## REVIEW

Development of standard protocols for biofilm-biomaterial interface testing

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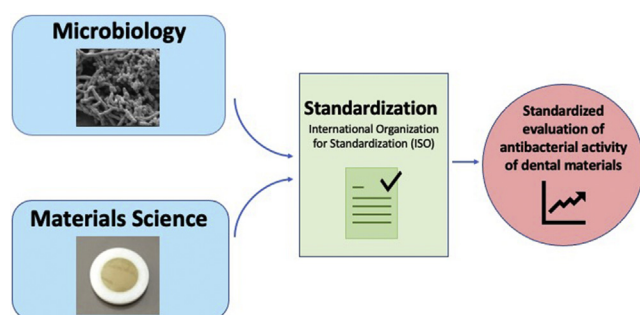
Abstract

The oral biofilm is associated with the most common oral diseases such as caries, periodontitis, and peri-implantitis. It is also linked to failures of dental treatment approaches (eg, direct or indirect restorations because of adjacent caries). Therefore, the development of materials with antibacterial properties is desirable. However, the design of meaningful tests to confirm such properties faces severe problems because of the complexity of the interaction of materials with the oral biofilm. Furthermore, owing to practical reasons, such tests need to be performed in vitro. In contrast, there is a need for predictive data that are comparable between different laboratories. Therefore, standardization of such tests has been advocated. The International Organization for Standardization (ISO) with its Technical Committee 106–Dentistry may be the relevant platform for this purpose. A standard (ISO 3990) is being developed for testing the antibacterial properties of dental restorative materials. This standard defines basic requirements for sample preparation, selection of bacterial strains, test methods and assessment, and reporting of results. It is considered to be the first step, and regular revisions are planned as new scientific evidence emerges. The support of the scientific communities providing multidisciplinary input is needed.

Key Words. Antibacterial; dental material; standardization; ISO standards; microbiology.

Introduction

Resin-based composites were one of the prominent research topics in dental material science in the past decade.[1] An estimated 800 million resin composite restorations were placed worldwide in 2015, further emphasizing the importance of this dental material.[2] After placement, dental materials are generally exposed to a diverse set of environmental conditions. This includes exposure to the abundant oral microbiota. Oral microbes are specialists in attaching to oral surfaces,[3] including dental restorative materials.[4] Moreover, the most relevant oral diseases such as caries, secondary caries, endodontic infections, periodontitis, and peri-implantitis are microbial biofilm–associated
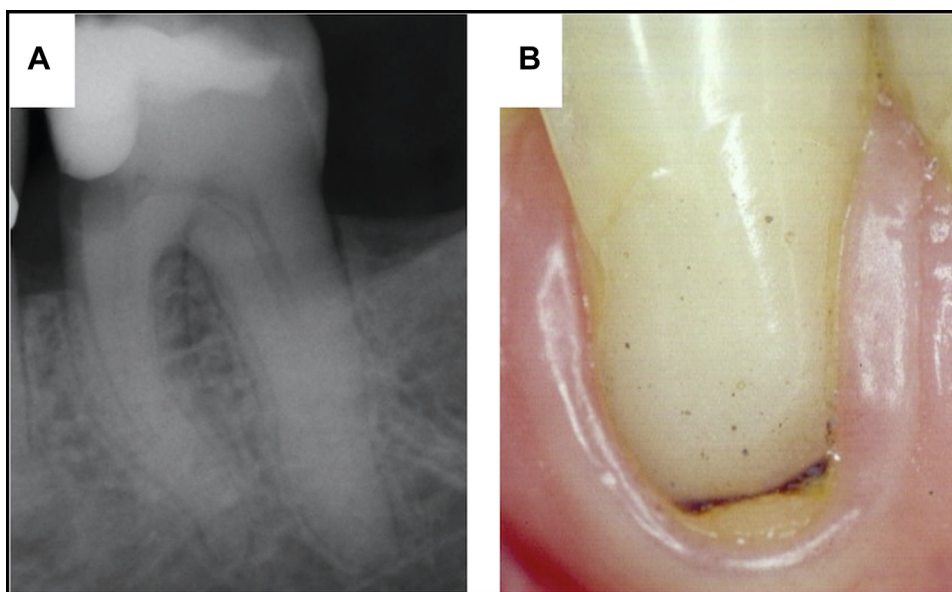
Figure 1 Caries adjacent to restorations. **A.** Radiograph of tooth no. 37, restored with a mesioocclusal composite restoration and caries adjacent to this restoration. **B.** Caries adjacent to the dentin margin of a Class V composite restoration on a tooth no. 34.

diseases.[4-8] It has further been reported that secondary caries is one of the most relevant factors for restoration failures (Figure 1).[9] Therefore, it may be of clinical relevance to develop dental materials that potentially prevent, counteract, or modify biofilm formation.[4,10,11] Since 2018, a number of mainly in vitro studies have been published on the development of novel dental materials claiming antibacterial effects.[4,12-14] In this context, the term bioactive material has been used, which indicates an intended, local and positive effect of such a material on the surrounding biological environment, including the oral microbes. In fact, interfering with the ability of the microbial community to interact with restorative materials is an important goal of bioactive dental material research. However, microbial attachment and biofilm formation can even be detected on modified surfaces designed to prevent attachment.[15] Therefore, it is a valid question to ask how we assess the microbial impact on dental materials?[10,11,14]

In the context of biocompatibility assessment, the interactions of dental materials with the eukaryotic cellular compartment of the oral cavity have been intensively investigated for decades,[16-18] and even legal regulations (eg, US Food and Drug Administration) and standard tests on biocompatibility have been developed to prevent material-related damages to patients, dental personnel, and the environment. However, this has not taken place in a similar way with regard to the interaction of dental materials and oral microbes.[4,10,11]

A major strength of dental material research is the availability of and long-term experience working with standardized testing mechanisms, which provide comparable data sets about the material or product properties and enable characterization of a given material or product.[19]

Some years ago, toxicologic aspects have been integrated into this system, starting with an initiative of the American Dental Association, which published their "Recommendations on standard practices for biological evaluation of dental materials" in 1972.[20,21] These recommendations have been the basis for the International Organization for Standardization (ISO) 7405 (Dentistry—Evaluation of biocompatibility of medical devices used in dentistry) with its last revision in 2018. The need for such standardization was also advocated for in 2019 for evaluating the potential antibacterial effects of dental materials.[10,11] In this context, a symposium was held during the 2021 annual meeting of the International Association for Dental Research, combining experts from oral microbiology, dental materials science, and standardization. In this article, we summarize the presentations of this symposium and aim to lay out the framework for a standards document that addresses the claim of antibacterial activity of dental restorative materials.

Standardization of Antibacterial Activity of Dental Materials: What Should We Consider?

For a standardized in vitro approach to model the biomaterial-biofilm interface, expertise from microbiology as well as materials science is needed. Figure 2 depicts the main aspects of both domains.

Microbiology

Pellicle Formation

It is unlikely that newly placed dental material is directly colonized by oral microbes. As soon as the material is placed
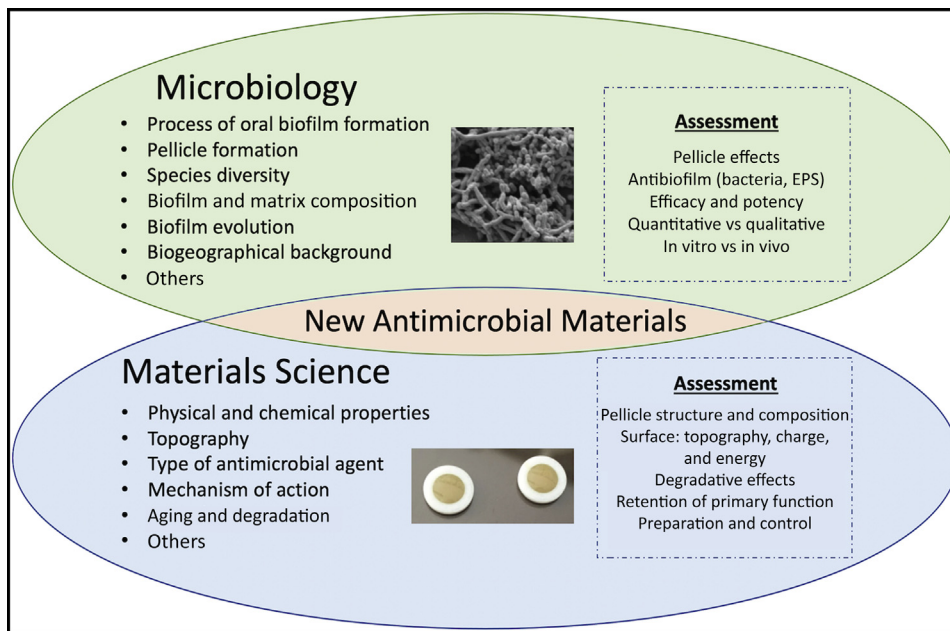
Figure 2 The interface between microbiology and materials science. Main aspects from microbiology and materials science that are crucial for developing a standard on antibacterial activity of dental restorative materials.

intraorally, saliva will cover the material, and a protein film, the so-called acquired salivary pellicle (Figure 3A, B), will form within minutes.[22–25] The acquired salivary pellicle is instrumental in several aspects of tooth surface–environmental interactions, including the presentation of specific proteins facilitating the attachment for oral microbes.[23,26] The pellicle further influences the antibacterial properties of dental materials[27] and should be included in any standard protocol for advanced bioactive material testing of the biomaterial-biofilm interface.[4,22,24]

The process of oral biofilm formation, starting with the formation of an acquired pellicle followed by bacterial colonization and finally ending in the maturation of the biofilm, has been reviewed elsewhere.[7,23,26] This basic principle also holds true for adhesion to dental material surfaces.[4] The challenge for testing potentially antibacterial materials is that they need to be exposed to biofilm formation (Figure 3C, D give examples of a microcosm biofilm on a resin-based composite surface). Biofilm formation itself is difficult to standardize because of the many ways in which biofilms can be grown in vitro (eg, static vs flow; selection of nutrient broth) and is further complicated by the choice of microbes (eg, single vs multispecies; microcosm vs defined community).[30,31] Test systems using solely planktonic cultures may only be used as a prescreening; the respective biofilms could respond differently to the material.[32] The process of biofilm formation is genetically controlled on the species level, thus being a spatial, temporal, and structural development process.[26,33,34] Therefore, biofilm formation cannot be forced but requires each member of the biofilm community to go through the developmental process. This

holds true for single-species biofilms and more complex defined or undefined biofilms directly grown from oral microbiological samples such as saliva or dental plaque.

Species Diversity and Biogeographic Background

A microcosm biofilm formed from subgingival plaque is not reflective of a biofilm grown on a dental material surface such as a dental restorative material, which is usually put on supragingival sites of teeth. Therefore, the selection of bacteria to be used for testing should reflect the anatomic site in which a newly developed dental material will be ultimately placed. Several studies have shown that the biogeographic distribution of species is site specific.[35] The consequence is that the species selection for any testing needs to be defined according to the aim of testing, and no one-serves-it-all selection of bacteria used for testing is possible. Furthermore, careful reporting and interpretation of test results is necessary, including a justification of the selection of the tested bacteria to put the results into the correct clinical perspective.

Biofilm Matrix Composition

Within the biofilm, microorganisms are embedded into a biofilm matrix (extracellular polymeric substances [EPS]), which contains polysaccharides, proteins, lipids, and nucleic acids[5,7] (Figure 3D; yellow arrows). This biofilm matrix is the main prerequisite for the adhesion and bond strength of the biofilm to the substrate; it guarantees mechanical stability and is a diffusion barrier for antibacterial substances.[5,7] Therefore, antibacterial strategies may also consider the EPS as a potential target.[5] However, it should be kept in mind that the composition of the EPS is on the one hand dependent on the
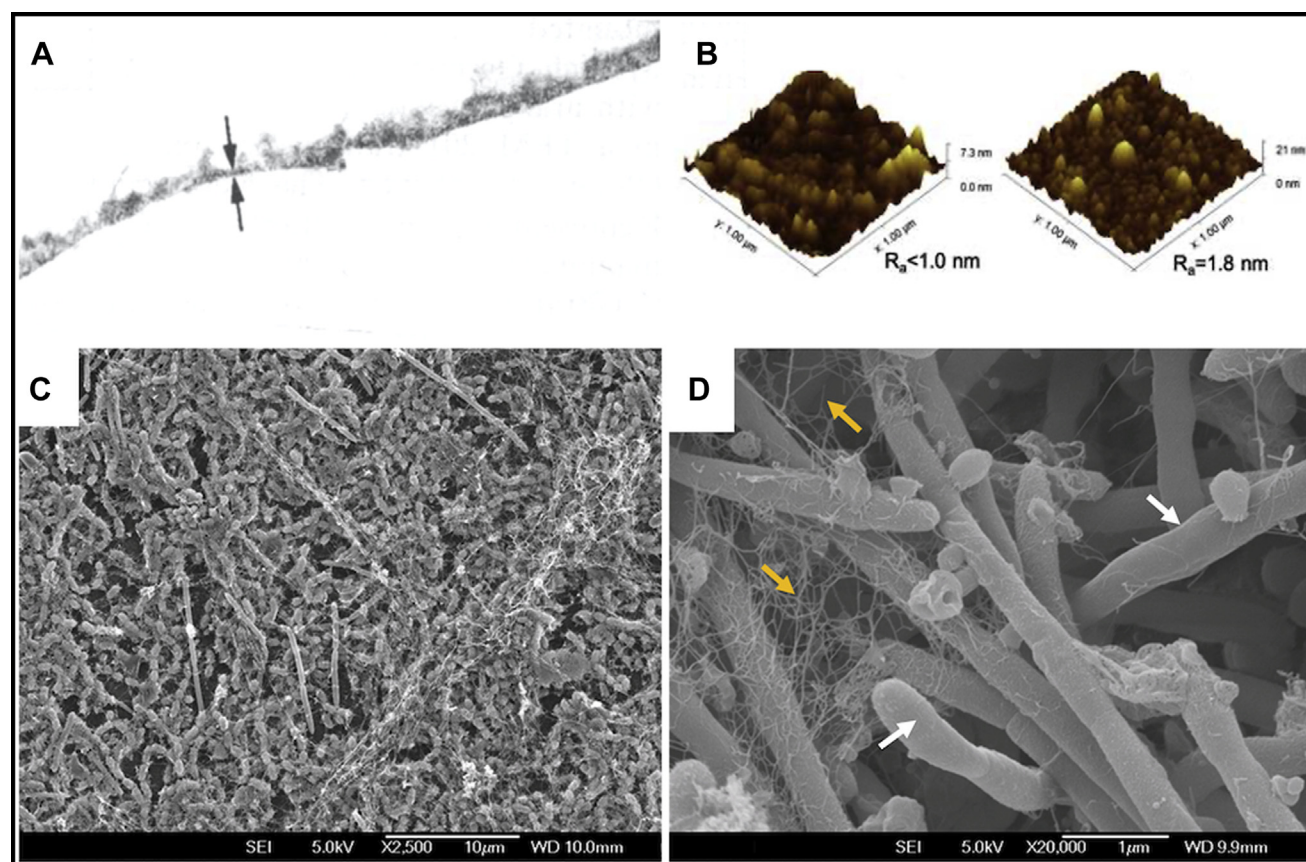
Figure 3 The salivary pellicle and biofilm formation on dental restorative materials. **A.** Transmission electron microscopic image of the salivary pellicle (black arrows) formed in vivo on dental amalgam. Adapted and reprinted from Hannig[28] with permission from Wiley. **B.** Atomic force microscopic images and roughness values (Ra) for zirconia surfaces without (left) and with (right) salivary pellicle in vitro. Adapted and reprinted from Sang et al.[29] with permission from Elsevier. **C, D.** Scanning electron microscopic images of microcosm biofilm: bacteria (white arrows) and remnants of extracellular polymeric substance (yellow arrows). Biofilms were grown from dental plaque inoculum aerobically in a CDC Biofilm Reactor (BioSurface Technologies) on a flat disk of a dental resin composite (3M Z100 Universal Restorative; 3M) for 48 hours.

species that are present in the biofilm and therefore heterogeneous in nature[30] and on the other hand dependent on the environmental conditions (eg, nutrition).[5,7,36]

Materials science

Physical and Chemical Surface Interactions

Surface physical and chemical properties of materials such as charge, surface energy and wettability, and topography (including roughness and surface chemistry) essentially determine the interactions of the material with the oral microbiota.[4,24] Therefore, surface physical and chemical properties, their evolution over time, and the composition of the test materials must be known for assessing potential antibacterial effects of dental materials, similar to testing biocompatibility. In addition, it is critical to adjust the characterization of all these properties and resulting interfacial interactions to the specific physical-chemical nature of the antibacterial agent, such as ions, molecules, biomolecules, (nano or micro) particles, and macroscopic surfaces. Furthermore, it must be kept in mind that the biofilm

may change material responses and interactions with biological agents and eventually change material properties.[4,37] For instance, biofilms on amalgam restorations affect the transport of ions to and from the surface. As a consequence, the environment on the restoration surface is modified.[38] Amalgam restorations may benefit from this biocorrosion because some of the solid by-products of these corrosive processes can seal gaps at the interface between the restoration and the tooth, thus preventing secondary caries.[37,39] In contrast, restorations from resin-based composites seem to be more vulnerable to biodeterioration, which can affect various material properties and foster degradation of the tooth-composite interfaces, which in turn may contribute to the development of secondary caries.[40,41] For example, *Streptococcus mutans* exhibit esterase activity at levels that are high enough to degrade resin-based composites and adhesives in vitro.[40]

Mechanism of Antibacterial Action

Different approaches of action for antibacterial dental materials have been developed so far. They are mainly based on either released (or eluted) substances from a material or

surface effects of materials to prevent the formation and growth of biofilms.[4] For example, 1 study investigated the incorporation of antibacterial substances into dental restorative materials and their antibacterial activities, including disruption of the biofilm EPS network and interruption of quorum sensing, because of the time-dependent release of these substances.[4] In contrast, surface effects are mainly based on surface roughness, topography, wettability, surface free energy, surface charge, and surface chemistry.[4] Known examples from nature are lotus leaves, shark or gecko skin, or cicada wings.[42] Protein adsorption may particularly decrease such surface effects because of pellicle formation on the material.[22,24] Therefore, pellicle formation needs to be stimulated in in vitro testing. In addition, a relevant but less explored mechanism of action for providing antibacterial potency to dental restorations is the use of active agents that are exposed at the interface with the restorative material and kill bacteria on contact, with no release of the agent from the surface.[43] Combinations of all these strategies have also been explored and pose additional challenges to characterize the claimed dental restorative materials and their surfaces.[4,43]

Sample Preparation and Controls

As outlined in the literature,[11] sample preparation has a decisive influence on the test results. Particularly, sample size, curing, surface treatment, and aging of the samples need to be defined. Appropriate positive and negative control materials must be chosen, and aseptic conditions or sterilization and sample cleaning protocols must be ensured.

Assessment of antibacterial activity

In choosing a quantitative method for investigating antibacterial effects from dental materials, a robust method such as assessing bacterial ability to replicate using colony-forming units (CFU) assays seems preferable. CFU assays (Figure 4) are considered the reference standard for assessing antibacterial effects but will not detect bacteria that are in a so-called viable but not culturable state and are dependent on the nutrient broth and growth condition selected for bacterial proliferation.[44,45] Furthermore, not all oral bacteria can be cultured in vitro, which might not be a problem for biofilms from defined consortia but certainly influences CFU results from microcosm biofilms cultured from microbiological samples such as dental plaque or saliva.[46,47] Special attention should be given to confirm that all bacteria forming the biofilm have been collected from the tested surfaces before plating and quantifying antibacterial activity.

In addition, to measure the bacterial replication ability, membrane damage (via flow cytometry) or metabolic activity (via MTT or XTT assay) can be investigated to get insights into the mechanism of action of a given material. In addition, a combination of different techniques can be used to narrow down the mechanism of a given antibacterial approach, as previously reported.[49]
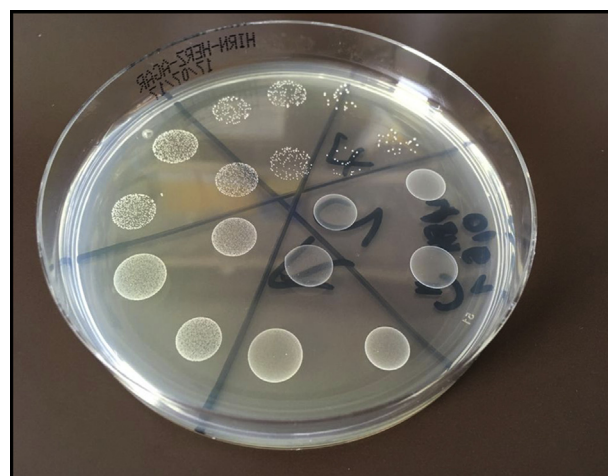

Figure 4 Quantitative assessment of antibacterial materials. Results from a colony-forming unit assay according to the method described by Miles et al.[48] An agar plate (here brain-heart infusion agar) is divided into sextants, bacteria (here: *Streptococcus mutans*) are serially 10-fold diluted, and 3 aliquots (20 µL) from each dilution step are plated in each sextant. Subsequently, the agar plate was incubated for at least 24 hours at 37 °C.

In contrast, viability staining kits such as LIVE/DEAD *Bac*Light Bacterial Viability staining (Thermo Fisher Scientific) are known to be prone to potentially produce false results and require extensive optimization for each bacterial species to be tested.[50] Therefore, these and other imaging techniques (confocal laser scanning microscopy, scanning electron microscopy, transmission electron microscopy) should mainly be applied for visualization but not for quantification purposes.

The salivary pellicle can influence the antimicrobial potential of a biomaterial. Therefore, assessment of antibacterial activity requires the initial deposition of the salivary pellicle, for which a combination of methods have been described.[27]

Impairment of primary function

Finally, it should be considered that antibacterial properties may also be associated with cytotoxicity; therefore, the evaluation of antibacterial activity must always be accompanied by a (standardized) evaluation of other biological effects, including cytotoxicity or mutagenicity. It should also be kept in mind that bacteria may be able to phenotypically adapt toward given agents released from dental restorative materials such as antiseptics.[51,52] Moreover, it is understood that antibacterial effects should not be at the expense of the overall performance of such materials (eg, physical, chemical, and mechanical aspects).[4]

Consequences

Because of the many variables described above (Figure 2), data from testing the antibacterial effects of dental materials

in vitro often fail to correlate with in vivo assessment.[4] Therefore, refinement of models and tailoring for specific applications is required, and particular emphasis must be given to design standard tests for assessing antibacterial effects with the aim that the generation of clinically nonrelevant data on antibacterial effects (eg, only a short peak of antibacterial efficacy in the first hours or days after setting of the material) is prevented.

Developing a New International Organization for Standardization Standard on Antibacterial Effects of Dental Materials

ISO standards

Standards can be considered as something established by authority, custom, or general consent as a model of example or as a rule for the measure of quantity, weight, extent, value, or quality,[53,54] or as a formula that describes the best way of doing something.[55] Generally, it is assumed that a standard accurately describes desired or defined properties, related requirements, and as exactly as possible the methods for demonstrating these properties. However, in the field of antibacterial dental materials and biofilm formation on such materials, many variables determine the outcome, as shown above. So, the question arises on how to select the most relevant variables? Furthermore, the experimental basis is highly heterogeneous.[4,10,11] However, a similar situation was faced when other biological standards related to dental materials such as ISO 7405 or the ISO 10993 series were developed.[21]

The ISO is an international nongovernmental organization made up of national standards bodies; it develops and publishes a wide range of standards and comprises representatives from various national standards organizations. ISO has installed specific technical committees (TCs) for the different areas. ISO TC 106, founded in 1962, covers the field of dentistry and comprises 8 subcommittees. To date, 188 dental ISO standards have been published. The scope of ISO TC 106 is the standardization in oral health care, including terms and definitions, performance, safety, and specification requirements of dental products. ISO TC 106 also formulates clinically relevant laboratory test methods, all of which contribute to improved global health.[56] ISO TC 194 covers the field of biological and clinical evaluation of medical devices, and those standards also are of interest for dental materials, as the latter are legally considered medical devices in most countries.

Different types of ISO standards have been developed:

- Composition and harmonization standards: defining the required level of components and ingredients and harmonizing terms and products (mainly descriptive)
- Performance standards: defining fixed tests and corresponding requirements

- Framework standards: defining cornerstones or basic requirements for certain tests but leaving space for individual adjustment
- Vertical standards: focusing on a specific group of materials (eg, polymer-based restorative materials)
- Horizontal and semihorizontal standards: being applied to larger groups of materials (eg, dental restorative materials).

Generally, reporting is an important aspect of all standards, especially in those considered to be framework standards such as ISO 7405 or the ISO 10993 series. ISO standards have mainly been developed to test market products, but they can also be used as a component of general material testing.

The advantages of ISO standards are that they are developed as a multistakeholder approach. All relevant parties, such as manufacturers, academia, or regulatory bodies, are invited to contribute, which should prevent the dominance of a single stakeholder. Furthermore, they are based on an international consensus, and the needs of different parts of the world are respected. In the special case of developing a standard for testing antibacterial activities of dental materials, available standards, experience, and expertise can be used (eg, from ISO 7405, ISO 10993-5, ISO 22196). In contrast, standards development can be time-consuming; accordingly, ISO has set time limits (eg, 3 years) until the completion of a given standard. It can also be seen as a drawback of such standards that they are mainly based on in vitro testing, raising the question of the predictability of the results for a clinical situation. Finally, the enforcement of single stakeholder interests should be prevented by encouraging the active participation of all interested parties, including academia, in the development of a standard.

Development of ISO 3990

The ISO TC 106 agreed in 2019 to start a new project (ISO 3990) related to the antibacterial effects of selected dental restorative materials, which is under development in ISO TC 106 Working Group 10, which also developed ISO 7405. A Draft International Standard is being prepared, which after agreement from national standard bodies, will develop into a so-called Final Draft International Standard and is planned to be published as ISO standard in 2023.

The title of ISO Draft International Standard 3990 is "Dentistry—Evaluation of antibacterial activity of dental restorative materials, luting cements, fissure sealants and orthodontic bonding/luting materials." This document specifies test methods for the evaluation of dental restorative materials, luting cement, fissure sealants, and orthodontic bonding and luting materials that are claimed by their respective manufacturers to exert antibacterial effects. The scope of the document does not include implants, nightguards, pulp capping materials, and proof of sterility of given medical devices used in dentistry, mainly to straighten and narrow down the standard and keep it to a reasonable
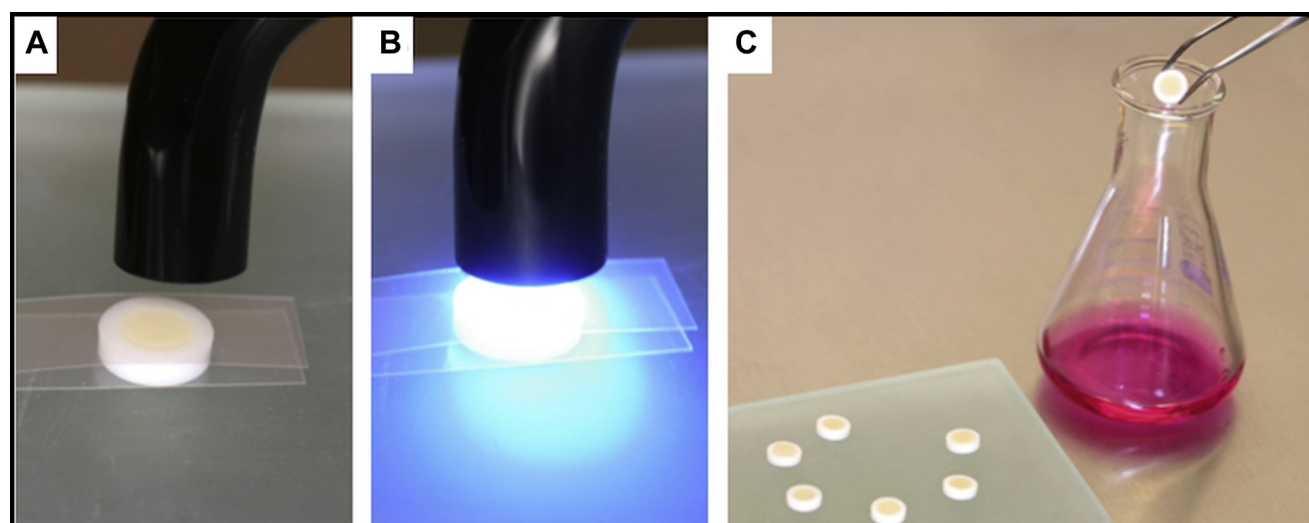
Figure 5 Sample and extract preparation. **A.** A sample mold (eg, from polytetrafluoroethylene) is filled with a resin-based composite, both ends of the mold are covered with a transparent oxygen barrier material (eg, polyester or mylar strips). **B.** The sample is light cured from both sides. **C.** Extraction is performed in sterile, chemically inert containers with a volume of extraction vehicle (eg, protein-rich nutrient broth) based on the exposed surface area for 24 or 72 hours at 37 °C.

size. Accordingly, the standard applies to antibacterial effects, not to antimicrobial effects, because the latter may also include antifungal or antiviral effects.

The standard is conceived as horizontal (covering a range of different product groups), framework (leaving space for individual adjustment), reporting (individual adjustments must be exactly reported and justified) standard. Because of the general applicability of in vitro tests for antibacterial activity and their widespread use in evaluating a large range of dental restorative materials, it is the purpose of this document to define a scheme for testing that requires decisions to be made in a series of steps rather than to specify a single test. For example, testing could be initially done with planktonic bacteria for screening purposes and continue with biofilms if antibacterial efficacy has been established. This should lead to the selection of the most appropriate test methods for a respective dental restorative material to be evaluated.

For sample preparation (Figure 5A, B) in terms of mixing, curing, surface treatment, use of molds, and so forth, the same rules apply as for other standards for the biological evaluation of medical devices such as ISO 7405 and ISO 10993-12. The tests described in this document shall be performed on extracts of the test sample (extract tests) and on the test samples themselves (direct-contact tests). The choice of 1 or more of these categories depends on the nature of the material to be evaluated, the potential site of use, and the nature of the use of the respective material.

Extract tests primarily focus on materials containing substances leaching out, whereas direct-contact tests are directed to leachables and surface effects. The choice of a test then determines the details of the preparation of the samples to be tested, the preparation of the cultured bacteria or biofilms, and the way in which the bacteria or biofilms are exposed to the samples or their extracts.

For testing the effects of leachables, extracts are prepared according to ISO 10993-5 by placing defined material samples into a given liquid like, protein-containing nutrient broth (Figure 5C). The extracts are prepared 24 hours after mixing and curing and additionally after 10 and 20 consecutive elution cycles to indicate long-term antibacterial activity, which may decrease because of the reduction of the release of leachables with an increasing number of elution cycles.[57]

For testing surface effects, the samples are likewise tested 24 hours after mixing and curing and additionally after 10 and 20 consecutive elution cycles to indicate long-term antibacterial activity and to ensure the formation of a covering protein-layer simulating pellicle formation.

Both extracts and the materials themselves could be first tested toward planktonic cultures of bacteria in a pre-screening assay.[32] When antibacterial activity is established, further testing would be done with bacterial biofilms. The selection of bacterial strains shall be based on the relevance of these organisms for the area of application of the respective material (eg, *Streptococcus* spp. for dental restorative materials or orthodontic bonding and luting materials).[35] Some examples for the recommended bacterial type or reference strains are listed in an appendix of the planned ISO standard along with their corresponding nutrient broths and solid growth media to be used for the set of experiments described in this document.

Negative and positive control materials need to be included in each assay. Preferably, controls should include materials with the same composition but lacking the active ingredient responsible for potential antibacterial activity (ie, negative control material). In addition, control samples should be prepared by the same procedures as the test samples. Furthermore, for tests on extracts, 0.2%

chlorhexidine digluconate is to be used as a positive control[58] and nutrient broth as a negative control (in addition to the extracts from the negative control material). For direct-contact tests, copper plates (purity ≥ 99%) are to be used as a positive control.[59,60]

For evaluation of antibacterial effects of a given material, it is mandatory to determine reductions of bacterial ability to replicate by CFU assay (Figure 4). In addition, bacterial membrane damage can be assessed by flow cytometry, or reduction in bacterial metabolic activity can be assessed by MTT test. There are strict requirements for passing the tests outlined in the standard, depending on whether extract tests or direct-contact tests have been conducted.

The median reduction of bacterial ability to replicate needed for a material to be considered antibacterial depends on whether testing extracts or direct material contact. For tests on extracts, an antibacterial material needs to exhibit a median reduction of bacterial ability to replicate at least 99.9% (3 $\log_{10}$ steps) compared with the negative control material, in accordance with the definitions of the American Society of Microbiology.[61–63] For direct-contact tests, an antibacterial material needs to exhibit a reduction of bacterial ability to replicate at least 99% (2 $\log_{10}$ steps) compared with the negative control material, per the definitions outlined in test JIS Z 2801.[64]

As the proposed standard will leave space for individual adjustments, special emphasis is placed on reporting the details of the finally chosen test design (reporting standard). Therefore, this document includes a detailed list of 17 items to report, including the respective justifications (eg, for the choice of bacterial strains to be tested). In addition, testing of antibacterial effects should always be accompanied by cytotoxicity testing of this material according to ISO 7405 and ISO 10993-5.

Synopsis

Informative and relevant preclinical assessment of clinically desired antibacterial dental restorative materials requires rationalization and selection of test conditions considering a large number of biological and material-related factors. Because of practical reasons, in vitro tests need to be defined. The complexity of the task challenges researchers and manufacturers in the field to properly determine the best experimental approach to get data that predict the presumed clinical antibacterial efficacy of dental restorative materials and which are comparable among different laboratories. Thus, there is a critical need for standardization.[10,11]

ISO is an established tool and maybe a suitable platform for that purpose. An attempt is being made to establish a specific standard for assessing the antibacterial activity of dental restorative materials (ISO 3990). Some essentials are planned to be fixed, such as sample preparation, planktonic followed by biofilm testing, defined requirements, suitable controls, and reporting obligations. However, there are

limitations, such as that the methods included in ISO 3990 are in vitro tests only and use a monospecies and static biofilm approach. Therefore, careful interpretation is of paramount importance, and clinical risk assessment in terms of biocompatibility testing (eg, according to ISO 7405 or ISO 10993-5) need to be performed in addition to testing antibacterial effects according to ISO 3990. The latter can be considered the first step to set up a standard for testing the antibacterial effects of dental restorative materials. According to ISO rules, it is a living document, which means that it requires regular revision. However, more research and commitment is needed in the future, as holds true for all other ISO standards.[18]

The general philosophy of such standards has been described as follows: "Standards bodies strive to base standards on the best available evidence, and to develop test procedures which are optimized for discriminatory power, reproducibility and comparability for use all over the world within constraints such as of expense, time, equipment and expertise availability, yet still sufficient for purpose with confidence."[19]

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