Ann Ig 2013; 25: 31-42 doi:10.7416/ai.2013.1904

Analytical techniques to study microbial biofilm on abiotic surfaces: pros and cons of the main techniques currently in use

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Key words: *Biofilm, microbial adhesion, microbial count, public health, quantitative analysis of biofilm*

Parole chiave: *Biofilm, adesione batterica, conta microbica, salute pubblica, metodi di conta microbica su biofilm*

Abstract

Biofilm is a bacterial lifestyle widespread in microbial world and represents a concern in health care. Despite the great life expectancy related to advanced health care, the increasing numbers of biofilm-mediated infections remain a significant public health challenge. Moreover, the problem of biofilm-mediated infections becomes much more severe when biofilm colonizes medical devices and biomaterials. The public health risk due to microbial biofilm-related infections is a concern that requires full attention. However, the complexity of biofilm makes difficult its exhaustive analysis. Although biofilm represents a major challenge in both microbiological and hygiene areas, at now methods aimed to analyse biofilm formation and development are not standardized yet. Different methods have been employed to qualitatively and quantitatively evaluate biofilm each of which is useful to estimate a peculiar aspect of biofilm lifestyle.

In the present review, fifteen assays for the qualitative and quantitative evaluation of bacterial biofilm colonizing abiotic substrates, such as medical devices, prosthesis or surfaces for food production together with advantages and limitations of each method were described and compared.

Some methods are suited to quantify biofilm matrix while others are capable to evaluate both living and dead cells or quantify exclusively viable cells in biofilm. In particular, colorimetric methods to evaluate biofilm matrix (crystal violet; 1,9-dimethyl methylen blue and fluorescein-di-acetate methods) or viable cells (LIVE/DEAD BacLight, BioTimer Assay, resazurin, tetrazolium hydroxide salt methods) and genetic methods to estimate the bacterial population (PCR and FISH) are reported. Moreover, a section is dedicated to examine the performances of advanced microscopic techniques employed to study microbial biofilms (mass spectrometry; confocal laser scanning microscopy; Raman spectroscopy and electron microscopy).

Because of its complexity, an exhaustive study of biofilm requires a combination of different experimental approaches as biochemical, genetic or physical ones.

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Introduction

Knowledge of the exact number of viable bacteria in a sample is a scientific information of primary importance for many microbiological studies, both in environmental and medical fields (1-6). For these reasons several methods have been adopted to count bacteria in different samples and for different purposes. The most common methods count free floating (planktonic) microorganisms. However, bacteria live predominantly adhering to a substrate in biofilm rather than in planktonic life-style (7). A biofilm can be defined as a surface-attached (sessile) community of microorganisms growing embedded in a self-produced matrix of extracellular polymeric substances (EPS). Typically biofilm shows an increased antimicrobial resistance as compared to planktonic bacteria (8-10). This aspect is of great significance in many areas as antimicrobial therapy, persistent and recurrent infections, medical device-related infections and more in general, for many public health considerations as prevention of nosocomial infections, quality control of drinking water, food processing and environmental hygiene with significant social and economic consequences (11, 12). Although biofilm represents a major challenge in both microbiological and hygiene areas, the enumeration of the actual number of bacteria in biofilm is still a great challenge for microbiologists (13).

Considering the complexity and heterogeneity of biofilm structure, the exact objective of investigation must be taken into account. The amount of EPS, the total number of bacterial cells embedded in biofilm or the effective number of "living bacteria" in biofilm must be considered as different "targets" requiring different experimental approaches. Taking in account these considerations, here, the most commonly used, as well as some innovative methods for analytical studies on biofilm are reviewed.

Staining assays

1) **Crystal violet (CV) assay**. CV staining is one of the first methods adopted for biofilm biomass quantification (14,15). In nutshell, this method consists in staining negatively charged molecules by the basic dye crystal violet. CV binds indifferently to negatively charged bacteria and polysaccharides of the EPS (16). After staining, the adsorbed CV is eluted using a solvent (e.g. ethanol or acetic acid). The amount of dye solubilised by the solvent (measured by optical absorbance at 590 nm) is directly proportional to biofilm size. A peculiar aspect is related to biofilm of violacein producing bacteria, in this case, CV can be replaced by a more appropriate dye e.g. Safranin (17). The limitations of this method are related to the low reproducibility of the method, i.e.: the experimental condition of biofilm growth, the specific nature and concentration of the solvent and the elution time are crucial steps. Moreover, because both living and dead cells as well as biofilm matrix are stained by CV, this method provides no information on the actual number of living bacteria and therefore it is poorly suitable to evaluate the anti-biofilm efficacy of antimicrobial substances. To overcome this problem in many laboratories it is practice to detach biofilm from surfaces and to count the detached bacteria by conventional Colony Forming Units Methods (CFU). Detachment procedure is the weak point of all these methods in fact, different disadvantages affect the results. Adopting a soft procedure of detachment, this less invasive approach does not ensure a complete detachment of all the bacterial cells (18). To overcome the low yield in

detachment, other methods adopted more radical procedures as sonication. Also in this case the experimental procedure, injuring cell viability, can compromise the bacterial cell count yielding false-negative results (19, 20). Moreover, the detached bacteria appear as aggregates of different size and not as independent cells, therefore the relative CFU counts show a very high standard deviation value as an index of low reliability.

2) **DMMB assay**. This is a colorimetric assay used to quantify specifically *Staphylococcus aureus* biofilm using the 1,9-dimethyl methylen blue (DMMB) (21). In nutshell, this method is based on the consideration that the main constituent of *S. aureus* biofilm EPS is the intercellular polysaccharide adhesin (PIA), composed of poly-b-1,6-linked-N-acetylglucosamine (22). Therefore PIA has been considered by some authors as a logical target for *S. aureus* biofilm detection. Taking into account that the cationic dye DMMB is commonly used to detect specifically glycosaminoglycans (GAGs) in biological samples (23) and considering the structural similarity between PIA and GAGs, DMMB has been used for the specific detection of *S. aureus* biofilm EPS. After complexation of DMMB with polysaccharides of *S. aureus* biofilm, the authors, adding a decomplexation solution, were able to quantify spectrophotometrically the amount of DMMB-dye released and, in an indirect way, the amount of matrix biofilm. This method is easy to perform, economic and requires little runtime. The main limitation of this method seems to be that it is limited to only those few bacterial species (i.e. some *S. aureus*) possessing the PIA-related biofilm matrix. Moreover, this test gives poor and inaccurate information on the quantity of living bacteria. To overcome this last limit, the authors propose to combine the described DMMB assay with companion experiment using resazurin to determine the viable number of bacteria in biofilm, thus making the assay more elaborate and time consuming (21).

3) **FDA assay**. Briefly, this technique uses the colourless fluorescein-di-acetate (FDA), a cell membrane soluble dye. After bacterial uptake, FDA is hydrolyzed by cellular esterases to fluorescein which is fluorescent yellow. The signal can be measured spectrophotometrically. Dead cells are not able to metabolise FDA so that there is no fluorescent signal. This method has been used to quantify *Candida albicans* biofilm growth at the surface of silicone disks. The fluorescence of *C. albicans* was measured using a microtiter plate reader equipped with excitation and emission filters of 486 nm and 535 nm, respectively (24, 25). Though easy to perform and not expensive, this method is not widely used. Considering the limited field of examination and the thickness of biofilm, this method is not particularly suitable for quantitative studies on mature biofilm, yielding only semiquantitative results (25).

4) **LIVE/DEAD BacLight assay**. This method is based on the use of two different nucleic acid binding stains. The first dye is the green fluorescent (Syto9), able to cross all bacterial membranes and bind to DNA of both Gram-positive and Gram-negative bacteria. The second dye is red-fluorescent propidium-iodide that crosses damaged bacterial membranes only. The stained samples are observed using a fluorescent optical microscopy to evaluate live and dead bacterial population. As matter of fact, live bacteria fluoresce in green and dead bacteria fluoresce in orange/red. The main drawback of this method is the need of observing statistically relevant portion of the sample, representative of the total population. In any case, as it is impossible a total count of bacterial cells, the method provides only semi-quantitative results (26, 27).

Metabolic assays

5) **Resazurin assay**. Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) is a biological dye that does not damage living cells. The blue-non fluorescent resazurin also known as Alamar Blue, can be reduced by cellular metabolic activity and converted in the pink-fluorescent resorufin (irreversible process) up to reach a completely reduced colourless state (reversible process). Pink-fluorescent resorufin can be measured spectrophotometrically. For these properties, resazurin has been used to detect viable microorganism in many studies on antimicrobials as well as to quantify the actual number of viable cells in biofilm (28-31). In this regard, however, some limitations must be considered. This test is highly susceptible to bacterial respiratory efficiency which in turn is related to the growth phase, and to age and thickness of the microbial biofilm. Moreover, as the time of resazurin reduction is species- and strain-related, some experimental conditions are difficult to standardize. In addition, a decrease in the resazurin reduction has been described in the presence of antibacterial compounds, thus reducing the reliability of this method in anti-biofilm researches (29, 32-34).

6) **XTT assay**. As described above for resazurin-assay, also this method uses a redox indicator to enumerate spectrophotometrically viable cells in biofilm. In nutshell, this method is based on the observation that microbial respiratory metabolism of viable cells is able to reduce the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide salt (XTT) to a water-soluble formazan. The number of viable bacteria in biofilm can be deduced measuring the absorbance of supernatant after the metabolic reduction of XTT (35). The main limitations of this method are related to the complexity and heterogeneity of biofilm structure and composition showing different metabolism gradients and to the predisposition of mature biofilm to slow down or partially retain the reduction and release of XTT and formazan, respectively (24).

7) **BioTimer assay (BTA)**. BTA is a colorimetric assay allowing counting viable bacteria in biofilm life-style. BTA employs a specific reagent containing phenol red. The colour of the specific reagent switches from red-to-yellow, thanks to microbial products of primary fermentative metabolism. The time required for colour switch of BTA specific reagent is correlated to initial bacterial concentration. Therefore, the time required for colour switch determines the number of bacteria present in the sample at time zero through a genus-specific correlation line. Noteworthy, BTA does not require sample manipulation. BTA is a low cost, easy to perform method and has been applied to count living bacteria in biofilm, to verify microbiological quality of foods and to evaluate antibiotic susceptibility of biofilm (13). Recently, a different specific reagent, containing resazurin has been employed to count non-fermenting bacteria as *Pseudomonas aeruginosa*. (36, 37). The main disadvantage relies on the difficulty in applying BTA for the evaluation of multispecies biofilm.

Genetic assays

8) **PCR; qRT-PCR**. Polymerase chain reaction (**PCR**) is widely used as diagnostic method. In biofilm, this method allows to identify efficiently the presence of specific genetic sequences related to individual bacterial species. Nevertheless, PCR as such, is not suitable for quantitative studies of biofilm and, as amplifying indifferently DNA of both viable and dead cells cannot be used for enumeration of

living cells. Moreover, due to its high sensitivity, false positive results can be expected from natural contamination.

To overcome these problems, "Real Time Quantitative-Reverse Transcription-PCR" (**qRT-PCR**) has been adopted. The qRT-PCR is one of the most powerful and sensitive gene analysis techniques available at now. While in traditional PCR analysis, results are collected at the end of the reaction, during qRT-PCR, the fluorescent signal is measured in real time at each amplification cycle and is directly proportional to the number of amplicons generated. Moreover, as PCR amplifies all DNA present in the sample, in qRT-PCR bacterial mRNA, showing short halflife, has been proposed as a promising indicator of cell viability (38), therefore qRT-PCR has been applied not only to detect but also to quantify a specific microorganism in Biofilm (39-41). Drawbacks of this method are related to sample preparation that must be free from contaminants and/or PCR inhibitors and to the choice of the primers sequence to overcome the amplification of gene sequence not functional for the purposes of the investigation. Other limitations of this method are due to the high costs and the difficulty of execution, requiring expensive scientific equipment and skilled technical staff.

9) **FISH**. fluorescence *in situ* Hybridization (**FISH**) technique is a genetic approach using oligonucleotide probes labelled with fluorescent dyes. These probes can be specifically designed to bind rRNA, particularly abundant in viable cells or to bind a specific molecule representative of a specific target of interest. Thanks to the genetic approach, this method can also be adopted for studies on multispecies biofilm. Worthy of note is that FISH can detect viable but not cultivable bacteria or bacteria with low metabolism (dormient) in biofilm. Combining FISH technique with Confocal laser scanning microscopy (see below) it is possible the identification and topographical visualization of different species in a multispecies biofilm. The principal limits are related to the complex preparation procedure and to the fact that the technique is time consuming and expensive. (42-44). Moreover this technique provides high quality but semiquantitative results.

Physical assays

10) **MS; DESI**. Mass spectrometry (**MS**) is a powerful analytical technique used to quantify known materials and to reveal chemical properties of different molecules. In nutshell, in this method, the substance to be studied is crossed by a beam of electrons so that all molecules are ionized with the production of gaseous ions. Ions are then separated in the mass spectrometer and are characterized by their mass/charge ratios and relative abundances. The data furnish a mass spectrum typical of each compound since it is directly related to its chemical structure. This technique has both qualitative and quantitative chance being able to identify and quantify unknown compounds. In biology MS plays a key role for analytical studies of metabolome. Taking into account the specific gene expression and post-translational modification in biofilm, MS can be considered an interesting approach to study bacterial biofilm. However, many steps in MS are highly invasive for the sample: high vacuum environment, aggressive chemical solvent etc. To overcome this problem, the Desorption-Electro-Spray-Ionization (**DESI**) assay has been proposed. DESI spectrometry analysis is carried out at atmospheric pressure and the sample is maintained under ambient conditions (45, 46). This characteristic allows direct and

non-destructive analysis of complex samples allowing chemical characterization of microbial biofilm in different growth state and conditions (47). However, MS and DESI methods are expensive and time consuming methods and require advanced equipment and skilled personnel.

11) **CLSM**. Confocal Laser Scanning Microscopy (**CLSM**) is an optical microscope equipped with a laser beam, particularly useful in biology and life sciences to study thick samples. Confocal laser scanning microscope technology make it possible to scan a thick biological sample, e.g. a microbial biofilm, by processing images, line by line, in X, Y and Z axes. Biological samples are often stained with specific fluorescent dye so that the fluorescent light from the illuminated spot is collected into the objective and transformed by a photodiode in electrical signal processed by a computer. The optical reconstruction of all the pixel information was assembled yielding a high contrast and high resolution three-dimensional image. This technique has been widely used in the study of biofilm, especially to study EPS components (48-50). The main limitations of this method are that CLSM allows only a semiquantitative investigation and that only few fluorescent stains can be employed simultaneously showing just a couple of component in the same image. These limits can be solved by coupling CLSM with other imaging techniques (see below).

12) **CRM (Confocal-RAMAN Microscopy)**. In Raman spectroscopy an electromagnetic laser beam with known wavelength hits the sample to be analyzed. Measuring its scattered radiation and the shift in energy, information on the chemical characteristics of the sample can be derived (51). Using Raman spectroscopy, we can acquire information on chemical fingerprint of different biofilms. However, a deep investigation inside thickness of the biofilm is a difficult challenge. Recently, combining Raman and CLSM (**CRM)**, non-invasive studies on microbial biofilm composition have been carried out (51-55). The main limitation is related to the set up of experimental condition to achieve high quality signals.

13) **EM**. Electron microscopy (**EM**) techniques take advantage of the higher resolution allowed by the use of an electron beam, i.e., of short-wavelength and high-energy radiation. In practice, magnification can be achieved from the one typical of optical microscopy to that needed to resolve nanometer details. Transmission electron microscopy (**TEM**) shows unique capability for the imaging of the inner of biofilms and intracellular features, but requires the sample to be prepared as ultra-thin slices (56-58). Conversely, scanning electron microscopy (**SEM**) has been widely used to visualize the surface of microcolonies as well as old biofilm (59-61). Moreover, SEM can be used in synergy with focused ion beam (**FIB**), the latter enabling the milling of selected areas of the sample, to investigate the inner of biofilms by removing the exposed surface layers and/or cutting cross-sections (59,60). In addition, SEM-FIB and TEM can be supported by energy-dispersive X-ray spectroscopy (**EDX**) to acquire local compositional spectra and maps of bacterial cells and biofilms (56, 59, 62, 63). The main limitation of SEM is the need for tedious samples preparation to dehydrate and make them suitable for vacuum operations. These limitations have been – at least partially – overcome by **cryo-SEM** and environmental SEM (**ESEM**). In the former, the sample is rapidly frozen so that vulnerable bacterial structures are preserved (58,61,64). Conversely, ESEM enabled the imaging in gaseous environment of hydrated and non-conductive bacterial biofilms (61, 64).

14) **XM**. In X-ray microscopy (**XM**) techniques, the sample is illuminated with a soft X-ray focused radiation, either mono- or poly-chromatic. Different XMs have been proved to enable high-resolution imaging and compositional mapping of biological samples without any specific preparation and with reduced radiation damage with respect to EM. In particular, scanning transmission X-ray microscopy (**STXM**) has been widely used to investigate the composition of bacterial cells and biofilms with nominal resolution of 25 nm (59, 65-69). STXM may also operate in synergy with other X-ray based methods, such as X-ray fluorescence. In particular, these two techniques have been recently used to detect arsenic in Fe(II)-oxidizing freshwater bacteria (70). Clearly, the need for complex and expensive instrumentations represents the major limit to the diffusion of XM techniques.

15) **SPM**. In scanning probe microscopy (**SPM**) the topography of the sample is reconstructed analyzing the signal from a sharp probe with nanometrical dimension, which scans the sample in close vicinity of its surface. Among the SPM techniques, atomic force microscopy (**AFM**) is the most widely diffused for the characterization of microbiological samples like bacterial cells and biofilms. AFM enables high resolution down to nanometer scale, nondestructive analysis, operations both in air and in water, and does not require for any specific sample preparation. Due to these characteristics, AFM has been used to visualize the surface of bacteria and biofilm (71-74), as well as to monitor (also in real time) the processes connected to the bacterial multiplication (75), EPS production (37, 76) and predation, e.g., by *Bdellovibrio bacteriovorus* and *Escherichia coli* (77, 78). In addition, the AFM probe can be used to exert ultra-low loads on the sample surface, thus enabling the quantitative measurement and mapping of biofilm elasticity (72, 76, 79). The adhesion between the AFM tip and the sample can be measured and mapped using both standard and functionalized tips: using the former, a-specific adhesion sites can be identified on the surface of bacteria (72, 80, 81); using the latter, molecular recognition can be performed (71, 82, 83). In addition, the adhesion properties of bacteria can be quantitatively studied by using the AFM tip to detach bacteria from the substrate (84-86). Finally, tip-enhanced Raman spectroscopy (**TERS**), a SPM-based technique that combines SPM with Raman spectroscopy, enables the single-point acquisition and mapping of Raman signal with the nanometrical lateral resolution of SPM (87, 88). The main limitation of SPM techniques is that only the sample surface and the inner portion immediately close to it can be analyzed. So, the ideal samples for SPM seem to be young bacterial colonies and biofilms, while old biofilms with thickness of tens of microns or more can be hardly studied except for their (near) surface layers.

Conclusion

Biofilm is a very complex biological community that acquires the properties of a multi-cellular organism. Therefore, the study of biofilm requires multiple approaches able to characterize the different aspects of biofilm. This short review reports only the most common methods used for analytical studies of biofilm. Different approaches are applied for the visualization and quantification of biofilm: biochemical, genetic, mass spectrometry and advances microscopic techniques. Each of them shows advantages and disadvantages and allows the evaluation of a peculiar aspect of the biofilm. It is evident, that a method allow-

ing a complete analysis of biofilm does not exist: quantification of EPS, viable bacteria, total viable number, thickness and roughness, elasticity of a specific biofilm require more than a single method. Therefore, the knowledge of the advantages and limitations of the different methods as well as the multidisciplinary expertise of the researchers are necessary pre-requisites allowing the right choice of the methodologies to be used.

In the next future, the new "lab-on-chip" technologies, able to measure multiple physical and chemical properties simultaneously, may represent a further interesting opportunity for future studies on microbial biofilm.

Riassunto

Tecniche quantitative per lo studio di biofilm microbici colonizzanti superfici abiotiche: pro e contro delle principali metodiche attualmente in uso

La tendenza dei batteri a sviluppare biofilm è una realtà ampiamente diffusa in natura. L'elevato numero di casi di infezioni correlate alla presenza di biofilm batterici rappresenta una problematica ben nota e una sfida aperta in campo medico, specialmente quando biofilm microbici colonizzano dispositivi medici quali protesi e biomateriali. E' necessario quindi disporre di tecniche affidabili per lo studio di questo fenomeno. Tuttavia, sebbene i biofilm microbici e le infezioni ad esso correlate rappresentino un problema aperto sia nel campo della microbiologia che dell'igiene, la complessa organizzazione strutturale e biologica dei vari biofilm ne ha reso difficile lo studio, tanto che ad oggi non esiste un metodo standard di riferimento. Diversi metodi sono stati impiegati nello studio qualitativo e quantitativo dei biofilm, ciascuno dei quali si è dimostrato utile per stimare un particolare aspetto della comunità microbica sviluppatasi in biofilm. In questa review, vengono descritti, valutandone sia i vantaggi che i limiti, quindici metodiche diverse per l'analisi qualitativa e quantitativa di biofilm colonizzanti substrati abiotici, quali ad esempio i dispositivi medici, le protesi o le superfici per la lavorazione di prodotti alimentari. Alcune delle metodiche risultano più idonee per lo studio della matrice esopolisaccaridica, mentre altre hanno come target diagnostico la presenza delle cellule batteriche nel biofilm stesso. In particolare, sono descritti metodi colorimetrici sia per lo studio della matrice (cristal violetto; 1,9-dimethyl methylen blue; fluorescein-di-acetate) che delle cellule batteriche vitali (LIVE/DEAD BacLight, BioTimer Assay, resazurina, sali di tetrazolio) o metodi per lo studio delle popolazioni microbiche in biofilm tramite un approccio genetico (PCR e FISH). Infine, una sezione specifica è dedicata alle più recenti tecniche di indagine microscopica (spettrometria di massa; confocal laser scanning microscopy; spettroscopia Raman e microscopie elettroniche). Concludendo, si evidenzia come, a causa della sua complessità, uno studio esaustivo del biofilm microbico richieda un approccio multidisciplinare combinando tecniche e competenze sperimentali fra loro complementari.

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