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SPECTROSCOPY AND MICROSCOPY EVIDENCES OF CANNIBALISM EVENTS IN THE *IN-SITU* ASSESSMENT OF LONG-TERM BIOFILM-ANTIMICROBIAL INTERACTIONS: A NEW VIEWPOINT ON ANTIMICROBIAL RESISTANCE?

M.C. Sportelli[1], R. Quarto[1], E, Tütüncü[2], R.A. Picca[1], A. Valentini[3], H. Barth[4], B. Mizaikoff[2], C. Kranz[2], N. Cioffi[1]

[1]*Chemistry Department, University of Bari Aldo Moro, Bari, Italy*
[2]*Institute of Analytical and Bioanalytical Chemistry, Ulm University, Ulm, Germany*
[3]*Physics Department, University of Bari Aldo Moro, Bari, Italy*
[4]*Institute of Pharmacology and Toxicology, University of Ulm Medical Center, Ulm, Germany*

Differently from planktonic state, bacterial biofilms can be considered the major cause of serious health issues in human medicine and food industry, due to their ensuing resistance against harsh conditions and pharmacological treatments [1]. Biofilms are defined as three-dimensional structures comprising cells rooted in a matrix build up by extracellular polymeric substances (EPS) [2]. This intricate system is dynamic, and its structure is strongly influenced by a plethora of parameters such as biofilm oldness and exterior conditions, like nutrients deficiency, and/or attack of various agents [3]. Biofilm formation is a chemically complex multi-stage process, in which bacteria transmute from planktonic form to sessile mode; the interaction that occurs between bacteria in a biofilm and its surrounding environment can largely determine the extent and the composition of the bacterial colonies. Moreover, bacterial colonies can activate survival strategies when subjected to stress conditions (i.e. presence of antimicrobial agents). Occasionally, cannibalistic behavior may occur [4], which involves the secretion of cannibalism toxins, which can kill sensitive bacteria of the same colony. Thus, generated lysed cells may then provide nutrients for the cannibals. Several new methodologies have been recently developed for or adapted to biofilm formation studies aiming at a comprehensive understanding of biofilm physiology, structure and composition, to find novel and more effective eradication strategies. Among them, Fourier transform infrared spectroscopy (FTIR) –especially in attenuated total reflectance (IR-ATR) mode – may provide *in-situ* and real time monitoring of biofilm lifecycles with molecularly specific details on the first attachment stages. Biofilm growth may occur during extended timespans; therefore, not only long-term effective bacterial treatments are required, but also appropriate analytical methods, to study the long-term behavior of biofilms. Due to the well-known biofilm antibiotic resistance [5], it is nowadays of increasing interest to develop innovative methodologies for the treatment of biofilm-related infections. In our laboratories, these new strategies mainly exploit inorganic nanoparticles (NPs) with antimicrobial properties, such as ZnONPs, AgNPs, CuNPs, etc. [6]. In this work, AgNPs have been embedded in various polymeric matrices (fluoropolymer, polyethylene oxide, polylactic acid, etc.), which allowed for the preparation of organic thin films with tunable loading of inorganic (i.e. antimicrobial) NPs. First, composite morphology was investigated by electron

microscopies and atomic force microscopy (AFM); then, x-ray photoelectron spectroscopy was employed to gather information about surface chemical composition. Kinetics of antimicrobial ion release were also investigated, and nanocomposite behavior was correlated for the first time with its swelling properties and 3D modification after immersion in liquid medium for a long-time span. The investigation of biofilm growth and inhibition by the antimicrobial material has involved both imaging (AFM and optical microscopies), and spectroscopic (IR-ATR analysis) techniques. In particular, the infrared spectroscopic analysis of the biofilm allowed gathering molecular information on the biofilm development and behavior during long-term contact with an antimicrobial surface. A detailed comparison between the IR data obtained at differently modified ZnSe crystal surfaces allowed for a decoupling of the effect of antimicrobial ionic release from the thin film, from the direct contact between bacteria and antimicrobial thin film. We demonstrated that Ag^+ ions, released in the surrounding solution, exert a biocide action: ionic release can slow down surface colonization and eradicate the bacterial biofilm within few hours. We also demonstrated that bacterial cells can re-colonize on dead biomass, when the latter is thick enough to prevent a direct interaction with the antimicrobial surface.

In summary, this study represents an excellent foundation for investigating the contact between nanoantimicrobials and nascent biofilms over extended periods of time. Moreover, it paves the way to further studies on the long-term exposure of biofilms towards antimicrobial surfaces, and it could be useful also for a better understanding of the early stages leading to antimicrobial resistance phenomena [7].

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