Recent progress in bio-inspired biofilm-resistant polymeric surfaces

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Any surface of human interest can serve as a substrate for biofilm growth, sometimes with detrimental effects. The social and economic consequences of biofilm-mediated damage to surfaces are significant, the financial impact being estimated to be billions of dollars every year. After describing traditional biocide-based approaches for the remediation of biofilm-affected surfaces, this review deals with more recent developments in material science, focusing on non-toxic, eco-sustainable nature-inspired biomaterials with anti-biofilm properties superior to the conventional biocide-based approaches in terms of addressing the biofilm problem.
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Introduction

Any surface of human interest can serve as a starting ground for biofilm development, limiting material application and increasing health risks and costs. The inclination of microorganisms to become surface bound is ubiquitous, suggesting that surface dwellers have a strong survival and/or selective advantage tendency greater than their free-floating counterparts, i.e. a certain degree of shelter and homeostasis that helps them persist in the environment (Dunne, 2002).

On the global scale, biofilm-related costs incur the spending of billions of dollars in different sectors of the economy including all clinical and industrial settings associated with surfaces (Plyuta et al. 2013). Most likely, the worst biofilm reputation belongs to biofilm associated with the medical and healthcare sectors, because they are responsible for more than 60% of all microbial infections in humans (Sadekuzzaman et al. 2015). Indeed, implantable medical devices applied to critically ill patients often become potential surfaces for biofilm formation, with devastating medical implications in terms of patient morbidity, mortality, prolonged hospitalization and increased healthcare costs (Hall-Stoodley et al. 2004; Darouiche 2007; Lo et al. 2014; Percival et al. 2015; Tenke et al. 2017).

The detrimental effects of biofilm can also be felt across numerous industries, including water treatment and distribution, food processing and marine-based industries. The result is a decrease in industrial productivity as well as the physical deterioration of industrial systems such as pipe plugging and corrosion (Stowe et al. 2011). In food-processing environments, biofilms are of special importance as they have the potential to act as a persistent source of microbial contamination, which can lead to the threatening of the microbiological quality and safety of food products, resulting in food-borne disease (Cappitelli et al. 2014). Approximately 95% of bacterial cells that grow in drinking water networks are attached to pipe walls, while less than 5% has been found in the water phase (Flemming 2002; Douterelo et al. 2016). Detrimental effects include microbe-induced corrosion, disinfectant depletion, color, odor and taste degradation and the microbiological deterioration of drinking water (Farkas et al. 2013).

Biofilm also affects the surface of buildings and monuments, both historic and modern (Polo et al. 2012; Villa et al. 2016; Vázquez-Nion et al. 2018). As a consequence of complex interactions within the microbial community and its substrate biodeterioration processes occur (Giacomucci et al. 2011; Cappitelli et al. 2012). The consequences are aesthetic and structural damage.

Historically, most strategies that attempt to mitigate the effects of biofilm focus on treatments aimed at killing the microbial cells in biofilm already present on solid surfaces. However, such strategies have limited efficacy owing to bacterial persistence and resistance in pre-formed biofilm (Feng et al. 2015). Indeed, sessile bacteria exploit features that make them up to 1,000-fold more resistant to antibiotic and biocide treatments than their corresponding planktonic counterparts (Stewart 2002). *In vitro* experiments have shown that young, less dense biofilm is more easily cleared away by antibiotic treatment than mature thicker biofilm (Stewart 2015). However, early diagnosis of biofilm infection is currently difficult, and most biofilm infections are caused by matured biofilm, thus making it difficult to eradicate them with antibiotic treatments (Wu et al. 2015). Additionally, biofilm treatment is hindered by the dramatic increase in antibiotic resistance among pathogens, reducing the possibility of treating infections effectively and increasing the risk of complications and a fatal outcome.

No less important are chemical treatments that often involve considerable amounts of potentially dangerous substances. Sooner or later biocides and antibiotics are released into the environment and, if they do not break down into safer constituent

parts, they persist intact over prolonged periods of time, raising severe environmental and human risks (Young et al. 2008; Schultz et al. 2011; Sousa et al. 2014). This is readily seen in the growing number of policies, directives, technical reports, strategies, recommendations and regulatory decisions designed to reduce the consumption of antimicrobial agents, ensuring their prudent use, and protect human and animal health and wellbeing (Directive 98/8/EC; Council Recommendation 2002/77/EC; SCENIHR report 2009; EFSA Summary Report 2012).

With regard to the severe adverse impact of biofilm on many human activities, this review provides an overview of current and advanced strategies employed to control and prevent unwanted biofilm on polymeric surfaces in recent years. Materials and coatings with antibacterial activity, as well as more recent biofilm resistant solutions based on non-toxic natural molecules, including advantages and disadvantages with respect to potential applications, are discussed (Tables 1 and 2). Finally, methods for assessing anti-biofilm performance of innovative polymeric surfaces are presented.

Surface modification

The resistance of biofilm cells to traditionally used antimicrobial agents has prompted researchers to focus on preventive strategies rather than on attempts that remove or kill microorganisms. The development of materials that can resist or prevent bacterial adhesion constitutes the most promising and emerging approach to deal with material-associated biofilm infection problems (Alves and Pereira 2016). These approaches aim at altering the polymer surface by using passive or active strategies that discourage microbial adhesion and thus biofilm formation (Coenye et al. 2011).

Passive surfaces

In passive strategies, the physiochemical properties of an existing material, such as composition, charge, hydrophobicity, roughness and porosity, are modified so as to minimize microbial adhesion upon contact, without releasing biocidal agents into the surrounding environment (Gbejuade et al. 2015; Romanò et al. 2015). These coatings are called passive, because their effect is not attributed to antimicrobially active functional groups.

Surface chemistry modifications

In the past, poly(-ethylene oxide) (Johnston et al. 1997; Roosjen et al. 2003; Roosjen et al. 2005), poly(ethylene glycol) (Holmberg et al. 1993; Park et al. 1998; Kingshott et al. 2003; Tedjo et al. 2007; Saldarriaga Fernández et al. 2007) and hydrophilic polyurethanes (Jansen et al. 1993; Nagel et al. 1996) were used extensively as passive coatings to increase surface protection against biofilm formation. Indeed, these anti-adhesive coatings reduce the adhesion force between the bacteria and the solid surface, enabling easy removal of bacteria before the formation of surface biofilm (Adhart et al. 2018).

These strategies are relatively simple and economic ways to counteract microbial colonization. However, despite their popularity in the academic literature, few commercially marketed biomedical coatings are available, perhaps due to the difficulties in creating surface-bound thin films amenable at industrial scale (Sjollema et al. 2018).

Additionally, these passive coatings have been shown to reduce the adhesion of bacteria and yeast *in vitro*, but after exposure to physiological fluids *in vitro* or *in vivo*, the reduction in microbial adhesion is usually small or soon lost (Roosjen et al. 2005; Saldarriaga Fernández et al. 2007). The anti-biofilm properties of the coating are quickly masked by an adsorbed conditioning film of bacteria-produced proteins that diminishes its effectiveness (Hetrick and Schoenfisch 2006) whereas coating degradation can also occur (e.g. hydrolysis, chain cleavage, surface removal) (Saldarriaga Fernández et al. 2007). Furthermore, surfaces that are non-adhesive to bacteria are often non-adhesive to tissue cells as well, making them less suitable for biomaterial implants and devices requiring tissue integration (Sjollema et al. 2018). Finally, the introduction of additional chemical species decreases biocompatibility (Dickson et al. 2015).

Surface topography modifications

Modification of surface topography with micro- and nanoscale features that minimize bacterial attachment is another passive strategy for preventing biofilm formation on abiotic surfaces. Indeed, surfaces with topographic features of dimensions much smaller than microbial cells, in the sub-micrometer or nanometer range, have been reported to inhibit attachment by reducing the contact area between bacteria and the surface (Hsu et al. 2013).

An interesting development in this area is the recognition that nature has developed numerous surfaces with highly optimized nanoscale topography able to minimize microbial attachment. Therefore, many studies have attempted to mimic the peculiar surfaces found in nature. Carman et al. (2006) developed engineered microscale surface design inspired by the topography of shark skin (with features 2 μm wide, 3 μm in height) able to disrupt biofilm formation on patterned poly(dimethylsiloxane) elastomer without the use of biocidal agents. Regularly spaced nanopillar structures, similar to those found on the bactericidal cicada wings, were reproduced on a black silicon surface by Ivanova et al. (2013) with optimal bactericidal effect against both Gram-positive and Gram-negative bacteria. A superhydrophobic and biocompatible micro/nano structure gecko skin-like surface with low adhesion, anti-wetting, self-cleaning and antibacterial properties has been developed by Watson et al. (2015) and Li et al. (2016). Other examples of bio-inspired surfaces with effective anti-adherence nanoscale feature include those resembling rose petals, lotus leaves, taro leaves, rice leaves and legs of water striders (Claudia et al. 2016; Barthlott et al. 2017).

According to these researchers, bacterial cells are killed through the mechanical rupture of their cell wall when they are in contact with the nanostructures (Tripathy et al. 2017). Therefore, recruitment of additional cells and biofilm build up are both prevented and resistance to the nanofeatures cannot evolve (Dickson et al. 2015).

Unfortunately, these discoveries have not been translated to technologically scalable processes yet. Indeed, most of nanostructuring methods available today require cleanroom technologies and are prohibitively expensive, slow and cumbersome for large-scale applications (Feng et al. 2015; Hasan et al. 2018). Additionally, although most studies have shown that biofilms are sensitive to nanoscale topographical details, no universal rules of attachment have been determined yet and some researchers have reported a greater level of attachment on some nanoscale surfaces compared to those with conventional topographies (Park et al. 2008; Hsu et al. 2013).

Active antimicrobial surfaces

In active approaches, the abatement of biofilm growth has been achieved by spreading a number of antimicrobials and disinfectants onto the surfaces or by incorporating them into synthetic polymer-based products, directly or by means of a

carrier. The anti-biofilm activity is the result of functional groups that interact with microbes in the surrounding area (Lichter et al. 2009).

Antimicrobial-releasing surfaces

In the antimicrobial-releasing surfaces, the biocidal agent is actively eluted from the surface when in contact with an aqueous environment (Coenye et al. 2011; Chen et al. 2013; Lo et al. 2014; Zanini et al. 2015). Such approaches are those most used to obtain devices with different antimicrobial spectra and duration. Indeed, a number of materials with entrapped antibiotics and disinfectants are commercially available, and are already used in clinical applications, especially for mitigating implant-associated infections (Antoci et al. 2008; Hockenhull et al. 2009; Francolini and Donelli 2010; Swartjes et al. 2015; Ashbaugh et al. 2016). Examples of active polymers for antimicrobial applications are reported in Gao et al. (2011), Chen et al. (2013) and Huang et al. (2016).

Release of antibiotics by coating degradation is also possible by using degradable polymers, such as poly(D,L-lactide), poly(ε-caprolactone) or poly(trimethylene carbonate) (Strobel et al. 2011; Guillaume et al. 2012). Shukla et al. (2012) found that application of vancomycin containing layer-by-layer assembled films increased drug loading by up to approximately 9 times the control. It is interesting to note that the approach enables the incorporation of different drug types in each layer, giving potential to engineered delivery system for drugs with a multitude release profile (Hammond 2012).

However, despite the considerable effort made, over the past 30 years there has been little progress, few products have become available on the market, and reviews are not unanimous about their benefits (Johonson et al. 2006; Nowatzky et al. 2012; Sjollema et al. 2018). The prerequisite for the good performance of such coatings is the continuous and constant elution of antimicrobial molecules from the surface, with a release rate sufficient to deter or slow down microbial attachment to ensure the long service life of the coating (Barrios et al. 2005). Unfortunately, such active coatings have been designed to release high initial fluxes of antimicrobial agents during the critical short term post-implantation period (several hours) so as to inhibit initial microbial adhesion through a biocidal mechanism. However, continued release beyond this short-term period (weeks to months) is not realized, making these systems less desirable for

long-term and extended applications (Hetrick and Schoenfisch 2006; Knetsch and Koole 2011; Gao et al. 2011).

Antimicrobial responsive surfaces

Several materials from which the antimicrobial substance release is triggered by the microorganisms approaching the surface have been proposed. Indeed, in such responsive approaches, both enzymes and acids excreted by the bacteria themselves have been used as triggers for antimicrobial release to combat their adhesion. Komnatnyy et al. (2014) introduced an enzyme-sensitive link into a poly(ethylene glycol) material. The bioactive compounds, i.e. quorum sensing signals and antimicrobial drugs, were only released in presence of the microorganisms that secrete the specific enzymes that cleave the sensitive linkage in the construction. Pavlukhina et al. (2014) constructed a pH/bacteria-responsive material providing a novel hydrogel-like montmorillonite/polyacrylic acid film able to keep gentamicin safely sequestered for months under physiologic conditions. When challenged with bacteria, the coating released gentamicin because microorganisms locally acidify the environment, e.g. by secreting lactic or acetic acid. Similarly, Wang et al. (2017) developed a new multilayer film with a high loading capacity for triclosan. In this system, the permeability of the films is altered in response to pH changes in the environment caused by bacteria providing the release of the antibiotic.

These responsive materials are supposed to provide new antimicrobial approaches with the following advantages: i) they exhibit the antibacterial activity only when and where needed; ii) they extend the useful life time of coatings, decreasing the premature depletion of the drug reservoir; and iii) they minimize side-effects related to continuous and uncontrolled molecule release, e.g. its accumulation in vital tissues.

Though these materials are interesting, they are seldom used as antibacterial coatings. The main challenges are: i) to achieve release of meaningful doses over multiple cycles; ii) to minimize non-triggered background leaching from surfaces; iii) the limited effect against multiple microbial infections; and iv) the altered efficacy of many antimicrobials due to changes in pH during microbial growth (Cloutier et al. 2015; Alvarez-Lorenzo et al. 2016).

Immobilization of antimicrobials

Important achievements have also been made to covalently immobilize antimicrobials on surfaces to completely overcome the problem of constant release (Antoci et al. 2008; Gharbi et al. 2015; Gerits et al. 2016; Peng et al. 2017). These so-called contact-killing surfaces are not intended to release antimicrobials into the surroundings, but to kill bacteria upon contact.

The active molecule, covalently bound to the polymeric chain, reaches the site of action on the bacterial envelope or inside the bacterium, e.g. by penetrating its cell wall. Therefore, the bond with the surface is generally performed by using flexible spacers that allow a certain degree of freedom of the bound antibacterial agents (Nie et al. 2016). Indeed, chain length and chain density are important parameters for polymer brush anchors (Adhart et al. 2018). Jose et al. (2005) used a double aminoethoxyethoxyacetate linker combined with a 3-aminopropyltriethoxysilane-modified titanium surface to provide vancomycin with a distance of about 4 nm from the polymer surface.

In contrast to the release coatings, surface binding technology of antibiotic agents creates a high local concentration, minimizing the risk of exposing bacteria to sub-inhibitory concentrations and thereby reducing the likelihood of resistance development (Nie et al. 2016). Little is known about the possible development of bacterial resistance against these materials and it remains to be seen whether or not this occurs upon their increasing usage. Certainly, it has been well documented that the constant use of antimicrobial agents inevitably leads to the development of antibiotic resistant strains and could even promote biofilm formation (Hoffman et al. 2005; Andersson and Hughes 2010). Moreover, the effectiveness of such material is most likely limited to infections caused by bacteria that are sensitive to the specific antibiotic (Hetrick and Schoenfisch 2006). No less important: the application of such surface-active systems is restricted to some surfaces for safety reasons, e.g. their use is less suitable for specific food contact materials as the carrying over of antimicrobials into food products might occur (Simões et al. 2010; Lucera et al. 2012; Cappitelli et al. 2014).

Metal-based antimicrobial materials

Metal coatings

Heavy metals have been used as an anti-biofilm agent, the metal being deposited on biomaterial surfaces by means of a coating technology (Stobie et al. 2008; Gallo et al. 2014).

Among the metals, the one that has long been the center of attention is silver (Knetsch and Koole 2011). There are indications that the antimicrobial activity of silver is dependent on the silver cation Ag+, which reacts with, and disrupts, the function of bacterial cell membranes, DNA molecules, crucial metabolic proteins and enzymes, and ultimately leads to cell death (Feng et al. 2000; Jung et al. 2008; Randall et al. 2013). Indeed, silver has been coated onto medical implants (Darouiche 1999; Devasconcellos et al. 2012), wound dressings (Heggers et al. 2005; Ip et al. 2006) and textiles (Sataev et al. 2014). However, such silver coating has its faults, including poor silver adhesion and lack of coating uniformity; it also requires special processing conditions (Kumar and Munstedt 2005). Furthermore, the incorporation of silver into polymers does not always result in efficient antimicrobial activity because of the poor solubility of most silver salts in polymeric materials (Knetsch and Koole 2011).

Metal-nanoparticles based materials

Over the last decade a great deal of interest has been shown in metal nanoparticles. This is because of the superior and unique features that make them particularly attractive for new and emerging nanoparticle-based anti-biofilm materials (Polo et al. 2011; Ahire et al. 2016; Mu et al. 2016; Qayyum and Khan 2016; Ramasamy and Lee 2016; Gambino et al. 2017). Among others, silver nanoparticle-based materials have been successfully proposed to limit biofilm formation on both medical and industrial applications, e.g. medical implants (Roe et al. 2008), air and water treatment filters (Mpenyana-Monyatsi et al. 2012; Gehrke et al. 2015), clothing (Zhang et al. 2009; Zhang et al. 2014), food processing surfaces (Araujo et al. 2013) and food packaging materials (Bumbudsanpharoke et al. 2015; Souza and Fernando 2016). Although it seems that bacteria are less prone to develop resistance against silver than they do against conventional antibiotics, concerns associated with the overuse of silver and the consequent emergence of bacterial resistance have been raised (Hobman and

Crossman 2015; Ebrahiminezhad et al. 2016). Moreover, these nanotechnology-based emerging novel anti-biofilm strategies are still in the nascent phase and more research is needed to clarify a number of safety, environmental, ethical, policy and regulatory issues (Fabrega et al. 2011; Gottschalk et al. 2013; Sajid et al. 2015; Gambino et al. 2015; Reed et al. 2016; Garuglieri et al. 2016; Hoseinnejad et al. 2017; Garuglieri et al. 2018).

Preventive green biocide-free surfaces

Numerous concerns have put pressure on the scientific community to develop alternative, more effective strategies; strategies perceived by the public as safe, and as posing negligible risk to human health and the environment.

Indeed, efforts are being directed towards developing innovative anti-biofilm materials with functional features targeting molecular determinants of biofilm genesis, instead of fighting biofilm with antimicrobial materials and coatings (Chen et al. 2013; Villa et al. 2013). In these approaches microorganisms are deprived of their virulent properties but their existence remains unaffected. Thus, selection pressure decreases, with the promising perspective of restoring the efficacy of traditional antimicrobial agents (Rasko and Sperandio 2010).

On considering the process of biofilm formation, different key steps can be identified as promising targets for the development of innovative anti-biofilm products. The first is to avoid surface microbial adhesion: this can be done by interacting with the surface sensing process in order to keep the pioneering cells in a planktonic form. Another good point of attack that has emerged is the disruption of mature biofilm. Indeed, interference with cell-to-cell communication processes inevitably results in biofilm matrix damage, leading to the destabilization of biofilm physical integrity. Finally, the promotion of biofilm dispersal by forcing the planktonic state is another interesting target (Kaplan 2010; Kostakioti et al. 2013).

Needless to say, the above strategies do not presume to be the solution to preventing biofilm development, but they could be used in combination with other conventional treatments to maximize the anti-biofilm performance of polymeric materials. Indeed, these strategies could be a step forward in applications where it would be advantageous to slow down contamination. Indeed, in many industrial and clinical activities, surface treatments that retard adhesion, and consequently biofilm

formation, could greatly enhance the efficiency of daily cleaning and disinfection procedures, because, once dispersed from the biofilm, free-floating microbes are more susceptible to detergents and biocides than those in the biofilm itself (Davey and O'Toole 2000; Dell'Orto et al. 2017). The synergic combination of antimicrobial and anti-biofilm agents is even recommended by the Infectious Diseases Society of America guidelines for the treatment of selected biofilm-associated infection (Pappas et al. 2004; Cui et al. 2015).

Already, to date, a number of natural and synthetic compounds based on this innovative biocide-free anti-biofilm strategy have been proposed. The latter includes a broad range of molecules, free in solution as well as coated or immobilized on a surface, that interfere with quorum sensing cell-to-cell communications and promote biofilm dispersal (Ding et al. 2011; Wu et al. 2015; Alexander et al. 2015; Brackman and Coenye 2015; Abraham 2016; Chen et al. 2016), as well as matrix-targeting enzymes able to effectively destroy biofilm architecture (Pavlukhina et al. 2012; Villa et al. 2015; Spadoni Andreani et al. 2016; Sadekuzzaman et al. 2015; Meireles et al. 2016; Spadoni Andreani et al. 2017; Snarr et al. 2017).

Surface modification with natural anti-biofilm compounds

Present day awareness of ecological problems, together with the increased number of safety laws, has prompted the scientific community to address the development of more eco-sustainable anti-biofilm materials with non-toxic and biodegradable properties. Thus, there is great demand for new approaches based on molecules displaying suitable environmental-fate parameters such as high water solubility, low partition coefficient, low bioaccumulation in biological systems, and no ecotoxicity; such molecules used in anti-biofilm materials would give them great potential as safe anti-biofilm agents (Qian et al. 2010). To date, a multitude of compounds found in nature have revealed promising anti-biofilm properties suitable for the development of improved effective eco-friendly, bio-inspired anti-biofilm materials able to replace, or integrate with, current dominating biocide-based strategies (Villa et al. 2013; Sadekuzzaman et al. 2015; Qian et al. 2015; Almeida et al. 2017). However, compared to the great amount of work devoted to the discovery of potent natural biofilm inhibitors, relatively little research has dealt with the design of anti-infective

bio-hybrid surfaces based on natural products and, of these, only a few materials display anti-biofilm properties without biocidal activity.

Passive and active natural molecule-based strategies

Barrios and coauthors (2005) and Newby and colleagues (2006) incorporated zosteric acid into silicone coatings, developing different strategies to achieve its slow release into the surrounding area. Bryers and collaborators (2006) and Rosenberg and colleagues (2008) proposed salicylic acid-releasing poly(anhydride-ester) polymers able to inhibit *Pseudomonas aeruginosa* and *Salmonella enterica* biofilms respectively. In a further study, Nowatsky and coauthors (2012) developed a new salicylic acid-releasing polyurethane acrylate polymer. Under aqueous environments, the polymer hydrolyzed and released salicylic acid, leaving the backbone intact and reducing biofilm formation of *Staphylococcus epidermidis, Bacillus subtilis, Escherichia coli, P. aeruginosa,* and *S. aureus*. Others examples are reported in Table 2.

Unfortunately, in most cases, these materials exhibited a discontinuous release, this release being initially high but soon followed by an exponential decrease. In addition, a very large number of molecules appeared close to the surface, reaching concentrations lethal for the microorganisms. The amount of released substance and its rate is influenced by factors like processing parameters, loading dose, applied technique, molecular size of the molecule and the physic-chemical properties of the polymeric material, all of which makes it arduous to carry out a strict monitoring of the anti-biofilm rate from the surface. Indeed, the problem of release rate in an aqueous medium can be attributed to the fact that most polymeric matrices and anti-biofilm compounds have incompatible physical characteristics. Most of the coating matrices are hydrophobic polymers, while some anti-biofilm compounds are hydrophilic, making their miscibility with the coating matrices difficult. The result is a non-uniform molecule distribution within the material, which increases the tendency of the surfaces to absorb and diffuse water through the polymer matrix; this allows the anti-biofilm molecules to diffuse out (Barrios et al. 2005; Newby et al. 2006; Nowatzki et al. 2012). To solve the problem, Barrios and coauthors (2005) investigated various techniques for incorporating zosteric acid into a model silicone material. When the zosteric acid distribution within the polymer was uniform, with small aggregates or even individual molecules, leaching took place at a reasonable rate, extending the material working

time. However, the optimal, and desired, constant rate was never reached as, at the beginning, the rate was quite high, reaching an almost constant value only later.

Covalent immobilization of natural molecules

The binding of natural molecules to surfaces could easily side-step the problem of constant release, guaranteeing the material long life as the molecules become permanently attached and integrated into the scaffold structure of the polymers. Indeed, grafting active compounds to polymers is preferred to covalent incorporation as biofilm development is a process that occurs at the surface and not in the bulk. This kind of approach requires very good knowledge of the exact functional groups required by molecules to exert anti-biofilm activity, identifying the molecular structure's binding site needed for the group's immobilization on the surface, without destroying the biological activity of the material. However, in most cases this information is not available. Hume et al. (2004) proposed two different methods for the covalent immobilization of the furanone 3-(1'-bromohexyl)-5-dibromomethylene-2(5H) on biomaterials: a furanone co-polymerization with a styrene polymer and a plasma-1-ethyl-3-(dimethylaminopropyl) carbodiimide reaction to produce furanone-grafted catheters. Biofilm formation by *S. epidermidis in vitro* was inhibited by 80% whereas in an *in vivo* sheep model immobilized furanones were found effective at controlling infection for up to 65 days. A study by Cattò et al. (2015) concerning the relationship between zosteric acid structure and anti-biofilm activity clarified that the carboxylic acid moiety conjugated to the double bond in trans configuration is necessary in the molecule to guarantee good anti-biofilm performance, while deletion of the sulphate ester group does not compromise molecule anti-biofilm activity. In addition, Cattò et al. (2017) showed that the para-position of the phenyl ring in the salicylic acid structure proved suitable for its immobilization on a N-hydroxysuccinimide polymeric matrix. On the basis of these two studies, Dell'Orto et al. (2017) covalently grafted modified cinnamic and salicylic acid on a low density polyethylene surface, previously activated by oxygen plasma treatment and using 2-hydroxymethylmetacrylate as linker. Both functionalized polymers displayed optimal anti-biofilm performance against *E. coli*, reducing biofilm surface coverage, thickness and cellular biovolume by more than 80%. Cattò et al. (2018) later confirmed that the reduction of biofilm biomass was achieved in both functionalized surfaces by a mechanism that did not affect bacterial viability,

which is an important factor in the challenge to limit the risk of developing resistant microbial strains. Additionally, the authors demonstrated that the functionalized surfaces strongly reduced polysaccharides in the biofilm matrix, maximizing the biofilm sensitivity to conventional antimicrobial agents. Noteworthy, the new polymers preserved their anti-biofilm activity over time.

Although anti-biofilm mechanisms are still poorly understood, current findings document that, in most cases, anti-biofilm materials affect microbial settlement by acting as an environmental cue that leads bacteria to global stress, providing conditions by which the best microbial strategy is to escape from the adverse environment rather than activate drug resistant sessile mechanisms. This response is often mediated by reactive oxygen species, used by cells as signals in adapting to changing environments (Gambino and Cappitelli 2016). For example, both zosteric acid and salicylic acid affect the bacterial oxidative balance by interacting with NADH: quinone reductase (WrbA), an enzyme belonging to a family of flavoprotein quinone reductases widespread in both bacteria and fungi (Cattò et al. 2015; Cattò et al. 2017). The outcome of this oxidative imbalance is the production of signal molecules that discourage the firm adhesion of bacteria on surfaces (Villa et al. 2012; Cattò et al. 2015).

Methods for evaluating the anti-biofilm performance of new bio-inspired polymeric surfaces

After the design and creation of bio-inspired polymeric surfaces, the validation of the new materials' anti-biofilm performance becomes a critical step for field applications. However, the *in vivo* testing of new anti-biofilm materials remains a difficult task due to the poor control over experiments and justified ethical concerns (Sjollema et al. 2018). Therefore, simplified *in vitro* systems have been developed to mimic different conditions encountered *in vivo* (Gomes et al. 2017).

While a number of industrial standard tests are available to assess the antimicrobial efficacy of medical and non-medical products, no accepted standard methods exist to properly evaluate the anti-biofilm activity of new surfaces with a mechanism of action that is different from simple killing or growth inhibition. Indeed, available surface evaluation standard tests are mostly intended to assess the efficacy of surface by testing the material ability to decrease microbial viability without considering differences in the mechanism of action (Sjollema et al. 2018). Therefore,

the only way to test is by adapting the standard lab-scale devices and procedures to reproduce the biofilms on the functionalized biocide-free materials.

Lab-scale systems for growing biofilms on anti-biofilm surfaces

The simplest experimental system relies on the use of microtiter well plates, a static assay particularly useful for examining early events in biofilm formation (Merritt et al. 2005). Microtiter plates allow surfaces to be modified or, alternatively, new bio-functionalized polymers can be further inserted into the wells. The general protocol allows for the inoculation of microtiter wells with a cell suspension for a desired period of time, after which the attached biomass is analyzed.

While microtiter tray based techniques are inexpensive and appropriate for large scale screening purposes, the static nature of these systems leaves them prone to nutrient exhaustion, thus limiting the generation of mature biofilm (Azeredo et al. 2017). As a consequence, the full effect of new tested materials on biofilm growth and dispersion cannot be evaluated with this system.

The dynamic solution lies in continuous-flow systems to produce mature biofilms on surfaces. Indeed, continuously pumping high shear and nutrients into the reactor provides stress conditions that promote biofilm development on the polymer surfaces (Goeres et al. 2005). Standardized protocols employing the Center for Disease Control biofilm reactor (CDC reactor), the rotating disk reactor (RD reactor) and the drip flow reactor (DF reactor), have been approved by the American Society for Testing and Materials (ASTM E2562-12 2012; ASTM E2196-02 2012; ASTM E2871-13 2013; ASTM E2647-13 2013).

The CDC reactor allowed Cai et al. (2012) to prove that diazeniumdiolate-doped poly(lactic-co-glycolic acid)-based nitric oxide releasing films applied in indwelling biomedical devices exhibit considerable anti-biofilm properties against gram-positive *S. aureus* and gram-negative *E. coli*. In another experiment, the CDC reactor was used to challenge dentin-composite and hydroxyapatite disks with multi-species oral biofilms, mimicking acidogenic meals and snacks (Li et al. 2014). Dell'Orto and colleagues (2017) tested a new anti-biofilm material obtained by the covalent grafting of *p*-aminocinnamic or *p*-aminosalicylic acids on low density polyethylene coupons against *E. coli* biofilm grown in the CDC reactor. Sawant and colleagues (2013) tested the anti-biofilm properties of silver nanocomposites against *E. coli* biofilm by the use of a DF

reactor. With the same device, Pérez-Díaz et al. (2016) evaluated the anti-biofilm capacity of chitosan gel formulations loaded with silver nanoparticles on strains of clinical isolates under conditions that mimic the flow of nutrients in the human skin.

All the described continuous-flow systems offer the advantages of the simultaneous use of different surface materials, the possibility of analyzing samples noninvasively, and standardized protocols, making it possible to compare different materials within one laboratory alone and among different laboratories (Gomes et al. 2017). Additionally, bioreactors allow to sample the materials aseptically at different time points during the sessile growth, without compromising the whole experiment.

Methods for quantification and structural assessment of biofilm on anti-biofilm surfaces

Once grown on a surface, the most widely used technique to estimate biofilm is the determination of viable cells by plate counting on agar media (Azeredo et al. 2017). Alternatively, flow cytometry, is a fast and precise way to count live and dead cells in a biofilm (Kerstens et al. 2015; Sgier et al. 2016). Quantification can be also achieved through colorimetric methods by staining biofilms and measuring the amount of desorbed dye by spectrophotometric measurement (Honraet et al. 2005; Welch et al. 2012; Larimer et al. 2016; Sabatini et al. 2018). Depending on the stain, this method enables quantification of the total biofilm biomass (e.g., crystal violet), the biofilm matrix (e.g. dimethyl methylene blue), or the metabolic activity of the biofilm cells (e.g. XTT 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt and resazurin). Furthermore, quantitative polymerase chain reaction (qPCR) could be used to account for the uncultivable portion of the biofilm microbial community (Dalwai et al. 2007).

Microscopy is one of the most powerful tools to assess the biofilm architecture on material surfaces. In particular, confocal Laser Scanning Microscopy (CLSM) allows the representation of the 3D architecture of the biofilm, and the acquisition of quantitative structural parameters such as biofilm bio-volume, thickness and roughness (Bridier and Briandet 2014) (Figure 1).

The amount of biomass retrieved on the surface is not the only indication of the anti-biofilm properties of a new material. In fact, functionalized materials might act by destabilizing biofilm organization and its physical integrity, compromising its structure

rather than decreasing the biomass on a surface (Villa et al. 2015). This mode of action might render the biofilm more prone to detachment and/or more susceptible to traditional antimicrobial agents. By using micro-bead force spectroscopy, it is possible to quantify biofilm adhesion and viscoelasticity at the micro-meter scale (Lau et al. 2009; Angeloni et al. 2016). Additionally, Atomic Force Microscopy was used to determine the adhesion forces between bacteria and goethite (Huang et al. 2015), and between biofilms and different plastics (vinyl chloride, silicone resin, Nylon 66, polycarbonate, polypropylene, polyethylene and polymethylmethacrylate) (Harapanahalli et al. 2015; Hirai et al. 2015).

The susceptibility of biofilms grown on functionalized materials to traditional antimicrobials is an important aspect to consider. In this respect, CLSM has been shown to be a powerful tool to analyze antimicrobial actions by a time course (minutes-to-hours scale) visualization of live and dead cells through the biofilm structure during a biocidal treatment (Rani et al. 2005; Daddi Oubekka et al. 2012; Singh et al. 2016). The general protocol consists in staining biofilm grown on the coupon with specific fluorochromes in order to detect live and dead cells (e.g. LIVE/DEAD BacLight bacterial viability kit) or enzymatic activities (e.g. esterase activity marker CalceinAM). The biofilm is then exposed to the antimicrobial treatment, and any changes over time in fluorescent intensity due to cell inactivation are recorded under CLSM (Figure 2 and Video S1).

Concluding remarks

The use of surfaces that prevent or limit microbial adhesion and biofilm formation by depriving microorganisms of their biofilm-specific traits, without affecting their existence, has been proved instrumental in combating surface-associated biofilm. The integration of such innovative strategies with conventional approaches also appear to be a good strategy as, once the biofilm is destabilized, microorganisms are more sensitive to biocide treatments. Now, the hard part is to translate these ideas into commercial reality: although the value of the previously described research is indisputable, it is equally true that a number of issues have yet to be solved. In contrast to the currently used solvent-based approaches, the design and synthesis of such surface materials needs to be focused on chemical approaches based on solvent-free, non-toxic reactions, adhering to the principles of green chemistry (Sheldon, 2016). In addition, the

diffusion of a natural-based surface commercially requires large amounts of natural materials, as, unfortunately, not all compounds are suitable candidates for commercial total synthesis due to their structural complexity (Dias et al. 2012). Advances in chemo-informatics have partially filled previous gaps: high throughput screening has shortened times whereas structure-activity studies have permitted a drastic reduction in the size and chirality of bioactive natural products. However, these approaches are often limited by the general lack of information concerning the mechanism of action, the cellular receptors and the active chemical scaffold of many bioactive natural products. This understanding is also necessary to achieve more efficient and better targeted anti-biofilm surfaces (Chen and Qian 2017).

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Disclosure of interest

The authors report no conflicts of interest.

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Table

Table 1. Comparison of various anti-biofilm strategies presented in the paper.

Anti-biofilm strategy	Mechanism	Advantages	Disadvantages
Surface modification			
1. Passive surfaces			
1.1 Surface chemistry modifications	Anti-adhesive coatings to reduce the adhesion force between bacteria and the solid surface.	- Simple and economic.	- Difficulties in creating surface-bound thin films amenable to industrial scale processing; - Anti-biofilm properties quickly masked by bacteria-produced substances; - Surface erosion during application; - Less suitable for long-term applications; - Less biocompatibility with living tissue; - Toxicity concerns.
1.2 Surface topography modifications	Modification of surface topography with micro- and nanoscale features that minimize bacterial attachment.	- No recruitment of additional cells and biofilm buildup; - No resistance against nanofeatures.	- Nano-structuring methods expensive and not available for large scale production; - Discordant results about the efficacy.
2. Active antimicrobial surfaces			
2.1 Antimicrobial-releasing surfaces	Biocidal agent actively eluted from the surface by contact with an aqueous environment.	- Incorporation of different drugs in separate sets of layers.	- Not controlled elution of antimicrobials from the surface; - Less suitable for long-term applications; - Resistance against antimicrobials. - Limited applications for safety reason.
2.2 Antimicrobial-responsive surfaces	Antimicrobial release triggered by microorganisms when approaching the surface.	- Antimicrobial activity only when and where needed; - Extended material life time by decreased premature depletion of drug reservoir; - Limited accumulation of antimicrobials in vital tissues.	- Not controlled elution of antimicrobials over multiple cycles; - Non-triggered background leaching from the surface; -Limited effect against multiple microbial infections; -Altered environmental condition by bacteria affect the release of antimicrobials. - Resistance against antimicrobials. - Limited applications for safety reason.

2.3 Immobilization of antimicrobials	Antimicrobials covalently immobilized on a surface.	- No release of antimicrobials from the surface; -Minimized risk to expose bacteria to sub-inhibitory concentrations; - Reduced likelihood of resistance development; -Long-term activity.	- Efficacy only against specific microorganism; - Limited applications for safety reason.
3. Metal-based antimicrobial materials			
3.1 Metal coatings	Heavy metal with antimicrobial activity, deposited on the surface or incorporated into a polymeric material.	-Broad spectrum of anti-microbial activity.	- Poor solubility of metal into polymeric materials; - Poor metal adhesion to the surface; - Lack of coating uniformity; - Resistance against metals.
3.2 Metal-nanoparticles based materials	Metals with antimicrobial activity, grouped onto nanoparticles and then incorporated into a polymeric material or coated or immobilized on a surface.	- Nanosized particles increase the antimicrobial potency	- Nano-structuring methods expensive and not available for large scale production; - Safety, environmental, ethical, policy and regulatory issues; - Resistance against metals.
Preventive green biocide-free surfaces			
1. Surface modification with natural anti-biofilm compounds			
1.1 Passive and active natural molecule-based strategies	Natural molecules with anti-biofilm activity coated on or incorporated into a polymeric material or eluted from a surface.	-No biocidal activity; -No development of resistance; -Enhanced efficacy of cleaning and disinfection procedures; -No toxicity concerns.	- Discontinuous release of the compounds; - Non-uniform molecule distribution within the material - Incompatible physical characteristics of anti-biofilm compounds and most polymeric matrices; - Mechanisms of action poorly understood.
1.2 Covalent immobilization of natural molecules	Natural molecules with anti-biofilm activity grafted on a surface or covalently incorporated into the polymer.	- No biocidal activity; - No release of compounds from the surface; -No development of resistance; - Long-term activity; -No toxicity concerns; -Enhanced efficacy of cleaning and disinfection procedures.	- Knowledge of the molecule functional groups required to exert the anti-biofilm activity; - Mechanisms of action poorly understood.

Table 2. Relevant examples of polymeric surfaces modified with natural anti-biofilm compounds.

	Coating agent	Polymer	Coating method	Target microorganism	Target mechanism	Remarks	Reference
PASSIVE STRATEGY	B-type proanthocyanidins	Permanox plastic slide	Spin coating onto a Permanox slide	*Staphylococcus epidermidis, Staphylococcus aureus, Enterococcus faecalis*	Bacterial adhesion	Treated biofilm composed by few cell clusters or single attached cells; compatibility with mammalian cells	Trentin et al. 2015
	Cinnamaldehyde, carvacrol	Poly(lactic-co-glycolic acid)	Incorporation in the polymer mixture	*E. coli, S. aureus, Pseudomonas aeruginosa*	Bacterial adhesion	Efficacy against *E. coli* and *S. aureus* biofilm	Zodrow et al. 2012
	Clove essential oil; Eugenol	poly(D,L-lactide-coglycolide)	Incorporation in the polymer mixture	*Escherichia coli O157:H7* and *K-12*	Bacterial adhesion, Biofilm maturation	Reduction of biofilm biomass, thickness, and substratum coverage by ≥ 90%	Kim et al. 2016
	Dibromohemibasta-din-1	Poly(ε-caprolactone-co-δ-valerolactone)	Varnish applied on the surface	*Paracoccus sp., Bacillus sp., Pseudoalteromonas sp.*	Bacterial adhesion, Biofilm maturation	39.6% biofilm inhibition for *Paracoccus* sp.; no effect on *Pseudolateromonas* sp. and *Bacillus*	Le Norcy et al. 2017
	Dihydrooroidin	PVC plastic	Mixed in a generic marine-based paint and applied on the surface	*Halomonas pacifica*	Bacterial adhesion	Active after 3 weeks in a marine environment	Melander et al. 2009
	N-vanillylnonanamide	Polyurethane	Dissolution in the polymer and sprayed on the surface	*Bacillus cereus, Bacillus thuringiensis, Pseudomanas stutzeri*	Bacterial adhesion	No anti-adhesion effect	Villa et al. 2009

	Rosmarinus officinalis essential oil	Catheter pieces	Functionalization on magnetite nanoparticles absorbed on the surface	*Candida albicans, Candida tropicalis*	Fungal adhesion, Biofilm maturation	Important reduction in adhering cells and biofilm development	Chifiriuc et al. 2012
ACTIVE STRATEGY	Salicylic acid	Poly(anhydride-esters)	Releasing of salicylic acid through the hydrolytic degradation of the polymer	*P. aeruginosa*	Bacterial adhesion, quorum sensing	47% reduction of bacterial adhesion after 3 h; reduction of biofilm formation after 3 days; resistant to cell degradation when implanted subcutaneously for 4 weeks	Bryers et al. 2006
	Salicylic acid	Poly (anhydrideesters)	Dispersion in the polimer	*Salmonella enterica* serovar *Typhimurium*	Biofilm maturation	No anti-biofilm effect at the air-liquid interface, no effect on cells attachment	Rosenberg et al. 2008
	Salicylic acid	Poly[1,6-bis(o-carboxyphenoxy)-hexanoate]	Built into the polymer backbone	*Salmonella typhimurium* MAE52	Bacterial adhesion, Biofilm maturation	Biofilm inhibition without affecting cells viability	Guinta et al. 2009
	Salicylic acid	Polyurethane acrylate	Co-polymerization with an acrylate-bearing urethane resins	*E. coli, P. aeruginosa*	Biofilm maturation	Reduction of biofilm formation for up to 5 days without affecting cells existence	Nowatzki et al. 2012
	Zosteric acid	Polystyrene poly[3-hydroxyalkanoate-co-3 - hydroxyalkenaote]	Dispersion and loading in polystyrene microcapsules	Activated sludge	Bacterial adhesion	Efficacy only in the first 48 h of biofilm formation	Geiger et al. 2004
	Zosteric acid	Polydimethylsilox-ane (Sylgard® 184)	Incorporation in the polymer mixture	Microbial consortium isolated from Lake Erie, *Pseudomonas putida*	Bacterial adhesion	70% reduction of bacterial attachment	Barrios et al. 2005
	Zosteric acid	Sylicon Sylgard® 184; Sylicon RTV11	Incorporation in the polymer mixture	Microbial consortium isolated from Lake Erie, *P. putida*	Bacterial adhesion	75% reduction of bacterial attachment on Sylgard® 184 and of 55% on RTV11	Newby et al. 2006

COVALENT ATTACHMENT	Furanone 3-(10-bromohexyl)5-dibromomethylene-2(5 H)-	Polystyrene; Silastic Tenckhoff catheters	Co-polymerisation with a styrene polymer; plasma-1-ethyl-3-(dimethylaminopropyl) carbodiimide reaction	*S. epidermidis*	Bacterial adhesion, Biofilm maturation	Biofilm inhibited up to 89%; effective *in vivo* sheep model up to 65 days	Hume et al. 2004
	p-aminocinnamic acid; *p*-aminosalicylic acid	Low-density polyethylene	Covalent grafting on the surface	*E. coli*	Bacterial adhesion, Biofilm maturation	Reduction of biofilm biomass up to 73 %; active after multiple use	Dell'Orto et al. 2017
	p-aminocinnamic acid; *p*-aminosalicylic acid	Low-density polyethylene	Covalent grafting on the surface	*E. coli*	Bacterial adhesion, Biofilm maturation, Antimicrobial susceptibility	Decreasing of biofilm thickness, roughness, substratum coverage, cell and matrix polysaccharide bio-volumes by > 80%; no biocidal activity; biofilm more susceptible to ampicillin and ethanol	Cattò et al. 2018
	Zosteric acid	Poly[3-hydroxyalkanoate-co-3-hydroxyalkenoate]	Covalent incorporation in the polymer backbone	Activated sludge	Bacterial adhesion	No cell attachment	Hany et al. 2004

Figures

Figure 1. 3D-reconstructed CLSM images of *E. coli* biofilm grown on non-functionalized (a) and functionalized with *p*-aminocinnamic acid (b) low density polyethylene surfaces. Biofilm grown on non-functionalized surface (a) shows a complex heterogeneous biofilm, with multi-layers of cells (green) organized in dense macro-colonies inside a well-structured polysaccharide matrix (red). On the contrary, biofilm grown on functionalized surface (b) shows a significant decrease in thickness with a uniform mono-layer of cells (green) and a significant reduction of polysaccharide matrix (red). Scale bar = 20 µm. © Cristina Cattò.

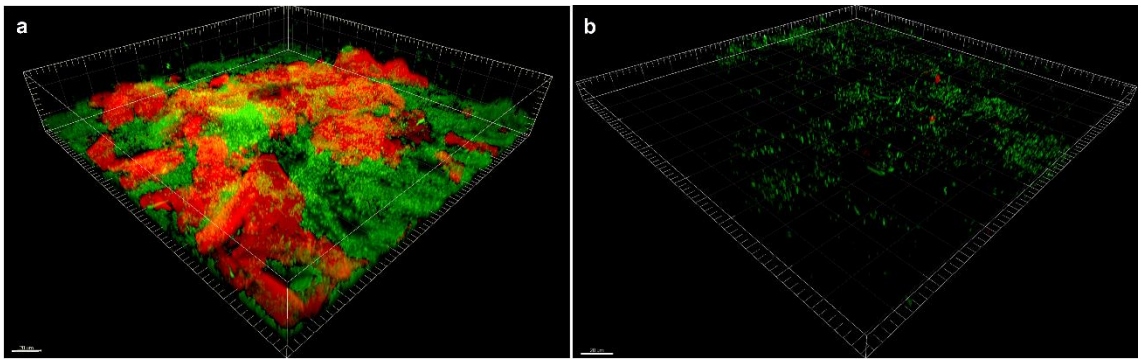
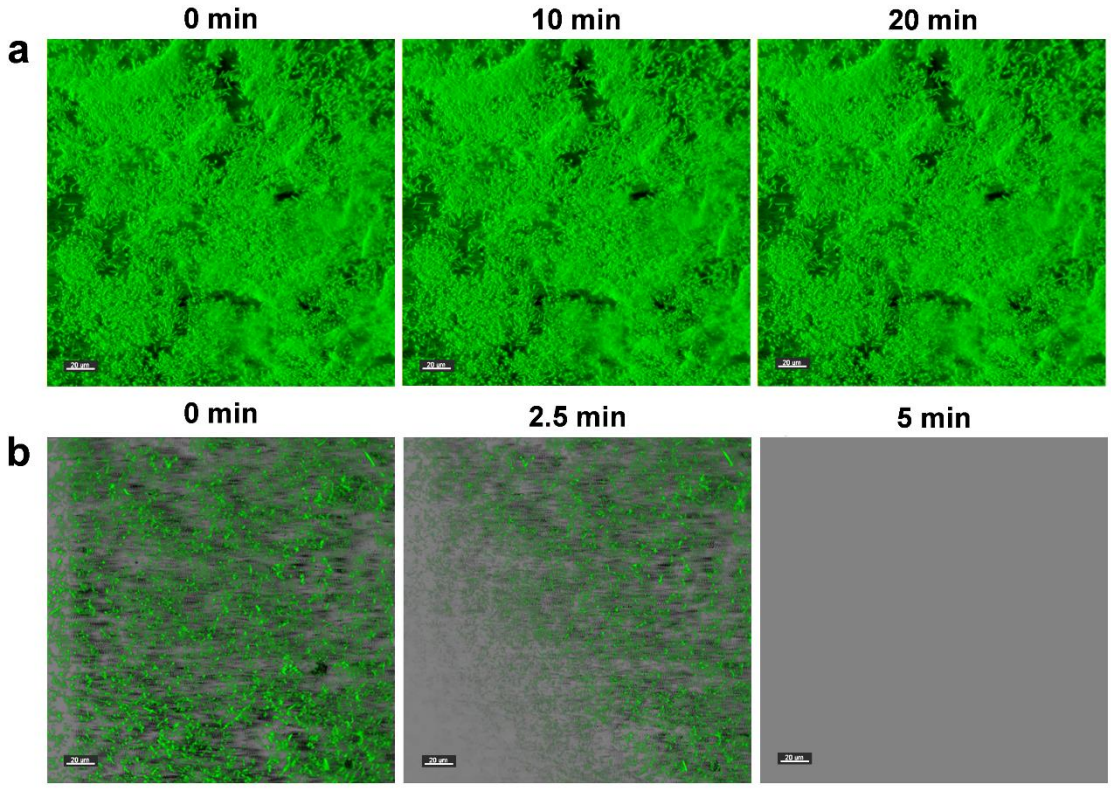


Figure 2. Time lapse CLSM of ethanol action performed on *E. coli* biofilm grown on non-functionalized (a) and functionalized with *p*-aminosalicylic acid (b) low density polyethylene surfaces. The fluorescence loss from stained *E. coli* cells is used to monitor real-time loss in cell viability during the biocide action. The images show that ethanol treatment did not affect green fluorescence of biofilm grown on the control surface within the 20 min of the experiment (a). On the contrary, ethanol treatment significantly affected the biofilm biomass integrity of biofilm grown on the functionalized surface, with a complete loss in fluorescent intensity in 5 min (b). Scale bar = 20 µm. © Cristina Cattò.



Supplemental material

Video S1. Time lapse CLSM of ethanol action performed on *E. coli* biofilm grown on low density polyethylene surface functionalized with modified cinnamic acid. The technique permits the direct visualization of cell inactivation patterns in biofilm structures during the biocide action. The method is based on the monitoring of fluorescence loss from stained *E. coli* cells, used to monitor real-time loss in cell viability. © Cristina Cattò.