At the Interface of Materials and Microbiology: A Call for the Development of Standardized Approaches to Assay Biomaterial-Biofilm Interactions

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A vexing problem with many dental restorative materials, such as methacrylate-based resin composites, is a higher than desirable failure rate and modest service life (Opdam et al. 2014; Demarco et al. 2017). The reasons for this are certainly multifactorial, but increasing evidence suggests that biomaterial interactions with biofilm-dwelling microbes and various host components are likely to play much larger roles than previously appreciated. Ultimately, these interactions can challenge the integrity of dental materials as well as promote various dental diseases such as secondary caries, especially at the interface between the biofilm and the restored tooth. As such, there is broad interest in the field to model biomaterial-biofilm interactions to assess the efficacy of novel material formulations or antimicrobial agents.

While there are many widely accepted standardized methods to evaluate the physical properties and biocompatibility of dental materials (de Souza Costa et al. 2014; Ferracane et al. 2017; Ilie et al. 2017), no similar consensus exists for the study of dental biomaterial-biofilm interactions. Consequently, dental materials are commonly assayed using vastly disparate biofilm growth conditions coupled with various approaches to examine biofilm viability. There is even little consensus in the field regarding the organisms that should be tested in *in vitro* models. Currently, most studies have employed single-species biofilms of *Streptococcus mutans*, while others have employed a variety of multispecies models containing a wide assortment of organisms. This makes it difficult at best to effectively compare outcomes between studies and can easily lead to dubious conclusions. It is also worth noting that the impact of biomaterials on the biophysical properties, structure, and composition of biofilms is rarely published, yet these are also potentially useful predictors of clinical efficacy. Likewise, *in vivo* biofilm challenge studies are severely lacking. In the following section, we highlight some of the major limitations identified in current research on biomaterial-biofilm interactions and call for a greater consensus of standardized testing techniques and conditions.

Currently, there is active debate in the research community whether single-species or multispecies biofilm models are appropriate. Ultimately, the model system employed should really depend on the goals of the study (Salli and Ouwehand 2015). For instance, high-throughput screening assays incorporating biofilms are likely to benefit most from highly reductionist single-species model systems. A common example involves

the use of single-species biofilms of *S. mutans* strain UA159. However, it should be recognized that any particular strain of bacteria is not representative of the species as a whole. Clinical isolates of *S. mutans* naturally exhibit a surprisingly broad diversity of important phenotypes, including major differences in growth rates, biofilm formation, acidogenicity/aciduricity, oxidative stress tolerance, and so on (Palmer et al. 2013; Liu et al. 2018). At best, UA159 should be viewed as an approximation of a typical cariogenic *S. mutans* isolate. For more detailed studies of biomaterial-biofilm interactions, complex multispecies models are likely to provide more accurate approximations of clinically relevant biofilms and thus the potential clinical efficacy of experimental biomaterials (Fig. A, B). In this case, the relevant biogeographical aspects of experimental biofilms (sub- or supragingival, for example) are critical considerations. These are consequential not only for materials directly exposed to the oral environment but also for those used in other locations, such as endodontic and implant materials. In addition, community interactions within multispecies biofilms have a tremendous impact on the metabolic and gene expression profiles of the individual organisms in the biofilm as well as the metabolites they excrete (Kreth et al. 2009; Marsh and Zaura 2017; Lamont et al. 2018). Furthermore, single- and multispecies biofilms also exhibit striking differences in their

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Figure. Key aspects of biomaterial-biofilm testing. **(A)** Overview of biofilm models to test biomaterial-biofilm interactions. The figure can serve as a guide about what kind of biofilm model can be used. Initial screening procedures with newly developed biomaterials might require only simple setups with single-species biofilms. More elaborate setups with increased clinical relevance should use multispecies biofilms and eventually intraoral appliances to grow in situ biofilms. **(B)** Species recommendation for biofilm models. This list can be used as guidance and contains examples of species most commonly associated with the respective disease condition. **Aggregatibacter actinomycetemcomitans*.

structural features (Klein et al. 2009; Ferrer and Mira 2016; Mark Welch et al. 2016). One could imagine how these fundamental differences between single- and multispecies biofilms could easily result in 2 vastly different interactions with the same materials. Similarly, there has yet to be a substantive debate in the field about the preferred species composition used in multispecies models, which is another key area where additional discussion is warranted.

Biofilm formation is a multistep developmental process requiring the genetic reprogramming of planktonic bacteria to adapt to a sessile biofilm state (Kolenbrander et al. 2010; Arweiler and Netuschil 2016). To replicate this process in vitro, initial biofilm inocula should consist of highly dilute cell cultures ($\leq 1 \times 10^6$ colony-forming units [CFU]/mL). This is particularly important since certain hallmarks of biofilm formation like the production of the extracellular matrix occur later in the developmental process (Kolenbrander et al. 2010; Arweiler and Netuschil 2016). Accordingly, the concentration of bacteria used in an initial inoculum can have a profound impact on the biophysical characteristics of the resulting biofilm and thus affect its behavior when grown on a test substrate. Likewise, biofilm behaviors can vary tremendously depending on whether they are formed in a static or flowing environment (Song et al. 2017). The most clinically relevant in vitro assay system would incorporate a constant flow of ~ 0.5 mL/min to mimic saliva production as well as nutrient pulsing to replicate the normal eating habits of the host.

Currently, there is an abundance of dental materials research focused on the development of antimicrobial materials to

inhibit biofilm formation. To analyze the antimicrobial efficacy of such materials, studies often employ microscopic techniques that incorporate fluorescent bacterial viability dyes like the BacLight LIVE/DEAD stain. A common misconception is that these dyes can be used as quantitative measures of cell death (Netuschil et al. 2014). Since the membrane-impermeable component of the stain (propidium iodide [PI]) can only cross compromised membranes, PI-stained cells are often falsely interpreted as indicative of dead cells. While these cells may in fact be dead, they are also just as likely to be injured or even starving viable cells. These cells will also exhibit PI staining, yet they might be easily recoverable and begin dividing if given the opportunity. An alternative assay that measures metabolic activity or direct viable cell counts should be employed as an adjunctive to independently verify the observed results.

In our view, the development of more standardized approaches to study biomaterial-biofilm interactions (a visual guide is presented in the Fig.) is likely to both simplify and improve outcome assessments of experimental and commercial materials. It is worth noting that similar issues are also being discussed for biofilm research outside of dentistry (Coenye et al. 2018). Thus, this is emblematic of the broader research trends occurring within the field. For dental materials research, we suggest the following to improve the standardization of testing procedures:

1. Materials should be assayed using biofilm models that simulate relevant disease conditions. For example, materials developed to reduce the prevalence of secondary

caries should mimic cariogenic conditions and minimally consist of *S. mutans* UA159 biofilms grown in the presence of 0.5% to 1% (wt/vol) sucrose. Depending on the goals of the assay, additional layers of complexity can be added to the model system to further increase its clinical relevance (Fig. A). Materials designed for use with other dental diseases, such as periodontitis, should employ a distinct set of relevant organisms (Fig. B).

2. Biofilms should be inoculated using low cell densities and allowed to develop to maturity to ensure that all biofilm developmental steps are captured. This time-frame can vary tremendously depending on the species employed. For fast-growing species like the oral streptococci, a mature biofilm can be obtained in ≥ 16 h, whereas fastidious organisms like *Porphyromonas gingivalis* might take several days.
3. Approaches used for antimicrobial testing of dental materials should be optimized and validated for each unique type of biofilm assay system prior to its implementation. For example, to achieve reproducible and accurate CFU measurements of single- versus multi-species biofilms, it may be necessary to employ 2 vastly different biofilm disruption protocols.
4. The biophysical properties of biofilms grown on dental materials should be assessed. Does the material composition affect biofilm attachment avidity and/or shear resistance?
5. Claims about the potential clinical efficacy of novel materials should be supported by more complex biofilm assay systems, such as microcosm biofilms or intraoral appliance biofilms.
6. Ideally, materials destined for industrial production or even licensing would first be tested in an independent facility. Independent verification of key material properties, such as its antimicrobial activity or biophysical characteristics, would greatly increase credibility, identify potential areas for improvement, and increase the likelihood of actual clinical efficacy.

With the recent explosion of research focused on developing novel materials designed to interface with bacterial biofilms, it is now an opportune moment to have frank discussions about how these materials should be assessed. Perhaps a symposium at the annual session of the International Association for Dental Research (IADR) or annual meeting of the Academy of Dental Materials (ADM) would be the appropriate venues to initiate such a discussion to ensure that a broad cross section of stakeholders can contribute to the conversation.

Author Contributions

J. Kreth, J.L. Ferracane, C.S. Pfeifer, S. Khajotia, J. Merritt, contributed to conception and design, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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