Topical: Considerations for Use of Low-Temperature Gas Plasmas for Mitigation of Biofilms in Microgravity Environments

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Introduction

NASA's future space exploration plans call for returning to the Moon and then travelling to Mars. Under the Artemis program, NASA in collaboration with commercial partners, will build increasingly larger human landers, not only to return humans to Moon, but also for establishing a future lunar base that can support human presence for months at a time. The future Moon base will serve as an ideal testing ground for developing and maturing technologies for deep-space exploration.

The long-duration nature of the missions potentially introduces a significant challenge, in form of microbial biofilms, that must be addressed to achieve mission success. Bacteria are ubiquitous in nature and have been well documented in space-craft environments, especially ones that support human habitation over prolonged periods [1–4]. Bacterial biofilms not only impact human health, but can also lead to bio-corrosion of space hardware and life-support systems [5]. To date, these challenges have been answered for the missions to ISS by continuously monitoring the affected systems and by delivering needed spare parts for maintaining system function. However, swapping out affected components with new parts may not be as simple for the farther located lunar bases and nearly impossible for a Mars mission. Achieving success in long-term missions thus requires developing technologies for mitigation of biofilms in microgravity environments. While there are numerous methods tested and used in attempts to eliminate or control biofilm growth there is no solution sufficient for all biofilm types. Earth-based experiments with cold temperature gas plasmas show highly promising results in controlling biofilm growth of diverse microbial species. We discuss possible application of low-temperature gas plasma for mitigation of biofilms in microgravity to prevent surfaces deterioration and disruption of functionality of spacecraft details. We also address current knowledge gaps about cold plasma's biocidal action and biofilms in microgravity and other considerations and concerns for use of low-temperature plasma in space exploration.

Biofilm as ubiquitous microbial lifestyle of microbes

Biofilms are structural communities of microbial cells that form on air-surface interfaces with solid, semi-solid, and liquid materials. In these communities, microbes are usually differentiated into distinct functional subpopulations and embedded into an extracellular polymeric substances (EPS) matrix consisting of polysaccharides, proteins, lipids, DNA, surfactants and others [6,7]. Due to the altered metabolic state of these cells and the protective effects of the matrix, the biofilms are notoriously difficult to eliminate once they are formed [6,8]. These difficulties apply to biochemical, chemical and physical methods for microbe elimination, such as for example antibiotic treatments, ultraviolet radiation, or mechanical scraping.

Nucleation of biofilms is a complex process that is regulated by quorum sensing and stress response mechanisms [7]. This ultimately starts with attachment of a microbial cell to the surface through adhesion between extracellular microbial structures and surface chemical moieties. This initial attachment is a critical step that is slow in comparison with further biofilm growth and maturation steps. Attachment of microbes strongly depends on the surface type: its architecture and topology, temperature, hydrophobicity, functional groups, charge and chemical composition in general. Biofilms on abiotic and biotic surfaces rely on different adhesion properties and only few are investigated to a great level of detail. As biofilms grow and mature, they undergo

significant changes that include cell type differentiation and formation of robust polymeric matrix around the cells that differs amongst species [6].

Biofilms in microgravity environments

Spaces occupied by humans are microbe-rich, due to shedding of normal microbiome organisms that can contain opportunistic pathogens, during everyday activities. This is true even in extreme extraterrestrial microgravity environments such as the International Space Station (ISS) and the Soviet/ Russian Salyut and Mir stations [3,4,9,10]. Surveys of the ISS have found in excess of 300 on-board microbial species including bacteria and fungi capable of causing human disease [4,11]. Surface and systems sampling in both the ISS and the Salyut/Mir stations has revealed biofilms in a wide variety of settings. A partial list includes rubber seals, navigation and viewing windows, thermal control radiators, water recycling systems, and even equipment shielded behind panels. Possible consequences include degrading or corroding materials, clogging of pipes intended for fluid flow, and lowering the efficiency or even disrupting the function of systems that are required for life support. Furthermore, these microgravity biofilms include pathogens capable of causing human disease. Thus, control of biofilm contamination in the microgravity environment is an important objective.

The majority of detected microgravity biofilms, similarly to the Earth environments, are multispecies communities. Recent studies use repositories of ISS-derived bacteria to model behavior of biofilm formers under normal gravity [12] which sets the stage for more details and realistic investigations in-flight. Formation of biofilms under altered gravity conditions has been investigated using several model systems over the last two decades [13–18]. The results of these studies imply that biofilms are growing differently in absence of normal gravity with some indicating the increased ability of pathogens to form biofilms. Examples of the observed changes include altered motility, better adhesion, thicker biofilm structures, and increased production of EPS [13,19–21]. Nevertheless, the studies are still sparse and some results are controversial due to the complexities of the in-flight experiments and microgravity simulation on Earth, in particular due to the long timescale required for biofilm nucleation and development under controlled conditions.

Effects of biofilms on materials

The growth of biofilms on material surfaces could have detrimental effect to both structural and functional properties of the materials [22]. For example, bio-corrosion in well studied phenomenon and can lead to pitting and crack formation in materials such as stainless steel [23] and aluminum [24] which are commonly used in space applications. Presence of biofilms can induce cathodic reactions on the metal surfaces that consume hydrogen and produce sulfate precipitates, or lead to generation of acidic conditions that erode the metal [25]. Bio-degradation is also an issue for plastic materials. Microbial depolymerases produced within biofilms can erode plastic materials [26].

Biofilms can also lead to functional changes of materials. An example is polyimides that are used widely in sensitive electronic applications such as controls and communications equipment where they are used as insulating materials. Biofilm formation can drastically alter the

dielectric properties of polyimide insulator films and can even lead to short-circuiting [26]. Another example of functional degradation is hydrodynamic blockages caused by biofilms formed in tubing and valves, that can either lead to increased pressure build-ups or improper sealing leading to equipment failure. Thus, control and mitigation of biofilms is essential.

Effects of low-temperature gas plasmas on surfaces and their interactions with biofilms

Low-temperature plasmas (LPT) can be generally defined as partially ionized plasmas with chemically reactive environment. LTP affects material surfaces in a multitude of ways depending on the surface material, plasma composition, plasma type, and treatment duration. For the purpose of biological applications, the plasmas used are typically "cold", i.e. at or near room temperature, and non-arcing. Thus, for cold plasmas, the effect on surfaces is predominately chemical based.

For soft materials such as polymers, LTP has been shown to increase the hydrophobicity or hydrophilicity of the surface. The plasma can modify the surface mechanical roughness and the chemical composition through three main pathways: functionalization, polymerization, and etching [27]. Functionalization is when the plasma adds new chemical groups to the material surface. The most common examples are fluorine (e.g. with SF6 of CF4 plasma) and hydrocarbons to improve hydrophobicity of a surface. Polymerization is where the plasma changes the surface chemistry of the material itself, without necessarily adding new species. For example, the hydrophilicity of polymer surfaces can be improved by increasing the number of C=O double bonds via plasma excitation [28]. Lastly, even cold plasma can etch surfaces and thus typically reduce the surface roughness. High aspect ratio micron or smaller features will generate high local electric fields which will cause high currents to collect and thus ablate or melt those features. One of the fields where plasma polymer surface modification has seen great interest is drug delivery, where the "dry" treatments by plasma are useful as they can modify just the surface properties, without changing the bulk structure [29]. For hard surfaces like metals, LTPs have primarily been studied for improving bonding with adhesives or in between layers such as with additive manufacturing. The plasma can remove the oxide layer on the metal surface and increase the surface free energy.

Due to these extensive and diverse changes that can be induced by low-temperature plasmas in different surfaces, the LTP effects were tested and applied to modify microbe-surface interactions. It has been shown that polymeric and metal surfaces can be pretreated to reduce or increase bacterial adhesion, that is the essential first step for any biofilm formation [30–33]. These effects are yet to be tested in microgravity environments that are known to alter multiple related physiological properties of microbial cells [19,34,35]. Therefore, LTP can potentially be useful for diverse approaches for reduction of biofilm formation and can be tested as a "microbial prophylactic" treatment for some of the high-traffic or moist surfaces at the crewed build environments in space (such as ISS or Moon settlement).

Biofilms elimination by low-temperature gas plasmas

It has been shown that LTP can successfully eliminate planktonic microbial cultures of diverse microbes. This included broad range of gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella spp*, etc), gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes, Enterococcus faecalis, Weissella confusa,* etc.) and

fungi *(Cryptococcus and Candida spp)* [36–43]. Interestingly, LPTs were also effective in eliminating highly resistant bacterial endospores of *B. subtilis*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus atrophaeus*, and *Geobacillus stearothermophilus* [44–46]. This bactericidal action is attributed to the collective effect of chemical constituents such as hydroxyl radical, peroxynitrate, nitrate, hydrogen peroxide, and others as well as physical constituents including ultraviolet, electric fields, charged particles generated by plasma [39,47]. Following the success of treating bacteria in suspension, multiple studies started assessing how well cold plasma can reduce survival of bacteria in biofilm structures or inhibit biofilm growth. The results of those studies indicate that different plasma setups work well to achieve strong reduction of viable cell counts in surface biofilms as confirmed by colony forming units plating (CFU) or live/dead staining microscopy methods. While biofilms of different species have different degrees of susceptibility, reduction of live cells was shown for such divergent organisms as *S. aureus, E. coli, B. subtilis, W. confusa, E. faecalis, P. aeruginosa* and others, and these include clinically relevant pathogens and environmentally important species. The range of species include many organisms that were identified in the ISS-derived bacterial isolations and in compositional surveys of spacecraft microbial communities [3,4,9]. There are initial studies that compare susceptibility of surface biofilms at different stages of their formation: pre-biofilm state of recently adhered cells to formed mature biofilms [40].

As mentioned above, most biofilms found in built and natural environments are mixed, polymicrobial biofilms often resulting in more robust and protected environment in such biofilms. Nevertheless, several studies address effects of cold plasmas on polymicrobial biofilms. For example, dental plaque models with *Streptococcus mutans, Streptococcus sanguinis* and *Streptococcus gordonii* mixtures [48] show significant but not complete reduction in biofilm viability (reviewed in Rao et al., 2020). Reassuringly the LTP effects are being seeing on biofilms grown on diverse surface materials that include semi-solid and solid; porous and non-porous surfaces.

Furthermore, LTP can "inactivate" fungal and dental biofilms. However, close reading and examination of data suggest that viability is greatly reduced but not zero and that physical presence of the biofilm may not be eliminated. It is unclear whether absence of mechanical disruption of the biofilm layer and leftover biomaterials continue affecting surface properties and processes (such as biocorrosion or hydrodynamic characteristics change).

Many current methods of biofilm disruption rely on mechanical or biochemical dispersion of biofilm communities. These methods can be a double-edged sword allowing a high number of viable bacteria to disperse into the new environments [50]. Upon incomplete biofilm disruption and cell killing, the residual biofilm can serve as an easier adhesion surface. It was shown in analyses of dental biofilms that partly disrupted biofilms regrow with a faster rate in comparison with *de novo* biofilm formation [51].

Use of low-temperature plasmas in microgravity environments

There are multiple currently employed at ISS disinfection protocols and some of those might be unattainable for long-duration missions without consistent ground support and resupply. Therefore, renewable methods for sterilization and sanitation are required. LTP might in theory serve as a "renewable" strategy but the energy costs are still unknown. In addition to this, successful use of LTPs for mitigation of microgravity biofilms in the ISS may depend on several

variables including compatibility, penetration, and toxicity. First, are materials to be plasma-treated compatible with such treatment? Does plasma treatment alter or damage the properties of the material being treated? Does such alteration or damage in turn alter the function of the material in question?

Second, will the plasma penetrate into all the nooks, crannies, and crevices of biofilm-contaminated electronic equipment, radiators, etc.? Liquid biocides may not penetrate well due to issues such as viscosity and surface tension, but plasmas may be expected to have more gas-like behavior and device-contact-free. Length, shape, and spreading of the plasma plume also might be important. While in this work we mainly discussed biofilms formed by different or mixed microorganisms, we have to mention that biofilms differ strongly based on what surfaces they form. In addition to obvious differences based on surface material's type and architecture, it is often overlooked that biofilms formed on dry, moist, or submerged surfaces vary drastically as well. Therefore, application of cold temperature plasma should be mainly considered for solid/air, semi-solid/air interfaces, while other methods might be more efficient for solid/liquid and semi-solid/liquid biofilms.

Third, will frequent and repeated operator exposure to plasma-generated toxic free radicals, UV radiation, etc., be a problem? It might be expected that biofilm mitigation on the ISS would involve frequent plasma treatment over large surface areas. Does plasma treatment at least partially vaporize biofilms? If so, are the vaporization products toxic or allergenic? Should protective gear be considered? There are several projects underway testing the potential LTP for disinfection of ISS-grown food plants and for sterilization of astronauts and equipment in a so-called 'sterilization shower'[52–55]. The results of these ongoing studies will contribute to understanding the safety questions around using plasma in the closed environment of a spacecraft. So far, a study showed that cold argon plasma is not toxic for primary normal human dermal keratinocytes and primary normal human dermal fibroblasts and does not increase mutagenesis rate in tested V79 Chinese hamster cell line [56,57]. Moreover, some novel studies appear to describe that cold plasma might have positive effects on multicellular organisms and initial data show speeding up the seed germination and wound healing [57–59]. While extensive basic or clinical studies are still lacking, it seems promising as no strong adverse effects yet reported for current suggested uses. Fortunately, additional investigation of these problem areas can be done in a terrestrial environment.

Cold gas plasmas have already been used for decontamination of spacecraft equipment on Earth and in studies in flight, therefore, it is likely that it is feasible to obtain at least some low temperature gas plasmas during a spaceflight [52,53]. Several LTP generating devices were created for proposed commercial use for different applications including some portable versions down to about a foot in their longest dimension. This opens up the possibility to do in-flight or perhaps even in CubeSats format experiments to address the feasibility of use of LTP for biofilm mitigation in microgravity [60,61].

Conclusions

As microgravity biofilms are disruptive to the existing and planned space missions, research on novel strategies of biofilm growth prevention and biofilm disruption is needed. With the high promise that low-temperature gas plasmas showed for disinfecting and biofilm

elimination tests under normal gravity, we need to test the behavior and efficiency of this treatment in the conditions of a space flight.

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