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TESTING METHODS FOR PHOTOACTIVATED ANTIMICROBIAL TEXTILES

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TIIVISTELMÄ

Riku Salomäki: Valoaktivoituvien antimikrobiaalisten tekstiilien testausmenetelmät
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Fotodynaamista antimikrobiaalista kemoterapiaa pidetään yhtenä mahdollisena tapana kiertää lääkeresistenttejä mikrobeja ja hillitä taudinaiheuttajien leviämistä. Se pohjautuu valoherkistimiksi kutsuttujen yhdisteiden kykyyn absorboida valoa ja siirtää virittynyt energiatila ilmassa lähellä olevalle molekylaariselle hapelle. Menetelmän antimikrobiaalinen teho syntyy *in situ* tuotetuista reaktiivisista happiyhdisteistä, joista menetelmälle tärkein on singlettihappi. Reaktiiviset happiyhdisteet tuhoavat mikrobeja hapettamalla mm. lipidejä, proteiineja ja DNA:ta johtaen lopulta solukuolemaan. Valoherkistimillä voidaan funktionalisoida esimerkiksi tekstiilejä tai muita pintoja.

Tämä työ on kirjallisuusselvitys, jonka alussa käydään lyhyesti läpi valoterapioiden kehitystä, hapen kemiaa, reaktiivisten happiyhdisteiden muodostumista valoherkistimien avulla sekä erilaisia valoherkistiminä toimivia molekyylejä. Pohjustuksen jälkeen esitellään antimikrobiaalisia tekstiilejä sekä niiden vaikuttavia aineita ja valmistusmenetelmiä. Työn päätarkoituksena on selvittää, mitä eri menetelmiä käytetään antimikrobiaalisten tekstiilien tehokkuuden testaamiseen ja miten ne vertautuvat keskenään. Myös testimenetelmien soveltuvuutta valoaktivoituven antimikrobiaalisten tekstiilien testaamiseen pohditaan.

Antimikrobiaalisia materiaaleja ja pintoja tutkitaan mahdollisina keinoina hillitä taudinaiheuttajien leviämistä. Suurin osa ihmisten kärsimistä taudeista tarttuu pinnoilla kasvavista biofilmeiksi kutsutuista mikrobiyhdyskunnista. Biofilmit ovat erityisen kestäviä ja hankalia tuhota niiden mikrobeja suojaavan rakenteensa vuoksi. Kaupallisissa antimikrobiaalisissa tekstiileissä vaikuttavana aineena hyödynnetään paljon mm. hopeayhdisteitä. Valoaktivoidut antimikrobiaaliset tekstiilit ovat uudempi keksintö, jossa kangas on funktionalisoitu valoherkistimillä.

Antimikrobiaalisten tekstiilien tehokkuuden testaamiseen on olemassa monia standardoituja menetelmiä. Testitulokset eri tutkimusten välillä ovat kuitenkin vaikeasti verrattavissa ja toistettavissa. Tuloksiin vaikuttaa mm. mikrobien kasvualusta, inkubaatio-olosuhteet, kontaktiaika mikrobien ja näytteen välillä, mikrobikonsentraatio sekä mikrobikanta ja tapa, jolla tehokkuus lasketaan. Lisäksi vaikuttavan aineen vähäinen diffuusio ja liukoisuus ympäröivään aineeseen saattaa tuottaa jopa vääriä negatiivisia tuloksia osassa testeistä. Valoaktivoitujen näytteiden kohdalla on myös huomioitava valon tasainen jakautuminen inkubaation aikana.

Työssä esitellään 6 kvalitatiivista testimenetelmää ja 8 kvantitatiivista menetelmää antibakteerisien ominaisuuksien testaamiseen. Lisäksi esitellään kaksi antifungaalisen, sekä yksi antiviraalisen tehokkuuden testausmenetelmää. Antibakteerisien ominaisuuksien testejä vertaillaan keskenään. Kvantitatiivisten testien tulosten analysointiin esitellään kolme tapaa, joista yhdyskuntia muodostavien yksiköiden laskeminen agarin pinnalla on yleisin käytetty tapa. Se on kuitenkin menetelmistä eniten aikaa vievä ja työläin. Kaksi muuta tapaa hyödyntävät valon absorption mittausta. MTT-analyysi mittaa mikrobien pelkistämän värilliseksi muuttuneen yhdisteen konsentraatiota spektrometrisesti. OD_{600}-analyysissä mitataan suoraan liuoksessa olevien mikrobisolujen määrää niiden absorboiman valon perusteella. Jokainen analyysimenetelmä mittaa hieman eri asiaa ja vertailu näiden välillä on mahdotonta.

On tärkeää löytää parhaat menetelmät valoaktiivisten antimikrobiaalisten tekstiilien testaamiseen, jotta niiden kehitys voisi edetä mahdollisimman tehokkaasti. Suunnittelemalla testit huolellisesti voidaan mahdollisesti saada tuotettua toistettavissa olevia ja vertailukelpoisia tuloksia.

Avainsanat: Fotodynaaminen antimikrobiaalinen kemoterapia, ftalosyaniini, valoherkistin, antibiottiresistenssi, antimikrobiaaliset tekstiilit

ABSTRACT

Riku Salomäki: Testing Methods for Photoactivated Antimicrobial Textiles
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Photodynamic antimicrobial chemotherapy is considered as a possible way to circumvent antimicrobial resistance and control the spreading of multidrug-resistant microbes. It is based on molecules called photosensitizers, which can absorb light and transfer the excited energy state to ambient molecular oxygen. The antimicrobial activity is mediated by *in situ* generated reactive oxygen species, of which the most important is singlet oxygen. Reactive oxygen species destroy microbes by a multipath oxidation of, for example, lipids, proteins, and DNA eventually leading to cell death. For example, textiles and other surfaces can be functionalized with photosensitizers.

This thesis is a literature review. In the beginning, a quick overview on the development of light therapies is presented, followed by brief chemistry of oxygen, light-sensitized generation of reactive oxygen species, and examples of different photosensitizers. Then antimicrobial textiles are discussed along with examples of active substances used and methods of production. The goal of this thesis is to find out the methods used to test the efficacy of antimicrobial textiles and compare them. The applicability of these methods for photoantimicrobial textiles is also considered.

Research on antimicrobial materials and surfaces aims for the development of methods to control the spreading of disease-causing microbes. Most diseases encountered by humans are caused by microbial communities, called biofilms, growing on surfaces. Biofilms are exceptionally resistant and difficult to destroy due to their protective structure. Commercial antimicrobial textiles utilise, for example, silver-based compounds as their active ingredient. Light-activated antimicrobial textiles are a more recent invention, which is based on functionalizing the textile with photosensitizers.

Many standardized methods are recognized for the testing of efficacy of antimicrobial textiles. Test results between different studies are, however, poorly comparable and difficult to reproduce. Results from tests are affected by the growth media, incubation conditions, contact time between the microbe and the sample, microbial concentration and strain, as well as the method of analysing the outcome. Also, minimal diffusion or solubility of the antimicrobial agent to the surrounding media may result in false-negative results in some methods. When considering photoantimicrobial textiles, the even distribution of light must also be considered. Three methods are described for analysis of quantitative tests.

Six qualitative and eight quantitative methods for antibacterial testing are presented. Also, two antifungal and one antiviral test methods are discussed. Test methods for antibacterial efficacy are compared with each other. Three methods for analysing the results from quantitative testing are described, of which the most often encountered method is the counting of colony forming units on agar. This method is also the most laborious and time consuming. The other two rely on measuring absorption of light. The MTT assay measures the concentration of a colourful substance produced by reduction via active microbes. In the OD_{600} assay the concentration of microbial cells is measured by direct measurement of optical absorption. Each of these methods measure a somewhat different quality of the microbes and thus must be compared with caution.

It is important to find the best methods for testing photoantimicrobial textiles for the development to move forward efficiently. Careful designing of the tests could possibly produce reproducible and comparable results.

Keywords: Photodynamic antimicrobial chemotherapy, photosensitizer, antimicrobial textile, antimicrobial resistance, antibiotic

ACRONYMS AND APPREVIATIONS

AATCC	The American Association of Textile Chemists and Colorists
AMR	Antimicrobial resistance
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
BODIPY	Boron-dipyrromethene
BPR	Biocidal Product Regulation
CFU	Colony forming unit
DBA	Dibenzylideneacetone
DNA	Deoxyribonucleic acid
DPP	Diketopyrrolopyrrole
ECHA	European Chemicals Agency
F	Fluorescence
IC	Internal conversion
ICS	Intersystem crossing
ISO	The International Organization for Standardization
JIS	The Japanese Industrial Standards
KB	Kirby-Bauer
KCTC	Korean Collection for Type Cultures
LED	Light emitting diode
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Nutrient agar
NAD(P)H	Reduced nicotinamide adenine dinucleotide phosphate
NB	Nutrient broth
NIR	Near-infrared
NMB	New methylene blue
NP	Nanoparticle
OD_{600}	Optical density at 600 nm
P	Phosphorescence
PACT	Photodynamic antimicrobial chemotherapy
Pc	Phthalocyanine
PDI	Photodynamic inactivation
PDT	Photodynamic therapy
PS	Photosensitizer
RLS	Resonance light scattering
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
TBO	Toluidine Blue
$TCID_{50}$	50 % tissue culture infective dose
TMPyP	Meso-tetrakis(N-methyl-4-pyridyl)porphinetetrakis(p-toluenesulfonate)
TTC	2,3,5-Triphenyl-tetrazolium chloride
UV	Ultraviolet
ZnTMPyP	Tetrakis(N-methyl-4'-pyridyl) porphyrinato zinc

CONTENTS

1. INTRODUCTION

Over 90 years ago penicillin was discovered, and countless of lives have hitherto been saved by antibiotics. However, the emergence of antimicrobial resistance (AMR) is forcing us to change the way we deal with pathogens. AMR is a globally recognized problem as the infections caused by drug-resistant pathogens are more difficult to treat, lead to increased morbidity and mortality, and are more prone to relapse. This also poses a significant economic burden on nations. [1] It is estimated that approximately 700 000 deaths are caused by drug-resistant bacteria globally each year and that this number could rise to 10 million deaths annually by year 2050. [2] In Europe approximately 25 000 deaths are caused by hospital acquired infections each year. [3] It is agreed worldwide that the main cause for the increased prevalence of AMR is the extensive and irresponsible use of antimicrobial substances. [1][2][4] Formation of biofilms makes the problem even worse. Biofilms are communities of microbes firmly embedded in a polymer matrix, and attached to a surface such as items, clothes, or teeth. The polymer matrix consists of extracellular polysaccharides, proteins, lipids, and DNA. Biofilms are also particularly resistant to antibiotic treatments. Of all bacterial and fungal infections in humans 80 % are caused by microbial biofilms. [5][6] It is of utmost importance to develop novel and effective broad-spectrum antimicrobial treatments which do not cause AMR.

Photodynamic antimicrobial chemotherapy (PACT) involves the use of a molecule called a photosensitizer (PS), a light source, and molecular oxygen to induce photodynamic inactivation (PDI) of microorganisms via oxidative stress. PACT is a physiochemical method and has received much attention in recent years due to its power of circumventing the AMR. [7] Other proposed modalities for antimicrobial treatments are, for example, antimicrobial chemicals, peptides [8] and metal oxide coatings or nanoparticles [9]. PACT is particularly interesting since it is effective against bacteria, fungi, viruses and protozoa, while also being able to destroy biofilms efficiently [10][11]. Application of PACT on textiles could possibly help controlling the spread of infectious disease, for example, in hospitals.

In this thesis a short general overview of PACT and related photochemistry is covered including a basic scheme for production of singlet oxygen. Also, some known photosensitizers will be described. Antimicrobial textiles have long been under development, and

an overview on them is given along with some commercial examples. Insight on photo-antimicrobial textiles is provided before examining the standards for safety, durability, and antimicrobial testing of textiles, and regulations in the European Union. Numerous standardized and non-standardized methods for antimicrobial testing are discussed and compared. Comparability between different testing methods is still a bit ambiguous and some clarity on the subject will be sought out. Especially the suitability of existing methods for photoactivated materials is considered. Thus, the main emphasis of this thesis is the comparison of existing testing methods for antimicrobial efficacy and the attempt of finding the most suitable procedure for testing of PACT functionalized textiles.

In section 2 a general overview of phototherapies is presented with main emphasis on photodynamic antimicrobial chemotherapy. The text begins from early history of medicinal use of light and quickly arrives at modern applications and prospects. A quick overview of related photochemistry is offered along with basic chemistry of reactive oxygen species (ROS) and the mechanisms of their antimicrobial properties. This is followed by a brief presentation and comparison of some known photosensitizers. Section 3 deals with antimicrobial textiles with focus on photoantimicrobial textiles. Description of antimicrobial testing methods and comparison of antibacterial tests is presented. Conclusion of the thesis is given in section 4.

2. LIGHT-ACTIVATED ANTIMICROBIAL EFFECT

The use of light as a treatment for various illnesses was already practiced in Ancient Egypt and India. Earliest clues of using such methods date back to 3000 BC. For example, phototherapy in Ancient Egypt involved making a powder from plants like *Ammi majus, Pastinaca sativa, Petroselinum crispum* and *Hypericum perforatum* that was applied on depigmented lesions as described in Ebers Papyrus 1550 BC. Exposure of treated body parts to sunlight resulted in skin pigmentation. Also, Atharava-Veda 1400 BC, one of India's sacred books, describes giving extracts of the plant *Psoralea corylifolai* to patients suffering from vitiligo and then being told to stand in the sun. In China phototherapy was first introduced by Lingyan Tzu-Ming in the Han dynasty during the first century BC. The Romans and the people of Ancient Greece are also known to have utilized phototherapy, or heliotherapy, as called by the Greek doctor Hippocrates. Romans built public open-air baths called "Therms" that were meant to utilize sunlight for treatment of skin diseases. [12, pp 4–5]

The modern journey of phototherapy begins with Arnold Rikli at the turn of 19th and 20th centuries when he reintroduced the use of light in medical applications. In the middle of 20th century phototherapy was already famous and widely used for treating lupus vulgaris, pulmonary tuberculosis and rickets. A Danish doctor Niels Ryberg Finsen's contribution to the development of modern phototherapy led to a Nobel Price of medicine as he used UV radiation to treat skin tuberculosis. [12, p. 6] The German scientists, Paul Ehrlich and Robert Koch, discovered that methylene blue is particularly good for staining bacteria. In 1897, Ehrlich developed stain from methylene blue that could stain both acidophilic and basophilic cells [13]. Later, Ehrlich used methylene blue to cure malaria with some success [14]. Also, the term "chemotherapy" was coined by Ehrlich [10]. Using dyes as photosensitizers, however, was an idea proposed by Oscar Raab in the end of 19th century. He observed the toxicity of acridine dye changing in relation to the amount of sunlight available. His hypothesis, and later the basis of photodynamic therapy (PDT), was that the dye converted light into active chemical energy. Oscar Raab was a student of Professor H. Tappeiner who introduced the term "Photodynamic Action" in 1904. Tappeiner's group started investigating the efficacy of PDT on tumours and other skin diseases. Later his group found out that the mechanism of PDT involved reactive oxygen species. The development of PDT continued, different photosensitizers were discovered, and progress was made with tumour treatments. [12, pp. 1–10] Nowadays PDT is used for treating various tumours, cosmetic defects, and infections.

Photodynamic antimicrobial chemotherapy (PACT) is similar to PDT but focuses solely on antimicrobial applications. The development of PACT halted in the beginning of the 20th century due to the discovery of antibiotics. [15] The so called “golden era of antibiotics” had begun and the development of PACT continued only after the emergence of AMR and the need for new strategies. Today PACT is a promising candidate for novel antimicrobial treatments in the so called “post antibiotic era”.

2.1 Reactive oxygen species and related photochemistry

Oxygen as a chemical substance appears in nature as a molecule of two atoms, which is properly called dioxygen (O_2 or $^3O_2$). Dioxygen is later referred to as molecular oxygen or oxygen. The ground state of molecular oxygen is the triplet form, where two electrons with parallel spins occupy separate π* orbitals (figure 1). [16, p.7] A system of particles (i.e. a molecule) with a total spin quantum number $S = 1$ is called a triplet. The total spin quantum number *S* is calculated by summing the values of electron spin magnetic quantum numbers $m_s = \pm 1/2$ of each electron in the system, where the sign is determined by the spin orientation. A system where $S = 0$ is called a singlet. The quantum numbers of molecular oxygen are defined by its electron configuration. [17, p. 115] Molecular oxygen is the only abundant paramagnetic molecule in existence with a triplet ground state. It is also able to react exothermally with all elements except gold. [18] In spectroscopic notation the triplet state of oxygen is denoted as $X^3\Sigma_b^-$. There also exists two singlet states for oxygen. The higher energy singlet state, denoted as $b^1\Sigma_g^+$, has both electrons with antiparallel spins in separate π* orbitals. This state quickly decays into the more stable singlet state $a^1\Delta_g$ where both electrons with antiparallel spins occupy the same π* orbital. The singlet state $a^1\Delta_g$ will later be referred to as singlet oxygen or $^1O_2$. [19, p. 2]

A group of very reactive molecules called the reactive oxygen intermediates (ROI) includes the superoxide radical $O_2^{\bullet-}$, hydroxyl radical $OH^\bullet$ and hydrogen peroxide H_2O_2. Together with ozone O_3 and singlet oxygen they form the group of reactive oxygen species (ROS). The definition of ROS may also sometimes include other oxygen bearing reactive molecules. [16, p. 5] The molecular orbital structures of some species of molecular O_2 are presented in figure 1.

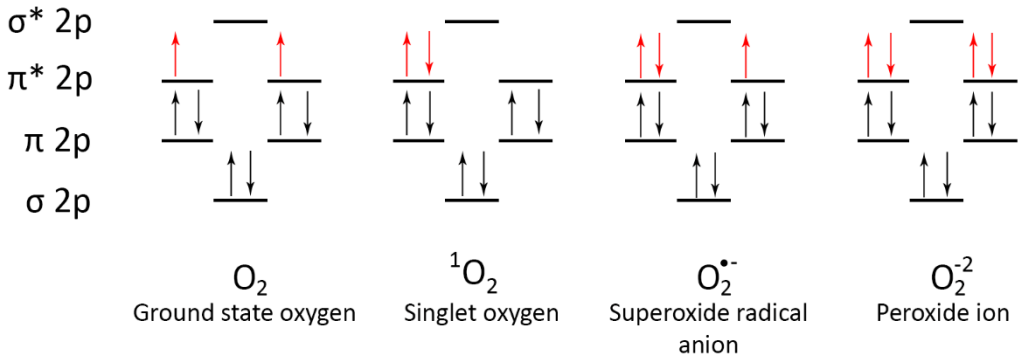


Figure 1. The molecular orbital structures of the 2p orbitals of ground state oxygen O_2, singlet oxygen $^1O_2$, superoxide radical anion $O_2^{\bullet-}$, and peroxide ion O_2^{-2}

Triplet oxygen participates in redox reactions with other molecules or atoms as it needs to accept a pair of electrons which have parallel spins. Singlet oxygen, on the other hand, accepts a pair of electrons with antiparallel spins. This results in higher oxidative ability as most nonradical organic molecules have electron pairs with antiparallel spins. Preferably, oxygen accepts one electron at a time and thus it is able to react fast by a single-electron transfer with other radicals. The one-electron reduction of oxygen generates a superoxide radical anion $O_2^{\bullet}$, and the one-electron reduction of $O_2^{\bullet-}$ leads to generation of other ROS, e.g. H_2O_2. Similarly, the reduction of H_2O_2 yields $OH^{\bullet}$ which further reduces to yield water or hydroxide. [16, p. 7]

ROS inflict oxidative damage to fatty acids, lipids, DNA, RNA, proteins, and enzymes, as well as other cellular organelles. Thus, it does not matter whether the ROS are inside or outside the cell as long as they are close enough to the target microbe. The fact that ROS attack multiple sites simultaneously makes resistance highly unlikely to develop since the required cellular changes are too numerous. Singlet oxygen is also able to denature antioxidant enzymes, such as superoxide dismutase and peroxidase. [10] The photodynamic inactivation of microbes relies mostly on the oxidative power of ROS. Oxidative stress can alter the permeability of the bacterial cell membranes leading to hindered proliferation and cell death. [20]

Generation of ROS may happen through many ways including intracellular or endogenous processes. Some examples of endogenous sources of ROS are pollutants, ionizing radiation, and ultraviolet light. Such pollutants may react to generate, for example, peroxides or ozone. [16, p. 11] Similarly, the singlet oxygen can be generated through either direct optical excitation of ground state oxygen, or chemical reactions. Another way to

produce singlet oxygen is the transfer of energy from an excited state of an organic molecule. Such organic molecules are called photosensitizers (PS). Photosensitizers absorb light of certain wavelengths and become excited to a higher singlet state ^1S_n in a one- or two-photon transition. Two-photon transition goes through one-photon and can happen if the photon density is high enough, or the excited singlet state is sufficiently long-lived to allow for a second photon to be absorbed. This singlet state can decay to the ground state $^1S_0$ via either fluorescence (F), internal conversion (IC), or intersystem cross (ICS) to the triplet state ^3S_n. This state can decay to ground state via phosphorescence (P) or internal conversion (r) unless it collides with molecular oxygen. A collision with molecular oxygen can result in the transfer of energy to the oxygen molecule exciting it to a higher singlet state. A Jablonski diagram for these processes is shown in figure 2. [19, pp. 3–4]

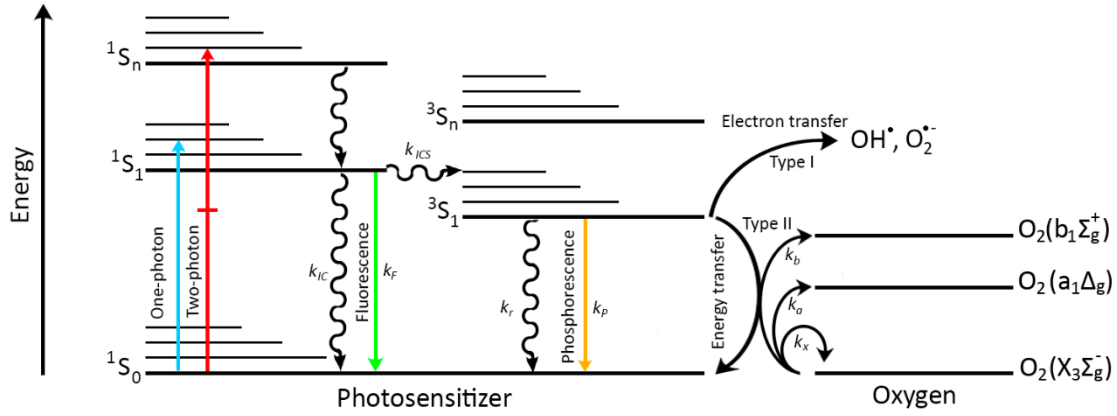


Figure 2. A Jablonski diagram of singlet oxygen production via energy transfer from an excited photosensitizer. Adapted from [19, p.4]

Fluorescence and phosphorescence both are types of photoluminescence. As seen in figure 2, fluorescence (e.g. $^1S_1 \rightarrow {}^1S_0$) does not involve the change of electron spin, and phosphorescence (e.g. $^3S_1 \rightarrow {}^3S_0$) involves a change in electron spin. Also, the transition from a singlet state to triplet state (e.g. $^1S_1 \rightarrow {}^3S_1$), called intersystem crossing, is energetically favourable, but generally happens rather slowly. The probability of ICS depends on the lifetime of the excited singlet state. [21, pp. 697–698] ICS and IC are both non-radiative heat-releasing transitions. [19, p. 23]

Quantum yield of singlet oxygen production, Φ_Δ, is defined as the fraction of absorbed photons resulting in the production of singlet oxygen. This depends on the quantum yield of triplet states, Φ_T, the fraction of triplet states quelled by oxygen, f_T, and the fraction of

these events that result in the production of singlet oxygen S_Δ. The equation for determining quantum yield of $^1O_2$ is as follows: [19, pp. 3–5]

$$\Phi_\Delta = \Phi_T f_T S_\Delta = \frac{k_{ISC}}{k_{IC} + k_{ISC} + k_F} \cdot \frac{k_{O_2}[O_2]}{k_{O_2}[O_2] + k_T + k_P} \cdot \frac{k_a + k_b}{k_a + k_b + k_X} \quad (1)$$

where the related rate constants k_n are presented in figure 2. The lifetime of $^1O_2$ in water is about 3 µs, and in the case of free diffusion the molecules move about 100 nm during this time [16, p. 89]. However, there is a lot of variation in the data on $^1O_2$ lifetime. The quantum yield of singlet oxygen can be used as an estimation of the antibacterial strength of a photosensitizer. It is, however, not the only parameter affecting it. The properties of photosensitizers are discussed further in section 2.2.

Light is electromagnetic radiation composed of energy quanta called photons. A single photon of a certain frequency ν or wavelength λ has the energy

$$E = h\nu = \frac{hc}{\lambda} \quad (2)$$

where $h = 6{,}63 \cdot 10^{-34}\ \mathrm{J\,s}$ is the Planck's constant and $c = 299\,792\,458\ \mathrm{m\,s^{-1}}$ is the speed of light. [22, p. 194] Absorption of a photon by a molecule can happen if the energy of the photon matches the energy difference of two energy levels in the molecule. Most often this is the energy between the ground state and some exited energy state. In the case of matter, or molecules, the spectrum is often continuous across the full wavelength range differing only in magnitude. This is due to the more complex nature of molecular energy states. [12, p. 29–30] The part of the spectrum visible to human eye ranges from 400 to 700 nm going from ultraviolet (UV) to near-infrared (NIR) light. [12, pp. 26–27] A typical photosensitizer absorbs light in the near-infrared, visible, or ultraviolet range in a one- or two-photon transition. [19, p. 4] Hence most photosensitizers known appear as colourful substances. In figure 3 the absorption spectra of a porphyrin and a phthalocyanine compound is shown as well as the spectrum of the lamp used.

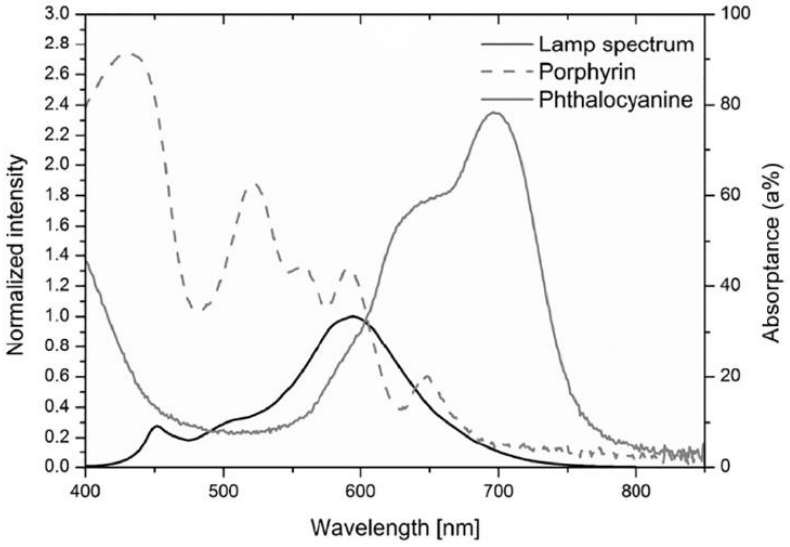


Figure 3. The absorption spectra of a porphyrin, a phthalocyanine compound, and the LED lamp used for illumination [23]

The regions of the absorption peaks in the spectrum are called absorption bands. The nature of the molecule determines the position, width and intensity of these peaks.

2.2 Photosensitizers

A good photosensitizer for PACT should possess good absorption and singlet oxygen production capabilities. It also needs certain amphiphilic properties and higher binding affinity to microbes in relation to mammalian cells. The charge of the PS also affects its antimicrobial properties. Positively charged ions such as Mg^{2+} and Ca^{2+} stabilize the outer membrane structure of bacteria. This membrane is weakened by a cationic PS competing with the ions and allowing the intake of the PS into the cell. [24] A cationic PS is thus also more selective towards microbial cells than mammalian cells. Gram-positive bacteria are readily susceptible towards PDI, but gram-negative bacteria are generally more resilient due to their more complex cell wall. Nevertheless, a positive charge on the PS allows the effective killing of the more resilient gram-negative bacteria by binding to their negatively charged cell wall. [25] It is worthwhile to note that photosensitizers used against cancer generally differ from those used as antimicrobials. Anticancer PSs are often anionic or have no overall charge, whereas antimicrobial PSs usually have pronounced cationic charges. Also, the locations of the absorption bands are not as important in antimicrobial PSs as in anticancer PSs. [26]

Many organic molecules that display PS qualities have been used and studied in various applications. Such molecules include for example porphyrins, phthalocyanines (Pc), in-

docyanine dyes, boron-dipyrromethenes (BODIPYs), diketopyrrolopyrrole (DPP) derivatives, curcumin, perylenequinone, furocoumarins, dibenzylideneacetone (DBA), Ru(II)-, Ir(III)- and Au(III)-complexes, methylene blue, new methylene blue (NMB) rose bengal, and toluidine blue (TBO). [24] Also, carbon quantum dots have been shown to work as effective photosensitizers for PACT *in vitro* [27]. The search for novel photosensitizers is never ending. A study testing 25 dyes used as cosmetic ingredients found out that 9 dyes belonging to pyrene, chlorophyll, xanthene and aminoanthraquinone groups showed potential as PSs [28].

Molecules containing four pyrrole or pyrrole-like rings are said to have a tetrapyrrole structure. Photosensitizers with such structure make up a large group of suitable molecules for PACT-applications. In nature tetrapyrrole backbones occur in biomolecules such as haem, chlorophyll and bacteriochlorophyll. They belong to porphyrinoid category, and many porphyrins have been tested for PACT. [26] One of the most studied porphyrins for PACT is the cationic meso-tetra(4-N-methylpyridyl)porphine (TMPyP). In *in vitro* studies it has shown to effectively reduce the growth of both gram-positive and gram-negative bacteria, and fungi. [25] As another example, efficacy testing regarding antimicrobial surfaces has been done with thermoplastic elastomer films containing ZnTMPyP (a zinc atom chelated in the central cavity of the molecule) [29]. The structure of TMPyP is presented in figure 4.

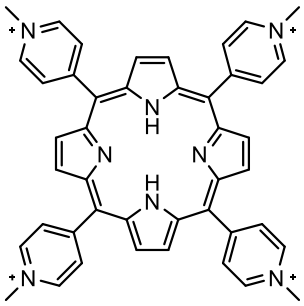


Figure 4. Molecular structure of TMPyP

Chlorins are another group in the family of molecules containing the tetrapyrrole structure. Compared to porphyrins, chlorins have one pyrrole ring reduced. Many important PSs belong to this category. As an example, chlorin(e6) is derived from chlorophyll and has been tested for PACT [30][31]. Its molecular structure is presented in figure 5. Other derivates of chlorins have also been tested for PACT and show potent antimicrobial properties [32][33]. As a further example, a photoactivated antimicrobial cotton textile material has been prepared using chlorin(e6) as the photosensitizer [34].

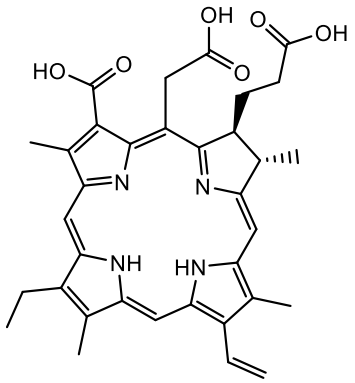


Figure 5. Molecular structure of Chlorin(e6)

Bacteriochlorins are another group with similar base structure. Compared to porphyrins, they have two opposite pyrrole rings reduced. They also have been tested for potential use in PACT [35]. As an example, the structure of a bacteriochlorin BC27 is shown in figure 6.

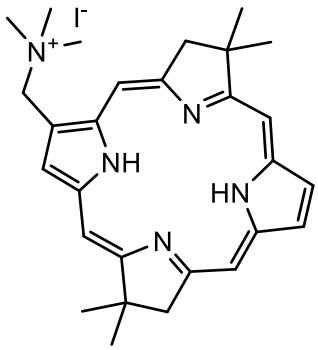


Figure 6. The molecular structure of BC27

Phthalocyanines are tetrabenzotetraazoporphyrins, yet they can also be classified as synthetic dyes [26]. Phthalocyanines have 16 possible places for substitution, so-called phthalo-positions, including both nonperipheral (α) and peripheral (β) positions. The nature of the substituents alters the chemical properties of the Pc. Also, metal atoms can form a complex in the central cavity helping in increasing the quantum yield and the lifetime of the triplet state. It has been shown that Pcs with a diamagnetic central metal such as zinc display higher antimicrobial activity than those with a paramagnetic metal like iron and vanadium. In addition to PDT and PACT phthalocyanines find applications for example in solar cells, catalysis, liquid crystals, and chemical sensors. Many phthalocyanines that show good inactivation of various microbial pathogens have been developed. [24] The structure of a cationic zinc phthalocyanine compound is presented in figure 7.

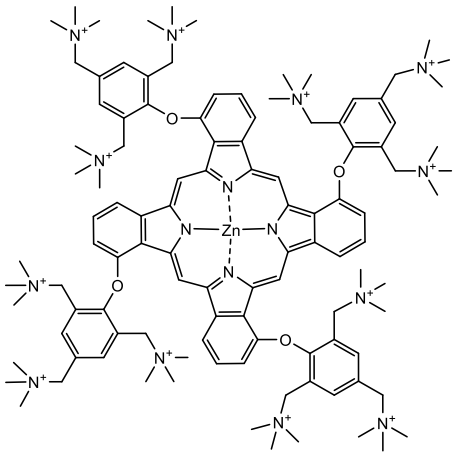
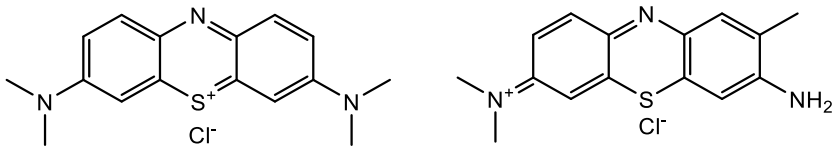


Figure 7. The molecular structure of a cationic zinc phthalocyanine compound [36]

Some PSs can be classified as synthetic dyes. These also include a large portion of PSs studied for PACT. A group within this class, called phenothiazinium salts, contains two popular dyes, Methylene Blue and Toluidine Blue, that have been extensively studied for antimicrobial applications. [26] Their structures are shown in figure 8.



Methylene Blue Toluidine Blue

Figure 8. The molecular structures of Methylene Blue and Toluidine Blue

Rose Bengal is another synthetic dye. It belongs to the xanthene class of fluorescent dyes. Rose Bengal, as a photoactive dye, has been known for a long time, and it has been tested for various applications including PACT. Heavy atoms, such as iodine and bromine, in the rings increase the probability of ICS. [26] The molecular structure of Rose Bengal is presented in figure 9.

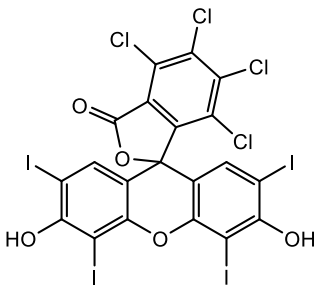


Figure 9. The molecular structure of Rose Bengal

Other synthetic dyes used as PSs include Squaraines, BODIPY dyes, Phenalenones and transition metal complexes. Some PSs can also be derived straight from the nature. Such examples are hypericin which is a perylenequinone isolated from St John's wort (*Hypericum perforatum*), hypocrellin A and B which are also perylenequinones isolated from the fungi *Hypocrella bambuase sacc* and *Shiraia bambusicola* P., riboflavin which is also known as vitamin B_2, and curcumin isolated from the plant *Curcuma longa*. [26] The structures of hypericin and curcumin are presented in figures 10 and 11, respectively.

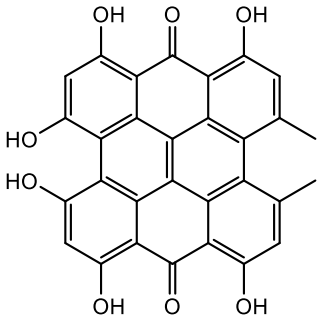


Figure 10. The molecular structure of hypericin isolated from *H. perforatum*

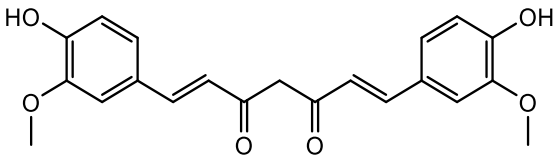


Figure 11. The molecular structure of the keto form of curcumin isolated from *C. longa*

The nature must have many unfound PSs waiting to be discovered. Plant extracts from *Lumnitzera racemosa* and *Alizia procera* have been demonstrated to work as photosensitizers inducing apoptosis in human cancer cells [37]. Also, in a recent study, the seed extract of the plant *Bixa Orellana,* which is also widely used as a dye, showed photoantimicrobial activity against bacteria causing halitosis (bad breath) [38].

Noble metal complexes like Ru(II) complexes and Ir(III) complexes display excellent singlet oxygen generation due to the long lifetime of their triplet state. Au(III) complexes have also been designed and exhibit long triplet state lifetimes and efficient singlet oxygen generation. [39]

3. ANTIMICROBIAL TEXTILES

Textiles are generally able to collect moisture and have a large surface area. This creates a suitable environment for various microorganisms to grow. The development and utilization of antimicrobial textiles is expected to address many issues related to microbial contamination. One important goal is to limit the spreading of infectious pathogens, especially ones expressing antimicrobial resistance, and controlling epidemics across the world. [40, p. 1] In addition to increased likelihood of user contamination, microorganisms on textiles can also cause unpleasant odours, stains, discoloration, and reduction in mechanical strength. About 100 000 tonnes of antimicrobial textiles were produced globally in 2000 and in Western Europe the increase in production between years 2001 and 2005 was reported to be 15 %. [41]

The active components used in antimicrobial textiles include, for example, silver compounds, zinc pyrithione, triclosan, polybiguanides, quaternary ammonium compounds and polymers like chitosan. N-halamines and light-activated compounds represent some of the more recent discoveries in antimicrobial textiles. [40, pp. 1–4][42] Some examples of commercially available antimicrobial textile fibres or structures are: ACTICOAT™ by Smith & Nephew [43] which has 2 layers of polyethylene coated with nanocrystalline silver and 1 layer of rayon and polyester and Crabyon© by SWICOFIL AG [44] which is a composite fiber of chitosan and cellulose viscose. Also, a Swiss company called SANITIZED AG produces antimicrobial hygiene function and material protection for textiles and polymers under the brand name Sanitized®. Some active ingredients they use are zinc pyrithione, isothiazolinone and silver. [45] BioCote Ltd is based in United Kingdom and provides antimicrobial functionalization on textiles, as well as antimicrobial testing based on internationally recognized standards [46].

Any commercial antimicrobial textile must be non-toxic to the consumer since they are often in contact with the human skin. Thus, it is agreed that testing for cytotoxicity, irritation and sensitization must be done. Also, the antimicrobial effect of the textile should not apply to human cells. Several methods for procuring evidence of the textile's safety for the human skin are available. The most relevant standards are offered by The International Organization for Standardization (ISO). The ISO 10993 standard is a set of methods for evaluating the biocompatibility of medical devices and it is also applicable for testing of textiles. The safety of the antimicrobial agent must be proven by testing for genotoxicity, reproductive toxicity and carcinogenicity (ISO 10993-3 [47]). Testing of the functionalized textile for irritation and skin sensitization is offered by standard ISO 10993-

10 [48] and in vitro cytotoxicity by ISO 10993-5 [49]. Also, the effect of the skin microbiota on the textile should be tested. [40, p.8]

With the consumer in mind, the durability of the product is also of interest. Washing and storage might prove detrimental to the antimicrobial effect of the textile. Antimicrobial efficacy must be tested before and after the durability tests. Standardized durability tests for work wear are offered by ISO 15797 [50] and for leisure wear by ISO 6330 [51]. The washing tests generally consist of 50 washes and the storage stability test is either 12–36 months at 25 ℃ and 60 % humidity or accelerated aging for 3–9 months at 40 ℃ and 75 % humidity. [40, p. 14]

There also exist some regulations regarding antimicrobial textiles available in the market. Only European markets shall be considered in this chapter. It is stated that all antimicrobial substances used in textiles must be listed by the European Chemicals Agency (ECHA). Also, a registration of an antimicrobial textile must be done as stated in the Biocidal Product Regulation (BPR) No. 528/2012 [52] and No. 34/2014 [53]. However, registration is required only if the antimicrobial activity is advertised. BPR states that compounds or compositions containing active substances which target harmful microbes biologically or chemically are to be considered biocidal products. Registered antimicrobial textile products have special requirements regarding labelling. It is also worthwhile to note that there are no requirements for evidence of antimicrobial efficacy. This means that any textile with or without any true antimicrobial capabilities could be marketed as having some. [40, pp. 15–16]

3.1 Functionalization of textiles with photosensitizers

There are many possible ways to prepare antimicrobial textiles. One is the functionalization during fibre formation. As all fibres are spun from a melt or a solution, addition of chemical compounds to the mixture could result in the fabrication of a functional yarn. However, the reality is not as simple and only very few such examples are known. No commercialized fibres made this way exist. Another way of textile functionalization is by surface modification. A very thin coating with, for example, an antimicrobial polymer protects the textile's surface and does not influence textile's properties such as thickness, tear strength and flexibility. [54, pp 305–306] For example, ZnO- and TiO_2-based nanoparticles (NPs) [40, p. 198], bactericidal metal ions trapped in alginates [55] and silver compounds [56] among many other antimicrobials have been studied for functional coatings on textiles. However, no studies using photoactivated coatings on textiles were found.

Photosensitizers are not directly consumed by their antimicrobial action. Thus, linking them covalently to the polymer could be a desirable method for functionalizing textiles. Porphyrins have been shown to keep their antimicrobial properties when grafted on to cellulose or chitosan, and such films and membranes exhibiting photobactericidal qualities have been made [57][58][59]. Photoactivated antimicrobial textiles have been made, for example, by linking porphyrins covalently to nylon fibre [60][61], Lyocell fibers [62] and cellulose via ester bonds [63]. Also, xanthene-derived photosensitizers, porphyrins and BODIPYs have been covalently grafted on cellulose [64][65].

Complicated ways of functionalization are not always necessary. Materials like cellulose could simply be impregnated with the photoactive dye. As an example, a cellulose filter paper impregnated with a phthalocyanine dye has been shown to provide a simple and efficient material for PACT. Even though the dye will bleach over time, the antimicrobial functionality can be replenished by soaking or spraying the material with the dye. [66] It has been shown that cationic substances are held tightly in a cellulosic matrix and only minimal leaching happens over time [67][68][69]. Also, a wool/acrylic knitted fabric has been dyed with a simple industrial dyeing process with Rose Bengal, and the antimicrobial activity of the dyed fabric was successfully demonstrated [70].

The antimicrobial functionalization can be achieved through many routes in the laboratory scale, but scalability should always be kept in mind. Since the goal should be to eventually produce antimicrobial textiles on the large scale, a relatively simple, inexpensive, environmentally friendly, and easily scalable process of functionalization should be chosen. This could potentially rule out complicated ways of covalent grafting in favour of simpler methods.

3.2 Evaluation of antimicrobial efficacy

Antimicrobial compounds either kill the target microbes or stop their growth. They are grouped according to the target microbe: antibacterial, antifungal, and antiviral. Antimicrobials can also be classified based on their function. It is generally thought that biocidal compounds kill microbes and biostatics inhibit their growth. Both can be divided according to the target organism, i.e. bactericides, bacteriostatics, fungicides, fungistatics, virucidals and virustatics. These definitions are, however, misleading, since biostatics do also kill microbes but in higher concentrations. The minimum inhibitory concentration (MIC) is defined as the smallest concentration required for inhibition of the visible growth of the microorganism after a 24-hour incubation. Minimum bactericidal concentration (MBC) is in turn the concentration required for 1000-fold reduction in bacterial density after a 24-hour incubation. By the formal definition, a bactericidal has an MBC-to-MIC

ratio of ≤ 4, while a bacteriostatic has an MBC-to-MIC ratio of > 4. [71] The definitions seem somewhat arbitrary (even more so in a clinical setting), and both “bacteriostatic” and “bactericidal” are frequently seen in the literature regarding photosensitizers. Generally, antibacterial properties are the most frequently studied properties of PACT-materials. Yet antifungal and antiviral tests are not neglected in the related literature.

Some tests involve the determination of zone of inhibition. Zone of inhibition is the area without microbial growth that forms around the edges of an antimicrobial specimen on agar. This involves taking three measurements and can be calculated as follows:

$$W = \frac{T - D}{2} \tag{3}$$

Where *W* is the width of clear zone of inhibition, *T* is the diameter of the test specimen and the clear zone, and *D* is the diameter of the test specimen. All measures should be given in mm. [72, p. 260]

It is not practical to count every single bacterium of a sample, and thus the sample is usually diluted and spread across a petri dish. The groups of microbes, called colonies, can be counted, and each is assumed to have grown from a single colony forming unit (CFU). The changes in CFU are expressed as a percentage reduction typically in factors of 10 using a logarithmic reduction scale. The so-called $\log_{10}$ (the subscript will be omitted in the rest of the thesis) reduction can be calculated in the following way:

$$\text{Log reduction} = \log_{10} \frac{N_0}{N} \tag{4}$$

where N_0 is the CFU before and N is the CFU after the application of antimicrobial treatment. Thus, a 1 log reduction means reduction of bacteria by 90 %, a 2 log reduction by 99 %, a 4 log reduction by 99.99 %, and so on. The percent reduction may be calculated as follows:

$$Percent\ reduction = \frac{(N_0 - N) \cdot 100\ \%}{N_0} \tag{5}$$

A relation between the equations 4 and 5 may be stated as follows:

$$Percent\ reduction = (1 - 10^{-L}) \cdot 100\ \% \tag{6}$$

where *L* is the log reduction. [73] CFU counting is not the only way for obtaining quantitative results. Some methods use spectroscopic measurements to determine the bacterial concentration. These methods are described in section 3.2.3.

Antimicrobial efficacy of textiles can be tested by various standardized methods. Both qualitative and quantitative methods are available. [40, p. 10] Qualitative results are usually enough when only the demonstration of antibacterial activity is needed. However, if the antibacterial activity is intended, or implied, quantitative testing is necessary in order to obtain a clearer picture of the capabilities of the material.

Many globally recognized standards for the testing of textiles exist. The International Organization for Standardization (ISO) is a non-governmental organization working in 164 countries [76]. Among all their standards the ISO provides methods for testing of textile safety, durability, and antimicrobial efficacy. ASTM International, formerly called the American Society for Testing and Materials, has over 12 000 standards operating globally. Among the ASTM standards are found methods for testing of antimicrobial efficacy of textiles. [77] The American Association of Textile Chemists and Colorists (AATCC) has been developing test methods for the textile industry since 1921. [78] One globally recognized standard for testing of antimicrobial activity of textiles comes from the Japanese Industrial Standard (JIS), which is coordinated by the Japanese Industrial Standard Committee [79]. While commercial production should rely on standardized testing, non-standardized methods of testing are often used in research publications to demonstrate the efficacy of the product.

Lack of comparability between different studies has been hindering the development of efficient antimicrobial textiles [74]. Some attempts on overcoming this problem have been done by comparison of results obtained via different methods of testing on certain functionalized fabrics [75]. It seems crucial to find the most reliable, reproducible, and comparable methods for testing photoactive materials.

The absence of contaminants in equipment and culture media is required for precise results. Often the method for sterilizing involves autoclaving the materials at 103 kPa (15 psi) for 15–60 minutes. However, not all textiles can be sterilized this way. Cotton, acetate, and many synthetic fibres can be autoclaved. However, wool, for example, should not be autoclaved and can instead be sterilized by ethylene oxide or intermittent (fractional) sterilization in flowing steam. [72, p. 146] Some bacteria and fungi used in the tests are possibly pathogenic to humans and special care must be taken when working with them. Control tests should always be made to ensure that no unwanted contaminations have occurred during the process. All individual tests should be prepared at least as duplicates, but triplicates are most often encountered in the literature.

Any test should involve a test with an unfunctionalized textile to test the effect of the plain textile on the microbes, plain inoculated growth media to serve as the base level for

calculations, a dark control with functionalized textile to test the photoactivity, and an irradiated functionalized textile to test the real photoantimicrobial efficacy. Also, regardless of the chosen method, a positive control with known antibacterial activity should be used to test the activity of the culture.

Generally, any microbial strain could be used for testing, but the scientific community seems to have adopted the use of standardized strains. The use of standardized strains of microbes increases the comparability and reproducibility of the tests since the microbial viability may be predicted to some extent. The bacterial strains used in the literature sources for the following standards usually come from the American Type Culture Collection (ATCC). Some examples are listed in table 1.

Table 1. Some bacterial strains used in antibaterial testing of textiles

Species of bacteria	**Gram type**	**Strain**	**Ref.**
Bacillus subtilis	positive	ATCC 6633	[80]
Staphylococcus aureus	positive	ATCC 6538	[81]
Listeria monocytogenes	positive	ATCC 19115	[82]
Kleibsiella pneumoniae	negative	ATCC 4352	[83]
Escherichia coli	negative	ATCC 11229	[84]

A single study comparing different testing methods used strains of the Gram-positive *Staphylococcus warneri* (DSM 20316) [85] and the Gram-negative *E. coli* (BL21(DE3)) [86][75]. Also, another study was used the strains from Korean Collection for Type Cultures (KCTC). The same study gave an example of an alternative for Gram-negative bacteria mentioned in table 1, namely *Pseudomonas aeruginosa.* [87]

Similarly, the strains used for antifungal tests are listed in table 2.

Table 2. Fungal strains used in antifungal testing of textiles

Species of fungi	**Strain**	**Ref.**
Aspergillus niger	ATCC 6275	[88]
Penicillium varians	ATCC 10509	[89]
Trichoderma ciride	ATCC 28020	[90]
Chaetomium globosum	ATCC 6205	[91]

Both antifungal testing methods described in section 3.2.4 used ATCC strains by default. However, it seems reasonable to assume that the strain selection is not that important when dealing with qualitative tests. With quantitative testing, it is unclear how well results obtained with different strains can be compared. The viruses used in antiviral testing will be discussed in section 3.2.5.

3.2.1 Qualitative antibacterial screening

Qualitative tests are usually fast and easy, and they are generally used to determine whether the sample is antibacterial or not. Several different samples can be quickly screened for the desired properties, and then tested further with quantitative methods. Three standards for qualitative testing stand out in the literature. They are the AATCC 147 (The parallel streak method) [92], ISO 20645 (Agar diffusion plate test) [93] and JIS L 1902 (Halo method) [94]. A fourth standard, AATCC 90 (Agar plate test) [95], was also found among the standards, but no studies were found to utilize it. JIS L 1902 also offers a quantitative method which is described in section 3.2.2. Tests are usually done with both the Gram-positive bacteria *S. aureus* and the Gram-negative bacteria *K. pneumoniae*. Other suitable microorganisms may also be used depending on the needs of the test. [40, p. 10] It is worthwhile to mention that some studies have used different concentrations of bacteria in the inoculum compared to what is mentioned in this thesis. Due to the qualitative nature of these tests, the exact bacterial concentration does not seem to be critical.

The parallel streak method, AATCC 147, involves inoculating nutrient agar plates with either *S. aureus* or *K. pneumoniae*. Another suitable test organism could be, for example, the Gram-negative *E. coli*. The procedure involves preparing a sterile nutrient agar (NA) plate and a liquid culture of the chosen microbe. The liquid culture should be incubated for 24 h and then diluted by transferring 1 ml of culture to 9 ml of sterile distilled water. Then the NA petri dish is inoculated with diluted inoculum using an inoculating loop. Five streaks approximately 60 mm in length and 10 mm apart should be made without refilling the loop or breaking the agar surface. The streaks allow for easier detection of contaminants. The textile specimen should be placed gently on top of the inoculated agar and intimate contact should be ensured. Incubation at 37 ± 2 ℃ for 18–24 h should follow. The possible antibacterial activity can be seen by examining the growth of bacteria in the contact zone between the agar and the textile sample. Zone of inhibition may be calculated. No criteria for passing the test exist, but absence of bacterial growth under the specimen is a good indicator of antibacterial activity. [72, pp. 259–260]

The agar plate diffusion test, ISO 20645, is very similar to AATCC 147. The procedure involves placing a sample of the functionalized textile on two-layer agar plates. The lower level is a culture medium free layer, which is prepared by pouring 10 ml of tryptone soy agar (TSA) into a sterile petri dish. After control of sterility, the upper layer is prepared by inoculating molten TSA (precooled to 45 ℃) with bacterial culture (1–5 x 10^8 CFU/ml), shaking the mixture vigorously to achieve even distribution of bacteria, and then pouring

it on the lower layer. The circularly cut (diameter 25±5 mm) test specimen is placed onto the upper layer with sterile forceps before it solidifies. Incubation of prepared samples is done at 37 ℃ for 18–24 h. Each type of sample should be done fourfold. The evaluation is done like in the parallel streak method. [96]

The Japanese Industrial Standards offer the JIS L 1902 standard which is titled *"Testing Antibacterial Activity and Efficacy on Textile Products"*. The official standard method includes 3 types of tests: 1 qualitative and 2 quantitative. The qualitative method is called the *halo method* and is similar to both aforementioned standards. The “halo” practically means zone of inhibition. However, it comes with a few modifications. The inoculum (10^7 CFU/ml) is added to the molten agar like in the preparation of the upper layer of ISO 20645 plates, but it is allowed to solidify before adding the test sample unlike in the ISO standard method. Also, only one layer of agar is used. [97] The antibacterial activity is evaluated by the absence of bacterial growth around the edges of the sample (halo formation). The size of the so-called halo provides some information about the antimicrobial activity of the sample. [98] Undoubtedly, zone of inhibition may be calculated in the usual manner.

The agar plate test, AATCC 90, is very simple as it involves embedding the specimen partially into inoculated molten agar. [95] Since no literature examples of the procedure were available for examination, only speculations can be made. Yet it is expected that the usual incubation for 24 h at 37 ℃ is suitable before determining the zone of inhibition.

There is also another, somewhat famous, and often used qualitative way to screen for antibacterial properties. The Kirby-Bauer (KB) test, also called the disk diffusion test or zone of inhibition test, was initially developed for testing of the efficacy of antibiotics towards specific micro-organisms. It is similar to some of the aforementioned standards involving agar techniques. It involves inoculating agar plates with bacteria and placing the textile sample discs on the agar. The antimicrobial substance is expected to diffuse from the samples to the surrounding agar and after incubation of 24 h at 37 ℃ the zone of inhibition in mm^2 is determined. [75][99] The KB test is not a standardized method, and it is possibly the simplest qualitative test found in the literature.

3.2.2 Quantitative antibacterial evaluation

The test methods which offer quantitative results are offered by standards ASTM E2149, AATCC 100, ISO 20743 and the absorption methods of JIS L 1902. Other quantitative tests and modifications are also available, and some of them will be discussed in further

sections. Like with the qualitative tests, it was found that differing bacterial concentrations were used among studies found in the literature.

The shake flask test, ASTM E2149, properly titled “Determining the Antimicrobial Activity of Immobilized Antimicrobial Agents Under Dynamic Contact Conditions”, is a quantitative method involving dynamic contact between the microbes and the textile. [100] It is designed for evaluation of antimicrobial strength of non-leaching antimicrobial functionalized specimens. It is suitable for routine quality control and screening tests and was developed to overcome certain difficulties in classical antimicrobial test methods for evaluation of substrate-bound antimicrobials. [101] An example procedure involves incubating a liquid culture of the microorganism and diluting it to the desired concentration of 1.5–3.0·10^5 CFU/ml. 1 g of the textile is transferred to each flask containing 50 ml of the bacterial solution. All flasks are shaken at room temperature for 1 h at 190 rpm. Afterwards the samples are diluted, and 1 ml of the solution is transferred on agar. Inoculated plates are incubated at 37 ℃ for at least 24 h and the number of surviving cells is counted. The results are presented as percentage of reduction obtained after comparing with a control sample. [100] More detailed descriptions of the procedure may be found in the literature [102][103].

Bacteria counting test, AATCC 100, is a method designed to further test the antibacterial efficacy of samples that have given positive results by AATCC method 147. In principle, it involves inoculating test and control samples with the bacteria and eluting the bacteria to a known amount of neutralizing solution after an incubation period. The number of bacteria present in the solution is determined, and the percentage reduction is calculated. The textile samples used should be circular with a diameter of 4.8 ± 0.1 cm. The number of samples capable of absorbing 1.0 ± 0.1 ml of inoculum should be stacked in a 250 ml jar for inoculation. The bacterial concentration of the inoculum should be 1–2 x 10^5 CFU/ml. The bacteria should be leached from the samples with 100 ml of neutralizing solution at time “0” and after incubation at 37 ℃ for a desired period. The neutralizing solution should be able to neutralize the antibacterial agent and take care of the pH needs of the fabric. Dilutions of 10^0, 10^1 or 10^2 are usually enough for the leached bacteria before incubation on NA plates. The percentage reduction and log reduction may be calculated from the incubated plates. When doing the calculations, however, the bacterial counts are to be stated as CFU per textile sample instead of CFU per ml of neutralizing solution. [72, pp. 145–146]

The ISO 20743 standard is titled "Textiles - Determination of Antibacterial Activity of Antibacterial Finished Products" and comprises of three so-called challenge tests, that are used to quantitatively evaluate the antimicrobial efficacy of the sample. The most suitable

of all three methods can be chosen depending on the textile. Also, all methods have specified limit values for antibacterial efficacy. [104]

The first method is the *absorption method*. Textile samples, weighing around 0.4 g, are placed in sterile flasks that can be closed. Six antibacterial and six control samples should be prepared. A suspension of bacteria is pipetted on textile samples and then incubated for 18–24 h at 37 ℃. Concentrations of bacteria are determined from three antibacterial samples at the beginning of the incubation and after incubation respectively. Percentage reduction and log reduction may then be calculated. [104]

The second method is the transfer method. Inoculation of NA plates is done with bacterial suspension and excess liquid is then removed from the surface of the agar. Three swatches of both the antibacterial textile and control are prepared by cutting each in discs with a diameter of 38 mm. The swatches are placed on the agar and weighed down with a 200 g steel weight. Then the samples are removed from the agar and placed with the inoculated side upwards on sterile petri dishes and incubated for 18-24 h at 37 ℃ in a humidity chamber. The percentage reduction in bacterial concentration is then determined. [104]

Printing method is the third option, in which the bacteria are printed on a textile with a special device. The samples should be circular with a diameter of 60 mm. The samples inoculated this way are placed on NA plates with the inoculated side facing up. Incubation is done at 70 % relative humidity at at 20 ℃ for 1-4 h and the percentage reduction in bacterial concentration is determined. [104]

A measure called the antibacterial efficacy *A* can be calculated with the following equation

$$A = (\log C_t - \log C_0) - (\log T_t - \log T_0) = F - G \tag{7}$$

where *F* is a value for bacterial growth on the control sample, *G* a value for bacterial growth on the antibacterial sample, $\log C_t$ and $\log T_t$ are logarithms of the arithmetic mean for bacterial count of all three samples after incubation for 18-24 h (*C* for control and *T* for antibacterial sample), $\log C_0$ and $\log T_0$ are the logarithms of the arithmetic mean for bacterial count on all three samples directly after inoculation. Limits of efficacy according to the standard are as follows: $A \leq 2$ is insufficient, $2 \leq A \leq 3$ is significant, and $A \geq 3$ is strong. [104]

For the absorption method of JIS L 1902, nutrient broth (NB) inoculum with bacterial concentration of 3 x 10^5 CFU/ml is prepared. A volume of 200 µl of this bacterial suspension is then added to each textile sample and incubated at 37 ℃ for 24 h. After the

incubation, the bacteria are leached from the samples with 20 ml of physiological saline solution at vortexing. The leached bacteria are then diluted serially and incubated on NA plates for CFU counting and further calculations. [98] There should be another quantitative method under this Japanese standard, but no clear descriptions of it were found in the literature and thus it will not be discussed in this thesis.

3.2.3 Spectroscopic methods

The Colorimetric MTT assay was first introduced by Tim Mosmann in 1983 and it measures the metabolic activity of living cells [105]. It is based on the reduction of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble formazan by cellular NAD(P)H-dependant oxidoreductase enzymes present in viable cells (figure 10). The resulting formazan has a purple colour and its concentration can be determined by measuring the absorption at 570 nm. [75] Resonance light scattering (RLS) technique has also been demonstrated as being a viable way of measuring the formazan concentration [106][107].

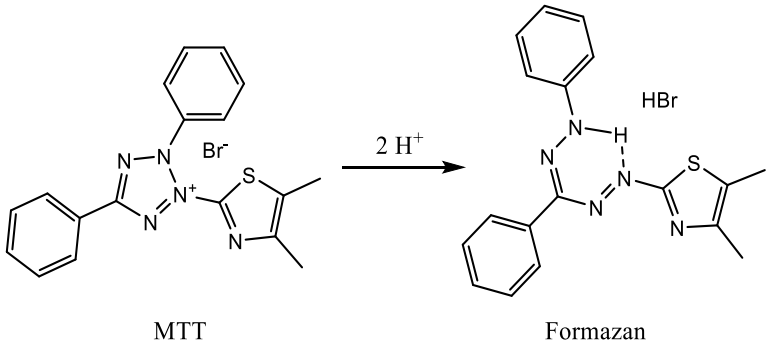


Figure 10. Reaction scheme for conversion of MTT to MTT formazan

2,3,5-triphenyl-tetrazolium chloride (TTC) can also be used in place of MTT. Even though MTT assay is generally used for testing the viability of mammalian cells, its use for measuring the antibacterial activity of textiles has been demonstrated several times and is commonly accepted [74][108][109]. [75] Also, when determining the safety of an antimicrobial textile product, it is possible to use MTT assay to test for cytotoxicity against human cells [106].

An example procedure involves growing bacterial suspension in 96-multiwell cell culture plates with the textile samples for 3 h at 37 °C, rotating at 250 rpm in an orbital shaker. Afterwards, the surviving bacteria are incubated for 5 min with 0.1 mg/l MTT in a culture medium. Then a 30 min lysis treatment is carried out in isopropanol and the absorption

at 570 nm using a reference wavelength of 630 nm is determined. Similar arrangement without the textile sample is measured for the control. [75]

Another way to determine the bacterial concentration is The OD_{600} assay. It is a kind of turbidity measurement used to measure the optical density of a sample at 600 nm, hence the name. It can be used to estimate the concentration of a bacterial suspension. As an example, bacteria can be grown in 96 microwell plates in the presence of textile samples. After incubation, portions of the bacterial suspension are transferred to new plates and the absorptions at 600 nm are measured. 100 % viability is determined by a control sample without the textile, and 0 % viability by a sample without bacteria. [75]

3.2.4 Antifungal efficacy tests

There are at least two recognized standards for evaluating antifungal activity of textiles. The AATCC 30 is a standard offering a semiquantitative method for both hydrophilic and hydrophobic textiles. DIN EN 14119 is a qualitative standard for classifying antifungal activity. [104]

The AATCC 30 consists of four different methods (I–IV) for assessing the susceptibility of textile materials to mildew (I) and rot (II) and to test their antifungal activity (III for hydrophilic and IV for hydrophobic textiles). The tests are said to give semiquantitative results. The standard does not specify limit values for inhibition of fungal growth or alterations in the textile properties. [104]

In test III swatches of both the antimicrobial textile and control are cut in discs with a diameter of 38 mm. A suspension of *A. niger* is spread on a NA plate and covered with the textile sample. Fresh *A. niger* suspension is then spread on the sample. Incubation for 14 days at 28 ℃ is done, and the percentage of surface area of the swatches covered with *A. niger* is determined. [104]

The test IV comprises of spreading a suspension of spores of *A. niger*, *P. varians*, and *T. ciride* on strips of antifungal and control textile samples. Strips should be 2.5 x 7.5 cm or 2.0 x 2.0 cm and saturated with nutrient solution. The inoculated strips are fixed in a square jar bottle with water and incubated for 14 days at 28 ℃. The fungal growth on the samples is evaluated after different time intervals. [104]

The standard DIN EN 14119 is called the *Evaluation of the Action of Microfungi*. Four procedures for evaluation of fungal effects on textiles are described by this standard. Of these, process B2 investigates qualitatively the antifungal activity of the textile. Only the inhibition of fungal growth is determined, but no limit values are stated for the antifungal

activity. The method involves inoculating liquid NA with a spore suspension of *A. niger* or *C. globosum* and pouring it on petri dishes. After the agar has solidified four textile samples (disc shaped with diameter of 30 mm) are placed on agar surfaces with either *A. niger* or *C. globosum*. and incubated for 14 days at 29 ℃. The inhibition zone around and the fungal growth underneath the textile is evaluated. [104]

3.2.5 Antiviral efficacy tests

Determination of antiviral activity of textile products is offered by the ISO 18184 [110] standard. The standard lists two example viruses, namely Influenza A H3N2 (A/Hong Kong/8/68/H3N2) and Feline Calicivirus which are of differing susceptibility and resistance. It is also stated that other viruses may be used for testing, and that results from any one virus cannot be extrapolated to other viruses. Also, to be justly called virucidal the European standard EN 14476 states that virucidal activity against Adenovirus, Norovirus and Poliovirus must be demonstrated. The number of infectious viruses may also be quantified with either the plaque formation method or the $TCID_{50}$ method (Infectious Units for cell culture 50 %).

The procedure involves inoculating the samples with the chosen virus and maintaining contact for 2–24 h. Then the viruses are eluted from the samples and number of viruses is quantified. Reduction value is calculated by comparison with control samples. [111] A more detailed examination of the methods described in this section is outside the scope of this thesis. Antiviral tests are only presented briefly in order to complete the picture of all antimicrobial tests on textiles. It is still worthwhile to mention that a single study focusing on virucidal properties of nanofiber textiles functionalized with the photosensitizer 5,10,15,20-tetraphenylporphyrin was found [112].

3.3 Comparison of test methods for antibacterial activity

A study comparing different methods for testing the efficacy of antimicrobial films concluded that reproducible and comparable results are difficult to obtain. However, among their repertoire of methods, the study used only one method, ASTM E 2149, described in this thesis. [113] The results from another similar study on antimicrobial textiles used only methods described in this thesis and yielded reasonably good comparability [75]. This suggests that comparability is achievable.

The results from antimicrobial tests are affected by several factors like the nutrient media, incubation conditions, contact time between the microbes and the sample, concentration

and kind of microbe strain, and the way of calculating the activity. All this makes comparison between different tests troublesome. In the case of photoantimicrobial materials, the light source and variation in the irradiation intensity should be considered. Variations in the angle of the incoming light and the path it travels through (e.g. glass, agar, and culture medium) could affect the generation of singlet oxygen. Naturally, preparing multiple equivalent samples is used to minimize such errors. Also, the diffusion and solubility of the antimicrobial agent into the surrounding medium can affect the outcome. To compare the qualitative tests, each method is are summarised in table 3.

Table 3. A summary of the methods offering qualitative testing of antibacterial efficacy of textile samples.

Method	Standard	Procedure
Parallel streak method	AATCC 147	Inoculation with five streaks on agar surface and placement of sample on top.
Agar diffusion plate test	ISO 20645	Preparation of a culture free lower agar layer and an upper layer with bacteria. Sample is placed on the top layer before it solidifies.
Halo method	JIS L 1902	Preparation of an agar layer with bacterial culture. Sample is placed on the layer after solidification.
Agar plate test	AATCC 90	Preparation of an agar layer with bacterial culture. Sample is partly embedded in the agar prior to solidification.
Kirby-Bauer test	no standard	Inoculation of agar plates with bacteria and placement of sample on top.

It seems reasonable to assume that a PSs bound covalently to the textile do not diffuse or dissolve into the surrounding agar. Similarly, PSs not bound covalently can possibly leach or dissolve to some extent. Generally, however, the PS should not be diffusible or able to dissolve to the surrounding media. It is thus possible that false-negative results can be obtained from tests relying solely on diffusion and dissolvation [74].

All the qualitative methods summarized in table 3 rely, to some extent, on diffusion or dissolvation of the antimicrobial agent. Qualitative screening of photoantimicrobial textiles is still possible since all methods include contact area between the textile and the agar. All qualitative methods seem plausible but may be unpredictable due to aforementioned reasons. Most notably, comparison of zones of inhibition between different antimicrobial materials should not be done based on qualitative tests, because they do not reflect the true antimicrobial potential.

The standard AATCC 147 offers easier detection of contaminants compared to others because of the spaces between the bacterial streaks across the agar. ISO 20645 and AATCC 90 seem to rely mostly on diffusion but also offer the best contact between the sample and the bacteria. JIS L 1902 and the Kirby-Bauer test seem very straightforward and simple. Also, the growth under the sample is easy to examine like in the AATCC 147 procedure.

Some studies done with novel photoactivated textiles use simple qualitative evaluation to demonstrate the antimicrobial efficacy of the product. Such process involves preparing inoculated agar plates and incubating textile samples on the agar surface upside down with light irradiation coming from the top. [62][63][64] This indicates that light traveling through the agar layer is not a problem and that standards like AATCC 147 and JIS L 1902 could good choices for qualitative testing of PACT-textiles.

Quantitative tests may be regarded as being somewhat more important regarding commercial applications. Comparison between the efficacies of PSs known to exhibit antimicrobial effects is important in development of PACT-textiles. A summary of the discussed quantitative methods is offered in table 4.

Table 4. A summary of the methods offering quantitative testing of antimicrobial efficacy of textile samples

Method	Standard	Procedure
Shake flask test	ASTM E2149	Sample is placed in a flask containing bacterial suspension and shaken for 1 h at room temperature. Then the samples are diluted, and a portion of the solution is transferred on agar to be incubated
Bacteria counting test	AATCC 100	Inoculation of textile samples with bacteria. After incubation, the bacteria are eluted from the sample and their number determined.
Absorption method	ISO 20743	Samples are inoculated with bacterial suspension and incubated. After incubation, the number of bacteria is determined.
Transfer method	ISO 20743	Agar plates are inoculated with bacteria and excess liquid removed. Samples are placed on the agar and pressed down with steel weights. Samples are incubated with inoculated side upwards in a humidity chamber.
Printing method	ISO 20743	Bacteria are printed on the sample with a special device. Samples are incubated with inoculated side upwards in a humid environment.
Absorption method	JIS L 1902	Textile samples are inoculated with bacterial culture. After incubation, the bacteria are leached from the samples and CFU counted after serial dilutions.
MTT assay	no standard	Textile samples are placed in 96-multiwell cell plates and incubated with bacterial suspension in a shaker. The surviving bacteria are then incubated with MTT in a culture medium following a lysis treatment in isopropanol. Absorption at 570 nm is measured.
OD_{600} assay	no standard	Bacteria are grown in 96-microwell cell plates with textile samples. Portions of the surviving bacteria are transferred to new plates and absorptions at 600 nm are measured.

As before, each method seems plausible. The ASTM E2149 offers dynamic contact conditions but possible issues with even distribution of light raise a question. Also, the transfer and printing methods of ISO 20743 seem unreasonably complicated compared to other available methods. Compared to the qualitative methods, none of the quantitative

methods rely on diffusion or solubility and could be effectively used for evaluating the true antimicrobial potential. It must be kept in mind, however, that the real conditions the textiles are intended to work in differ greatly from anything achieved in the laboratory. The way of inoculating the textiles directly with the bacterial culture seems efficient and offers freedom in choosing the method of irradiation during the incubation.

There are also three methods of analysis. The bacterial concentrations can be measured via calculating the number of CFU:s on an agar after serial dilutions, or optionally MTT or OD_{600} assays can be made. Calculating the number of CFU:s on an agar plate gives an estimation on the number of living bacterial cells, but does not address their metabolic activity. MTT, on the other hand, gives an estimation on the metabolic activity in the sample. OD_{600} measurements give an estimation on the number of bacterial cells, living or dead, in the sample.

CFU counting is widely used but relies on laborious dilutions and is more time consuming than spectroscopic methods. The MTT assay and OD_{600} assay offer a quick way to measure the bacterial concentrations and make possible the screening of many samples at a relatively short time. Reliable results from spectroscopic methods require, of course, that no substance capable of absorbing light at the measurement wavelength is leached from the textile during incubation. Spectroscopic methods have been shown to work well for determination of the bacterial concentration [74][75].

Some studies use a simple quantitative method involving incubation of the textile samples in 24-well plates with the bacterial culture following a leaching of the bacteria with phosphate-buffered saline solution and counting of CFU on agar plates [34][65][114][115][116]. The procedure shares similarities with standards AATCC 100 and ISO 20743.

4. CONCLUSION

Photoantimicrobial textiles are a promising candidate for first line of defence against multi-drug resistant bacteria and novel pathogenic threats. The reactive oxygen species generated by irradiated photosensitizers attack the target microbe via many routes and thus makes resistance unlikely to develop. A great variety of photosensitizers are known and many more are yet to be discovered.

There are many ways to functionalize textiles with photosensitizers, but simple, economical, environmentally friendly and scalable processes should be favoured. Textile materials must pass the required safety tests and regulations. Durability testing is also often done by commercial producers.

Standards for testing the antimicrobial efficacy of textiles include both qualitative and quantitative methods. While these methods were not originally developed for photoactivated antimicrobial textiles, they seem to be applicable. The diffusibility, solubility and light requirements must be considered when testing photoantimicrobial textiles.

All qualitative methods rely on diffusion and solubility and are thus unpredictable. However, they can provide means for successful qualitative screening while not reflecting the true antimicrobial activity of the sample. The discussed quantitative methods have also proven to work well, while two methods were seen to be unnecessarily complicated. Three methods for calculating the bacterial concentrations were discussed. Counting the CFU is often used but is time consuming and laborious due to required serial dilutions. Two spectroscopic methods were also described. They offer fast screening of samples and are simple to conduct. However, each of the three analysis methods measure somewhat different qualities of the bacteria. Hence, the results from CFU counting, MTT assay, and OD_{600} assay are not comparable with each other.

Some properties affecting the outcome of these tests were described, and it was concluded that comparability and reproducibility is attainable. However, this requires careful planning of the experiments and controlling the setup. Very few studies comparing different methods have been done [74][75][98][113], and more research is required to find the most accurate procedures.

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