows the same data, but divided into type of light source. It can be seen that LEDs, lasers and lamps can all be very effective sources for APDT, with the highest reductions seen for LEDs. An important point is that large reductions are seen for low radiant exposure of light and that the highest radiant exposures do not give the largest reductions. There is not a clear optimal radiant exposure, because there is not enough data at low radiant exposure to identify the minimum that is still effective. Further studies at low radiant exposure would therefore be useful. Fig. 13 is similar to Fig. 12, but shows the effectiveness of APDT as a function of the irradiance of light used. Fig. 13a shows that effective reduction of pathogens can be achieved using a wide range of irradiance, including low irradiance – there is no clear correlation with light irradiance. Fig. 13b shows that lasers, lamps and LEDs can all be effective, and again there is no clear correlation with irradiance.

Since in the literature statements concerning the difference in sensitivity of Gram-positive and Gram-negative bacteria are common, an analysis depending on this classification was performed. For both, a broad range of radiant exposure (Fig. 14) and irradiance (Fig. 15) was applied. Up to 90 $J cm^{-2}$ no correlation between radiant exposure and bacteria reduction was observed. Above 90 $J cm^{-2}$ Gram-positive bacteria show better reduction, however, the gap in research between 90 and 160 $J cm^{-2}$ for Gram-negative bacteria should be here taken into account (Fig. 14a).

Additional analysis of reduction as a function of radiant exposure (Fig. 14b) and irradiance (Fig. 15b) was performed for the four most common bacterial species. A relatively wide

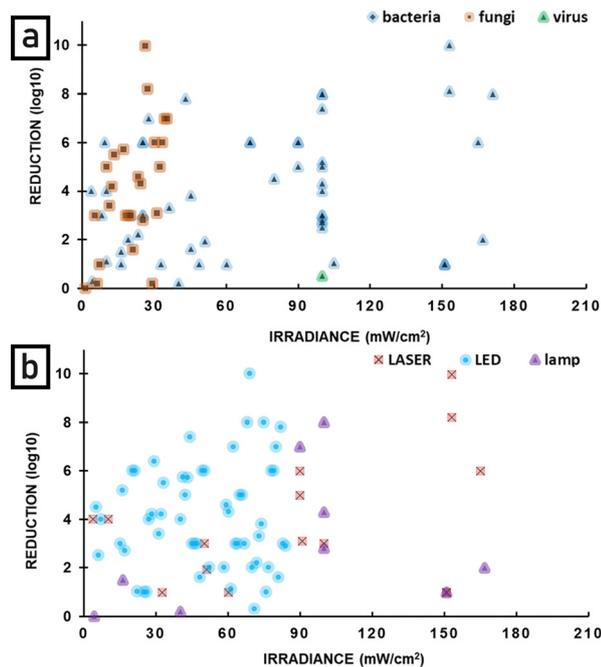

Fig. 11 The reduction of targeted pathogen number *via* antimicrobial photodynamic therapy with the use of different light sources.

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Fig. 13 The reduction of targeted pathogen number *via* antimicrobial photodynamic therapy as a function of applied irradiance and pathogen (a) and light source (b). The overlapping of the markers on the graphs shows the most common values.
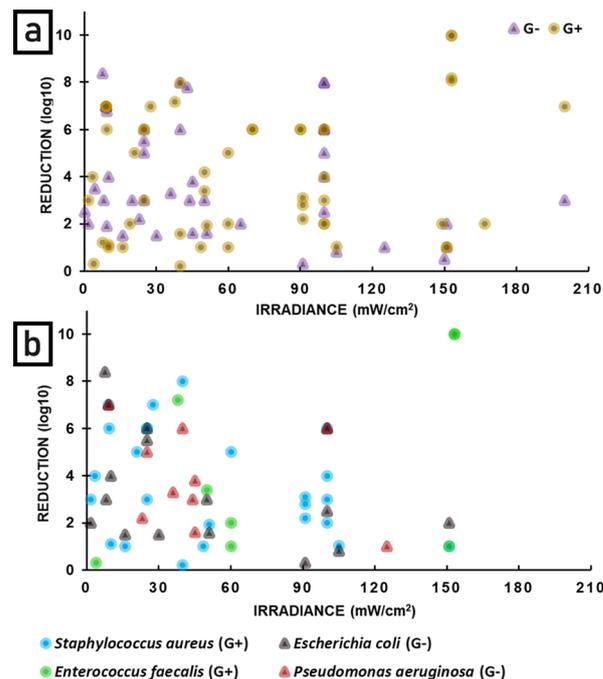

Fig. 14 The reduction of targeted pathogen number *via* antimicrobial photodynamic therapy as a function of applied radiant exposure divided into Gram-positive and Gram-negative bacteria (a), and the four most popular bacterial species in APDT (b).

range of reduction is observed for *Staphylococcus aureus* and *Escherichia coli* at radiant exposures up to 50 J cm^{-2}. Higher

Fig. 15 The reduction of targeted pathogen number *via* antimicrobial photodynamic therapy as a function of applied irradiance and pathogen (a), and four the most popular bacterial species in APDT (b). The overlapping of the markers on the graphs shows the most common values.
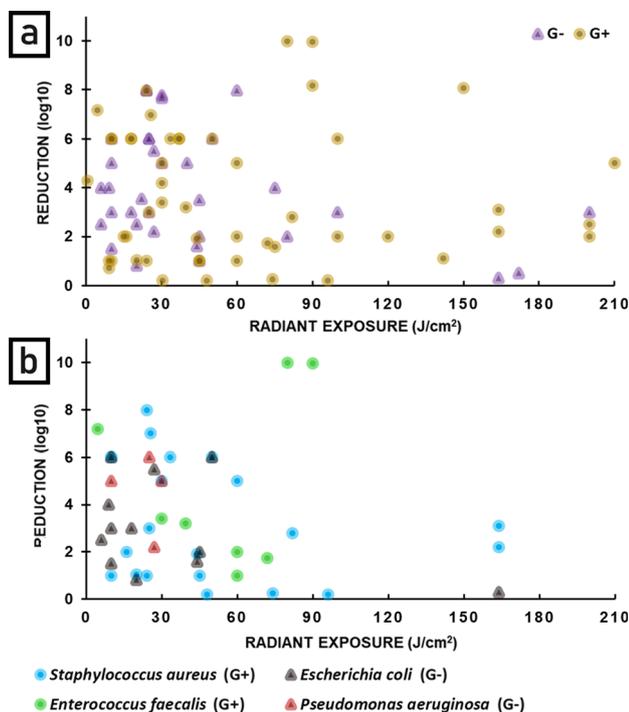
radiant exposures do not appear to significantly improve the reduction of Gram-positive bacteria. A similar pattern is observed for irradiance, where values above 60 mW cm^{-2} do not increase the bactericidal effect of APDT.

5.5 Implications for study design and reporting

Upon reviewing the literature, we have noticed key parameters are often missing in APDT studies, making comparison between studies difficult. APDT studies should be appropriately designed and methods fully described to enable comparison and ensure that other researchers can independently repeat the procedure.

Light parameters. As light plays an essential role in APDT, causing photosensitiser photoactivation, the reporting of light source parameters is of vital importance. Due to the direct influence of the light source on APDT results, its description should be full (appropriate calculations and units) and clear (exact values if possible) to avoid mistakes that could dramatically change experiment outcomes. For this reason we suggest reporting the following six parameters in the methodology:

1. **Radiant exposure** (J cm^{-2}) – the main parameter, reported in nearly 50% of analysed publications,

2. **Irradiance** (mostly mW cm^{-2}) – the second most reported parameter, allows the calculation of radiant exposure if the time of irradiation is known,

3. **Irradiation time** – reported in most publications, but needs the other light parameters to be useful. Whilst two out of three of radiant exposure, irradiance and irradiation time enable the third parameter to be calculated, it is helpful to the reader to provide all three parameters.

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4. **Fractionation** – in some cases the radiant exposure of light is delivered by turning the light source on and off. If this is done the details should be provided.

5. **Spectrum of the light source or wavelength if a laser** (nm) – its overlap with the photosensitiser is crucial for determining the amount of light absorbed. In the case of lasers, the exact wavelength is shown, studies using other light sources tend to present ranges,

6. **area illuminated and distance between light source and sample** – these are helpful for understanding the experimental configuration including likelihood of heat transfer from the source to the sample.

Biological parameters. The APDT effect also strongly relies on the biological (target) and chemical (photosensitiser) parameters of the study. Not only the targeted pathogen but also the amount of pathogen used (if *in vitro*) affect the final reduction, understood as a decrease of the target amount in comparison to the untreated group/sample. Another issue is methylene blue toxicity even in dark conditions. The biological and chemical parameters listed below should be presented in the methodology due to their direct influence on outcomes:

1. **Initial amount of photoinactivation target** (cells, CFU or PFU) – strongly influences the result, some studies present it as the amount of target before photosensitiser addition which leads to its dilution,

2. **Photosensitiser concentration at the start of illumination** – studies should present the amount of photosensitiser during irradiation, some publications refer only to the initial concentration,

3. **Solvent used for photosensitiser solution** – some solvents (including methanol and ethanol) may be toxic for the pathogen itself, their use requires additional control,

4. **Preincubation time** – its presence depends on an additional step where samples are stored in the dark with photosensitiser before irradiation, sometimes with additional shaking,

5. **Additional washing before irradiation** – included usually when preincubation is implicated.

6. **Appropriate control experiments** – In the case of APDT, three control experiments are needed. The most fundamental is a control sample without light and photosensitisers stored in the same environmental conditions as the sample exposed to APDT. In addition, due to methylene blue having some toxicity in the dark, a control experiment without light but with photosensitiser is needed. This is often referred to as a "dark control." The dark control should not be the main basis for sample comparison or calculations due to the actual or potential toxicity of the photosensitiser even without light. The third control that should be performed is a "light control" in which the sample is illuminated, but no photosensitiser is present. The high radiant exposure of energy delivered to the target in a short period of time is a contentious issue. It may lead to pathogen death, which is the primary goal of APDT and an indisputable benefit. However, it may also lead to microbe destruction without photodynamic process participation and promote photobleaching which results in the photosensitisers permanent inability to produce ROS.

Details of temperature and temperature control – The temperature of the experiment needs to be reported and the effect of heating by the light source also needs to be considered and mitigated. This may involve having appropriate distance between the light source and the sample and/or active temperature control of the sample.

6. Conclusions

There is growing interest in APDT due to the steep rise in antibiotic resistant pathogens. Light is a crucial component of PDT because it excites the photosensitiser, leading to the generation of reactive oxygen species. This review has shown that PDT can be effective against bacteria, fungi and viruses, including the emerging problem of SARS-Cov2.[299,463,464] A large range of light sources, irradiance and radiant exposure values have been used for APDT. By collecting all these results together, we have seen that effective APDT can be achieved using many different light sources. We have also seen that low radiant exposure of light can be at least as effective as high radiant exposure, and also that low irradiance of light can lead to effective PDT. We found some problems comparing published work, and have therefore suggested key optical and biological parameters that should always be reported. At present the vast majority of studies are *in vitro*, making it a priority for the field to move towards clinical studies in the future.

The fact that a wide range of light sources, irradiance and radiant exposure values can be effective is important for the future development of APDT and opens a path to light sources that are more cost-effective and convenient for clinical studies. It means that advances in the rapidly developing field of optoelectronics can feed into the development of APDT. We expect a move away from lasers to more robust, efficient, simpler and less expensive light sources, such as those based on LEDs, thereby removing a crucial barrier to the adoption of APDT. This trend is already apparent in the recent literature where in the period 2020–2022, there are more reports of LEDs for APDT than of lasers. Furthermore, finding that high powers are not required for effective APDT creates the possibility of using compact and even wearable light sources for APDT. In this context organic light-emitting diodes (OLEDs) are particularly attractive as they provide flexible light sources that emit over an area. The ready availability of practical light sources can be expected to facilitate the adoption of APDT, providing a powerful tool in the battle against microbial resistance. Reaching this goal will require not only advances in light sources, but also a move from *in vitro* studies to clinical trials.

Conflicts of interest

IDWS is a founder and shareholder of Lustre Skin Ltd which sells products for skincare, including a wearable light source for the treatment of acne.

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Acknowledgements

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