# Strategies to overcome biofilm resistance

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Biofilm have been found to be involved in a wide variety of microbial infections in the body, by one estimate 80% of all infections. A number of anti-biofilm strategies have emerged to prevent the colonization and formation of biofilms. However, there are four main intervention strategies in clinical practice for biofilm-associated infections. The first strategy is to prevent the initial microbial cell attachment through the material surface modification of the medical devices. Second, for an established infection, removal of the infected device is the definitive treatment strategy that is common usually; nevertheless this can cause adverse collateral effects in the patient. Third, agents such as liposomes, polymer-based and nanoparticles are used to penetrate and enhance the antimicrobial transport into the biofilm matrix in order to kill the embedded organisms. Lastly, therapies that can prevent more than one biofilms mechanism simultaneously, as antimicrobial photodynamic therapy (aPDT) that being a multi-target process, there is no development of microbial resistance. So, this review will focus on the mechanisms of biofilm resistance and the therapeutic strategies to overcome that.

**Keywords** microbial resistance, biofilms, therapeutic strategies, aPDT.

## 1. Introduction

Biofilms are increasingly recognized as being responsible for most of human infections [1]. The National Institute of Health (NIH) reported that 65-80% of all microbial diseases are biofilm-based, causing many deaths and high health costs worldwide [2]. Infectious diseases for which microbial biofilm have been held responsible include lung infections of cystic fibrosis patients, colitis, urethritis, conjunctivitis, otitis, endocarditis and periodontitis. The biofilm in medical devices are of particular concern because once the device is colonized, infection is virtually impossible to eradicate [3]. This difficulty occur due two distinct characteristics of biofilms: i) they are highly resistant to host immune clearance and to treatment with antimicrobial agents, ii) protected biofilms might be capable of shedding individual bacteria and sloughed pieces of biofilm into surrounding tissues and the circulatory system causing acute illness [4]. So, the microbial pathogenesis and antibiotic/host immune resistance is related to the ability of the microorganism to form a biofilm.

Biofilms are formed when unicellular organisms come together to form a community that is attached to a solid surface and encased in an exopolysaccharide (EPS) matrix [5]. Many steps are involved in biofilm formation including i) transport of microbes to a surface, ii) initial attachment, iii) formation of microcolonies, iv) biofilm maturation, and v) detachment of cells from the biofilms [6]. The knowledge of these processes helps to understand the mechanisms of biofilm resistance in order to create strategies to control biofilm development.

Currently, wide range of anti-biofilm approaches have been proposed, their focus is specially to inhibit biofilm adhesion and biofilm growth. The strategies in the first case usually, have proposed the surface modifications of medical devices using chemical functional groups as adhesins (key regulators of adhesion stage) that prevent protein adsorption [7]. In the latter approach, traditional techniques such as quaternary ammonium salts, silver ions and antimicrobials are generally used. However, the production of different types of polysaccharides that are specialized to form the structural components of the biofilms as extracellular matrix (ECM), hamper the penetration of these substances [8]. In addition, the quorum sensing (QS) that besides to be involved with the biofilms formation also maintains the biofilms intact [9]. These facts make the ECM and QS, targets of new approaches.

Therefore, this review will be followed by a discussion of the numerous approaches that have been applied to overcome the mechanisms of biofilm resistance.

## 2. Correlation between biofilms growth and mechanisms of resistance

As commented above the biofilm growing differ from planktonic cells with respect to their genetic and biochemical properties. Basically the biofilm-forming cells coaggregate with each other forming coordinated groups attached to a biotic or non-biotic surface; they surround themselves with EPS matrix, communicate effectively via QS mechanisms and express low metabolic activity limiting the impact of conventional antimicrobials acting against actively metabolizing cells [1, 10, 11].

Although the structure and function of biofilms are as varying as the type of microorganism, many process steps of biofilm formation are similar. There are many papers that divide the biofilm development into five stages (Fig. 1) [11-

15]. The first stage involves reversible attachment of planktonic cells to a biomaterial surface and/or host cell surface [6, 16]. This attachment starts by a physical contact between microorganisms and the surface that is governed by both long- and short-range interactions[17]. Long-range interactions position cells near the surface and include van der Waals forces, electrostatic forces and the hydrophobic effects [3, 17]. The proximity of microorganisms with the surface promote a short-range interaction including hydrogen and covalent bonding between microorganism extracellular moieties and the device or host cell surface. At this stage, the cells are still susceptible to antimicrobial agents [5].

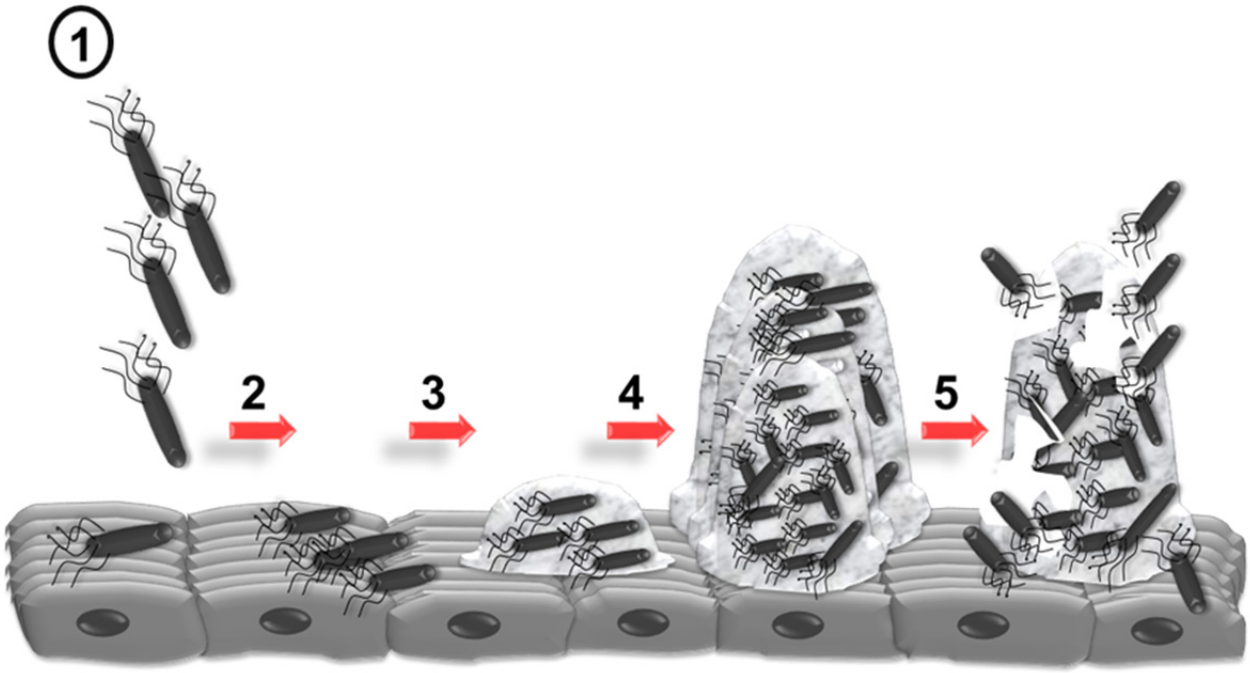


**Fig. 1** Five stages of biofilm development: reversible stage (1), irreversible stage (2), maturation stage (3), biofilms mature (4), and detachment stage (5). Adapted from Kostakioti *et al* [18]

Subsequent microorganism adhesion to surface becomes irreversible and the cells begin to secrete EPS (second stage) [8]. Capsules and fimbriae act as adhesins bridging the microorganism to the material surface via nonspecific hydrophobic and electrostatic interaction and/or ligand-receptor interactions [19]. The EPS matrix are certainly more than only polysaccharides; they comprise a wide variety of proteins, nucleic acids, phospholipids, lipids, amyloid fibers, humic substances, and in some cases, surprising amounts of extracellular DNA (e-DNA) [2, 20]. It is thought that EPS is the first mechanism of biofilms resistance once contributes to the biofilms vitality, acting as a virulence factor and conferring to the bacteria both enhanced antimicrobial resistance and protection from host immune responses [1, 21]. The EPS act as an adsorbent or reactant, reducing the amount of antimicrobial available to interact with biofilm, as well as their structure physically reduces the penetration of antimicrobial agents by walling off access to regions of the biofilm (Fig. 2) [1]. In the host immune system the microbial antigens stimulate the production of antibodies, which cannot effectively kill cell within the biofilm and may cause immune complex damage to surrounding tissues [18]. Furthermore, the aggregation of microorganisms into EPS-coated biofilms might make them less susceptible to phagocytosis [18].

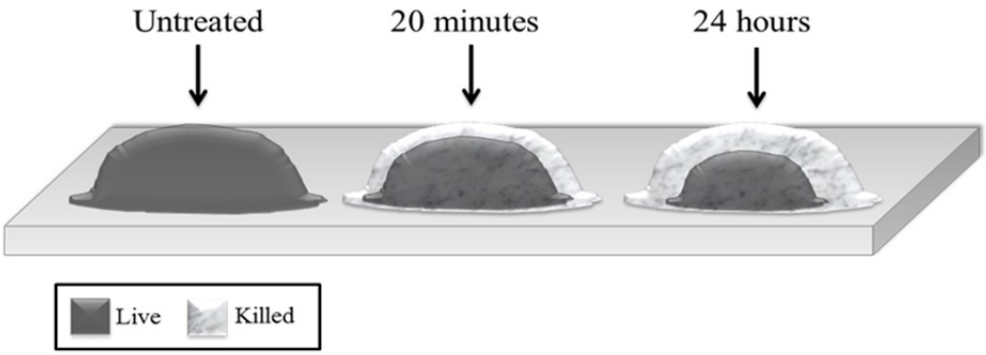


**Fig. 2** Biofilm resistance to antibiotic addition. Adapted from Davies [1].

After the irreversible attachment, starts the maturation stage where the amount of ECM increases (stage 3) around the microcolonies, due a continued secretion of EPS [11, 12]. This stage is controlled by QS that is an intracellular communication system designated for cell-density and population based gene regulation. There are four well indentified and characterized groups of QS molecules: N-acyl-L-homoserine lactone (AHL) QS-system in Gram (-) bacteria, the autoinducing peptide (AIP) QS-system in Gram (+) bacteria, the autoinducer-2 (AI-2) QS-system in both Gram (-) and Gram (+) bacteria and the farnesol, (aromatic alcohol) systems in fungi [22, 23].

In the fourth stage is possible to observe a mature biofilm containing cavities that serve as transport channels of water and planktonic cells throughout the biofilm community, and also provides a unique environment for optimum nutrient absorption and waste disposal [6]. In spite of these open channels, some microorganisms located deep inside of the biofilms structure experience nutrient limitation which contributes to a slow-growing, in other words the cells divide infrequently. This reduced growth-rates result in a state of cells-dormancy that justifies another mechanism of biofilm resistance, once the majority of antimicrobials are active against dividing cells [1]. These observations indicate that nutrient availability is a crucial factor influencing metabolic activity of biofilms, so cells in the centres of the largest microcolonies have reduced metabolic rates compared with cells at or near the surface (Fig. 3) [1].

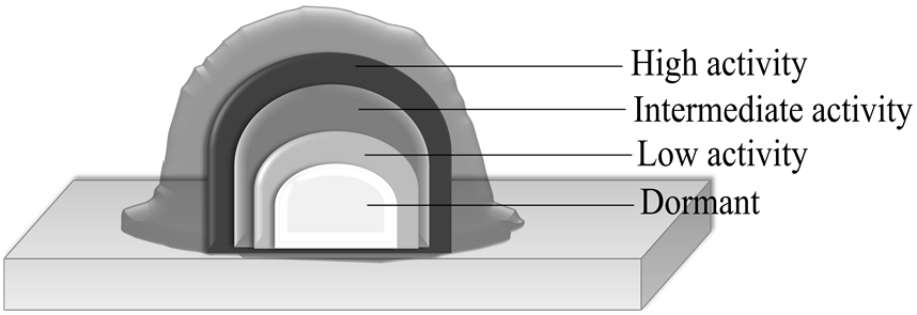


**Fig. 3** Metabolic activity in a biofilm microcolony. Adapted from Davies [1].

The last stage involves the biofilms detachment which can occurs in two ways: erosion, described as the continual dispersal of single cell or small portion of the biofilms; and sloughing, where large pieces of the biofilms are significantly lost [11]. This disruption can expand the infection once the microbial cells are liberated and can colonize another location/surface. However, these cells are more similar to planktonic cells making them more susceptible to eradication.

In addition, the biofilms present a third and still speculative mechanism of resistance that involves genetic adaptation of different conditions. This hypothesis suggest that at least some of cells in biofilms adopt a distinct and protected phenotype that is responsible to growth on surface, contributing to the stage 1 and 2 of the biofilms formation [10, 11].

## 3. Antibiofilm strategies

Under the back-ground of biofilms resistance a range of approaches have been proposed to overcome the huge diversity and flexibility of these microbial communities. The targets of anti-biofilm strategies are especially to prevent the biofilms attachment, disrupt the QS and EPS matrix, and reach the dormant cells.

At the adhesion stage of biofilms formation the surface modification of medical devices is one of the principal strategies. Surface characteristics, such as surface roughness, surface free energy, and chemistry can influence the type and the feature of the biofilms [18, 24]. So, special interest has been set on the alteration of surface hydrophilicity, surface tension or surface energy of the devices that may reduce the force of attraction between the microorganism and the biomaterial [25, 26].

The principal ways to make the biomaterial modification are by physico-chemical methods to obtain anti-adhesive devices, and by incorporation of antimicrobial substances into the materials to obtain anti-proliferative, colonization-resistant devices [27]. Physical modifications of surfaces include changing the hydrophilicity to reduce adsorption of bacteria, increase the attraction or retention of antibiotics at the surface or allow the attachment of a drug delivery system [28]. A versatile physical method is plasma-technique where the surface of a material is exposed to a glow discharge under reduced pressure, without changing bulk properties like mechanical stability and elasticity [29]. Other technique used is ion-beam modification that involves impingement of a high-energy ion beam, as the silver deposition on polyvinylchloride (PVC) or polyester (PE) and sulfonate and carboxylate distributed along polystyrene [29]. This mechanisms is interesting due to the bactericidal property of the metal and specific interactions with adhesive proteins leading to inhibition of microorganisms, respectively. The surface photo-grafting is a process where the high-energy electrons as gamma radiation, UV and visible light are used to manipulate the chemical structure of biomaterials in order to improve their haemocompatibility and affect the adhesion behaviour of microbial cells [30]. In addition, a low percentage of additives on surface as the insertion of carboxylic groups in polyurethane using thioglycolic acid can

make significant changes in the surface properties; increasing surface hydrophilicity and consequently reducing the microbial adhesion [31].

As the QS are actively involved in controlling biofilms formation, studies have focused in a way to inhibit this system. The furanones and RNA III are two classes of leading candidates reported as quorum sensing inhibitors (QSI) [4]. Furanones are analogs of homoserine lactones that can act on both AHL systems and AI2 systems, interfering in biofilms growth and also increase the biofilms susceptibility to antimicrobial agents [32, 33]. The RNA III is a linear Agr QSI that up regulates the production of toxins at post-exponential phase of growth, repress microorganism virulence and biofilms formation [34].

As EPS matrix delays and prevents anti-microbial agents from reaching target microbial cells by diffusion limitation, bioelectric fields and ultrasound applications have been used to enhance the efficacy of antimicrobial agents [1]. These methods have two way of action, the biofilm penetration enhancement and killing of bacteria through abrasive sterilization processes [35]. In bioelectric processes a small "dc current" is used to enhance the efficacy of biocides. As a example, photomechanical waves (PW) generated by ablation with high-pulsed lasers has been used as an extension to the electric field to cause a synergic effect on biofilm killing [36]. Regarding the ultrasound effects, studies have showed enhance in biofilms killing when combined with antimicrobial agents [35, 37].

Another way to inhibit the biofilm formation is disrupting the biofilm matrix via degradation of the biofilm-enzymes [12]. It has been described that the DNase I induced biofilm degradation by 66.7-95% once the average of biofilms thickness reduced 85-97%, indicating lower amounts of extracellular DNA [38, 39]. Moreover, the DNase I increases the biofilm susceptibility to antimicrobial. Alpha-amylases also are enzymes that have an activity against biofilms, especially *S. aureus* biofilms [12]. These enzymes can reduce a formed biofilm as well as decrease a biofilm formation [40]. The most effective alpha-amylase reported was isolated from *Bacillus subtilis* that induced 89% degradation of *S. aureus* biofilms [40]. Others important enzymes are: lyase [41] that causes biofilms matrix liquefaction, lactonase [42] that decreases significantly the biofilms virulence, and lysostaphin [43].

The potential of nanoparticles to combat infection has increased markedly over the past decade, once they present function as biocide, anti-adhesive, and delivery capabilities. As reported that silver can inhibit DNA replication, expression of ribosomal and other cellular proteins, and interferes with the bacterial electron transport chain, nanoparticles of silver have been applied to inhibit the biofilm formation disrupting the EPS matrix or preventing the microbial adhesion [44, 45]. Chitosan nanoparticles and lipid nanoparticles also showed promissory results [12, 46, 47].

Antimicrobial peptides are a bactericidal strategy that is produced by the innate immune response system and have been used for the development of novel types of antibiotics [48]. Lytic constitute one of the main important classes of antimicrobial peptides that bind the LPS (lipopolysaccharide) moieties of the bacterial cell membrane, disrupting membrane stability [49]. However, as the most of antimicrobial agents, their activity spectrum and mechanism of action is restrict and need to be more precisely defined. So, due to this restriction and the increasing antimicrobial resistance, the attention by scientists to ethnopharmacology become an interesting approach. Basically, this strategy use plant extracts as antibiofilm activity, once they have anti-QS and antivirulence factor properties that easily inhibit biofilms formation disrupting the EPS matrix [12]. Some example of these plants are *Melia dubia* [50], *Terminalia catappa* [51], *Allium sativum* [52], *Croton nepetae-folius* [53] and so on.

Therefore, several physical, chemical and biological techniques are being practiced to achieve biofilm killing. Each one of these methods has its own advantages and disadvantages that provide different effects in the biofilm inactivation (Table 1). A combination of these approaches as well as the developments of new innovative strategies may potentiate the treatment of biofilms. The aPDT is relatively a new approach that can improve the efficiency of other inactivation strategies used in a combined protocol.

 

**Table 1** Effect of different approaches against microbial biofilms.

| *Biofilm-target* | *Approach* | *Microorganism* | *Reduction of microbial biofilms CFU ($log_{10}$)* | *References* |
|---|---|---|---|---|
| **Attachment** | Plasma | *E. coli* | 4.00 | [54] |
| | | *P. aeruginosa* | 7.00 | [54] |
| | | *S. Aureus* | 6.00 | [55] |
| | Photo-grafting | *C. albicans* | 6.44 | [56] |
| | | *E. coli* | 5.00 | [57] |
| | Nanoparticles | *S. aureus* | 3.22 | [58] |
| | | | 4.13 | [58] |
| | | | 3.43 | [58] |
| | | *E. coli* | 5.18 | [58] |
| | | | 4.97 | [58] |
| | | | 5.32 | [58] |
| **Quorum sensing** | Furanones | *S. epidermidis* | 4.76 | [59] |
| | | *P. aeruginosa* | 4.80 | [60] |
| | RNA III | *S. aureus* | 4.52 | [61] |
| | | *S. aureus* | 4.50 | [62] |
| | | *S. epidermidis* | 6.20 | [34] |
| | Plant extracts | *E. coli* | 4.00 | [50] |
| **EPS matrix** | Bioeletric process | *P. aeruginosa* | 6.02 | [63] |
| | | *K. pneumonia* | 3.9 | [63] |
| | | *E. coli* | 4.27 | [63] |
| | Ultrasound | *P. aeruginosa* | 3.96 | [64] |
| | DNAse I | *E. coli* | 5.78 | [64] |
| | | *A. baumannii* | 5.29 | [38] |
| | | *H. influenzae* | 5.00 | [38] |
| | | *K. pneumoniae* | 5.21 | [38] |
| | | *E. coli* | 5.13 | [38] |
| | | *P. aeruginosa* | 5.10 | [38] |
| | | *S. aureus* | 4.92 | [38] |
| | Alpha-amylase | *V. cholerae* | 5.11 | [65] |
| | | *S. aureus* | 4.27 | [65] |
| | | *P. aeruginosa* | 4.34 | [65] |
| | Plant extracts | *P. aeruginosa* | 6.00 | [66] |
| | | *S. epidermidis* | 6.99 | [67] |
| | | *K. pneumoniae* | 2.70 | [68] |
| | | *C. albicans* | 3.00 | [68] |
| | | *E. coli* | 5.16 | [68] |
| | | *S. aureus* | 6.00 | [68] |

### 3.1. A new and promissor strategy: aPDT

Antimicrobial photodynamic therapy appears to be endowed with several features for the treatment of biofilm. This approach is characterized by broad spectrum of activity, being effective toward both wild strain and antibiotic-resistant Gram (-) bacteria, Gram (+) bacteria and yeasts. Two main properties make this therapy promissory: aPDT is a multi-target process that almost no development of resistance mechanisms in known pathogens due to the mode of action and type of (biochemical target) and present selectivity (few undesired side effects) [69]. The reason for the first advantage is the generalized action of the light-activated drug on vital cell structure once the drug has accumulated inside the target cell [69].

Basically aPDT employs a non-toxic dye, termed a photosensitizer (PS), and low intensity visible light which, in the presence of oxygen, combine to produce cytotoxic species. Photosensitizers have a stable electronic configuration,

which is in a singlet state in their lowest or ground energy level, $^{o}PS$. Following absorption of a light-photon, the molecule is promoted to an excited singlet state, $^{o}PS^*$ and may then convert to the triplet state, $^{3}PS^*$, that occurs via intersystem crossing involving a change in the spin of the excited electron. The singlet state has a short lived between $10^{-6}$ and $10^{-9}$ seconds and triplet PS has a sufficiently long lifetime to allow it to undergo chemical reactions. These states can interact with surrounding molecules in two different pathways: Type I – singlet sate and Type II - triplet state. Type I mechanism involves electron-transfer reactions from one molecule to another, resulting in the formation of toxic oxygen species that causes cellular damage directly. Type II mechanism involves energy transfer to molecular oxygen, producing excited state singlet oxygen ($^{1}O_2$) that induce irreversible cell damage [70].

The microbial damages occur mainly in DNA, cytoplasmatic membrane and in the cell wall [71]. Generally, DNA damages can be repaired by the action of DNA repairing systems, nevertheless some microorganism are easily killed by aPDT [72]. The damages to the cytoplasmatic membrane can involve leakage of cellular contents or inactivation of membrane transport systems and enzymes [73]. This membrane and cell wall are an important aPDT-target once the PS do not necessarily needs enter the cell to kill the microorganism. In addition, thus target cells have no chance to develop resistance by stopping uptake, increasing metabolic detoxification or increasing export of the drug [69].

It has been shown differences in susceptibility to aPDT among different microorganisms. This is due to the different outer membrane structure of Gram (-) bacteria, Gram (+) bacteria and yeasts. Normally, many PS are more effective against Gram-positive bacteria than against Gram-negative bacteria and fungi [73]. The high susceptibility of Gram (+) species can be explained by the presence of porous peptidoglycan wall with no significant amount of lipids or proteins [74]. In contrast the Gram (-) bacteria has a many-layered outer structure located outside the peptidoglycan layer composed by strongly negatively charged lipopolysaccharides (LPS), lipo-proteins and proteins with porin function [74]. Fungi presents much more complex targets than bacteria, due have a rigid cell wall composed by a glucan, mannan, chitin and lipoproteins and separated from the plasma membrane by a periplasmic space [70]. Besides, the similarities of yeast cell with mammalian cells make the selectivity photoinactivation more difficult.

To overcome these differences several PSs are being development to dwelling bacteria and fungi, especially when these microorganisms are presented as biofilms. The **table 2** shows some in vitro and in vivo studies of aPDT against microbial biofilms.

**Table 2** aPDT studies against biofilms

| *Microorganism* | *Photosensitizer* | *Reduction of microbial biofilms CFU ($log_{10}$)* | *References* |
|---|---|---|---|
| *P. aeruginosa* | Methylene blue | 6.00 | [75] |
| | TMPP[a] | 4.20 | [76] |
| | pL-ce6[b] | >6.00 | [77] |
| | δ-ALA[c] | 9.00 | [78] |
| | PEI-ce6 [d] | >6.00 | [79] |
| *E. coli* | pL-ce6 [b] | >6.00 | [77] |
| *S. aureus* | TBO[e] | 5.00 | [80] |
| | Hypericin | 5.00 | [81] |
| | mTHPC[f] | 5.50 | [81] |
| | Merocyanine | 7.00 | [82] |
| *S. epidermidis* | Methylene blue | 1.20 | [83] |
| | pL-ce6[b] | 1.50 | [83] |
| | TBO[e] | 6.00 | [80] |
| *C. albicans* | TBO[e] | 5.00 | [84] |
| | SiPc1[g] | 3.00 | [85] |
| | NMB[h] | 4.50 | [86] |

[a]5,10,15,20-tetrakis(1-methyl-pyridino)-21H,23H-porphine; [b] poly-l-lysine - chlorin(e6); [c] aminolevulinic acid; [d] polyethylenimine-chlorin(e6); [e] toluidine blue; [f] m-tetrahydroxyphenylchlorin; [g] silicon phthalocyanine1; [h]new methylene blue.

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