

**Adhesion of oral streptococci to enamel and dental materials —
Studies using a flow chamber and microcalorimetry**

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gewidmet.

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Abstract

Dental plaque formation on human teeth is the crucial etiologic factor in the pathogenesis of oral diseases: caries, periodontal diseases, and peri-implantitis. Dental plaque has been defined as a diverse community of microorganisms found on teeth as a biofilm, embedded in an extracellular polymer matrix of bacterial origin and including host components. Bacterial colonization starts with the adhesion of early colonizers, called pioneer bacteria, to the salivary pellicle on teeth as well as on dental materials within minutes after tooth cleaning. The early colonizers, mostly streptococci, contribute to plaque development and ultimately to oral diseases. Investigations of dental plaque, including bacterial adhesion, employ various *in vivo* and *in vitro* models and use microscopic methods to assess surface phenomena. The complexity of the oral environment makes it difficult to generate an *in vitro* system including all relevant aspects.

The studies presented (paper I-III) were aimed to adapt two *in vitro* models, a flow chamber system and a microcalorimetric technique, for investigating adhesion of oral streptococci to human enamel, glass and different dental materials.

The dental materials used for the flow chamber experiments (paper I + II) were titanium (Rematitan®M), gold (Neocast 3), ceramic (Vita Omega 900), composite (Tetric Ceram), and four different all-ceramics. The early colonizing *Streptococcus sanguinis*, *S. oralis*, and the caries-inducing *S. mutans*, and *S. sobrinus* were used as the model organisms.

The saliva-coated materials were incubated with the bacteria in the flow chamber during one hour. Number and vitality of adhering bacteria were determined microscopically after staining. The results suggested that variations in the number and vitality of the adherent oral streptococci depend on the surface characteristics of the substrata and the acquired salivary pellicle. It also depended on the bacterial species, as *S. mutans* and *S. sobrinus* adhered about 10 x less than *S. sanguinis* and *S. oralis*.

Isothermal Microcalorimetry (paper III) indeed allowed evaluation of initial bacterial adhesion of *S. sanguinis* to glass. Maximum heat flow measurements during adhesion were about 10 fold lower than during bacterial growth. Experiments showed that increased surface areas, provided by increased amounts of glass beads, were associated with higher energy release. Heat flow was higher when cells were suspended in human saliva than in PBS.

Based on these results both methods appear to be applicable to study bacterial adhesion to new dental restorative or implant materials. Advantages and disadvantages of the methods are discussed.

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Introduction

The oral cavity represents a very complex biologic system including soft and hard tissue, forming diverse ecological niches, and a multitude of microorganisms. A recurring uptake and removal of nutrients, saliva and microorganisms operate in the mouth accompanied by shear forces due to chewing, flushing of saliva, speaking, and swallowing as well as frictional removal by tongue and oral hygiene. In order to resist these forces and to achieve a stable colonization, bacteria need to adhere to hard or soft tissues or to other bacteria.

Adhesion, colonization, and growth of oral bacteria on tooth surfaces lead to a multispecies biofilm, called dental plaque (Rosan and Lamont 2000; Hicks et al. 2003; Marsh 2004). It is the cause of common oral infectious diseases such as dental caries, gingivitis, periodontitis, and peri-implantitis. Plaque is not only present on natural teeth but also on dental materials and implant components as well.

Saliva

Saliva plays an important role in oral health, regulating and maintaining the integrity of the oral hard and soft tissues (Humphrey and Williamson 2001; Hicks et al. 2003; Dodds et al. 2005; Llana-Puy 2006). Several functions can be defined for saliva: lubrication and protection, buffering action and clearance, maintenance of tooth integrity, antibacterial activity, and digestion. Saliva provides a protective surface coating of mucosa and teeth. Several salivary proteins are involved in the antimicrobial defence, and are able to eliminate or inhibit bacteria, fungi and viruses that enter the body via the oral cavity. Other salivary components induce aggregation of microorganisms and debris for oral clearance. Certain enzymes initiate digestion of ingested nutrition, and saliva has been shown to be the most important biological factor in the prevention of dental caries and erosion due to its inhibition of the demineralization and promotion of remineralization of enamel and dentin. Salivary

components have overlapping, multifunctional roles which can be beneficial and detrimental. Whole saliva consists of 99% water and 1% of inorganic and organic constituents (Schenkels et al. 1995). Inorganic constituents include bicarbonate for buffering and calcium and phosphate for maintenance of tooth mineral integrity. Calcium, phosphate and fluoride are key ions in the resistance of exposed tooth surfaces against microbial acids because they decrease demineralization and favor remineralization. Degradation of arginine-rich proteins by bacterial enzymes to urea and ammonia contributes to maintaining a neutral pH. Organic constituents of saliva include a variety of proteins (Schenkels et al. 1995). Several proteins bind calcium and phosphate ions and contribute to a supersaturation at the tooth surface, thus inhibiting demineralization and promoting remineralization. Mucins are high-molecular-weight proteins that give saliva the typical visco-elastic character and protect against dehydration. Mucins also promote the clearance of bacteria by masking their surface adhesins and inhibiting bacterial colonization. Secretory Immunoglobulin A (sIgA) is the dominant immunoglobulin of the mucosal immune system while IgG and IgM are present at much lower concentration in saliva. The protective role of sIgA is based on neutralization of viruses and binding bacterial antigens like adhesins, toxins, and enzymes. Proline-rich proteins are involved in different functions of saliva: they have antimicrobial activity, induce aggregation and clearance of microorganism. Several other salivary proteins, like histatins, cystatins, statherin and amylase or lysozym possess antimicrobial properties (Schenkels et al. 1995).

The average daily production of whole saliva in health varies between 1 and 1.5 L with enormous individual variations in quantity and quality of saliva. The volumes of saliva produced vary depending on age, diet, time of the day and the type and intensity of stimulation (e.g. chewing, acid). The importance of sufficient salivary flow (amount and quality) is documented in people experiencing very low flow rates, e.g. due to advancing age,

certain medications or head and neck radiotherapy. These have a high risk of rampant caries (Dodds et al. 2005; Giannoni et al. 2005; Jham and da Silva Freire 2006; Kielbassa et al. 2006).

Saliva has also become useful as systemic sampling measure for medical diagnosis and research due to its easily collected, non-invasive source of information (Sreebny 2000; Llena-Puy 2006).

Salivary pellicle formation

Microbial adhesion to oral hard surfaces occurs rapidly after tooth cleaning and is always preceded by the adsorption of an acquired pellicle containing components from saliva (Hannig 1999a; Lendenmann et al. 2000). Pellicle formation on solid surfaces can be detected as early as one minute after exposure to the oral environment (Hannig 1999b). It is a mainly proteinaceous film, formed through selective adsorption of salivary constituents (Vacca Smith and Bowen 2000). Acidic proline-rich proteins are the predominant pellicle proteins. Other major proteins in the pellicle are secretory IgA, cystatin, high-molecular-weight mucin, lactoferrin, lysozyme and amylase. The proteins in the pellicle originate from whole saliva, but there are some distinct differences between the composition of proteins in whole saliva and in the pellicle (Yao et al. 2003). The pellicle forms on natural tooth surfaces as well as on restorations. However, protein composition of the salivary pellicle may vary with the location in the dentition, the chemical properties and surface free energy of the substrates (Carlén et al. 1998a; Carlén et al. 2001). Streptococcal glycosyltransferase was also detected in pellicle, indicating that bacterial proteins may be incorporated into the pellicle as well. The acquired pellicle may function as a diffusion barrier protecting enamel against acid-induced demineralization. The pellicle also serves the initial adhesion of bacteria to the tooth or dental material surface, which is the first step in plaque formation.

Oral microbiota

More than 700 different bacterial species or phytotypes have been detected in the oral cavity over 50 % of which have not been cultivated. The microflora on teeth, the tongue, buccal epithelium, soft and hard palate, and the vestibulum revealed 20-30 different predominant species at each site, and the number of predominant species per individual was in the range of 30-70 (Mager et al. 2003; Aas et al. 2005). The most common species belonged to the genera *Gemella*, *Granulicatella*, *Streptococcus*, and *Veillonella*. Based on a few individuals analyzed there appears to be a distinctive predominant bacterial flora of the healthy oral cavity that is highly diverse, and site and individual specific. Species typically associated with oral diseases (caries and periodontitis, see below) were not found (Aas et al. 2005).

In addition to the plaque-associated oral diseases, oral bacterial species have been implicated in several systemic disorders, such as bacterial endocarditis (Douglas et al. 1993; Lockhar 2000), aspiration pneumonia (Scannapieco 1999), non-oral abscesses (Corson et al. 2001), and cardiovascular disease (Beck and Offenbacher 2001; Mattila et al. 2005; Al-Zahrani et al. 2006). Furthermore, maternal periodontitis may be a risk factor for preterm birth and other adverse outcomes of pregnancy (Jeffcoat et al. 2001; Boggess and Edelstein 2006).

Development and composition of dental plaque

Subsequent to pellicle formation the development of the bacterial biofilm into a mature dental plaque can be described in distinct processes: primary interaction of bacteria with the surface, initial adhesion through unspecific forces followed by attachment of the so-called first colonizers or pioneer bacteria to the surface by specific ligand-receptor interactions, coadhesion of other (secondary) bacteria and growth associated with the synthesis of exopolysaccharides (Rosan and Lamont 2000; Marsh 2004).

The primary interactions of bacteria with the tooth surface can result from diffusion,

sedimentation, liquid flow, or due to active bacterial movement. Interactions of the surface and the bacteria include electrostatic, hydrophobic, and van der Waals forces, and lead to firm but reversible adhesion of the bacteria to the pellicle. Subsequent interactions between specific molecules on the bacterial cell surface (adhesins) and complementary receptors in the pellicle result in an irreversible adhesion. Other bacteria may adhere to already attached early colonizers by specific interbacterial adhesin-receptor interactions (often involving lectins) a process called coadhesion (Kolenbrander 2000). This and cell division of the attached microorganisms lead to an increase in the thickness and diversity of the plaque. Concurrent polymer production results in the formation of a complex extracellular matrix made up of glucans, fructans and heteropolymers. Such a matrix is thought to form an open architecture with channels and voids and retains nutrients, water and key enzymes within the biofilm (Allison 2003). This mature plaque, formed during several days, functions as a true microbial biofilm community with metabolic interactions among the microbes. These include interactions to catabolize complex host glycoproteins and to develop food chains. In addition cell-to-cell signalling can occur which can lead to coordinated gene expression within the microbial community. Cells also communicate with one another via horizontal gene transfer. These interactions in biofilms often result in increased resistance to antimicrobial agents as compared to planktonic cells (Donlan and Costerton 2002; Gilbert et al. 2002; Henderson 2002; Kolenbrander et al. 2002; Petersen et al. 2005; Suntharalingam and Cvitkovitch 2005). The microbial composition of the mature dental plaque is dependent on the primary binding of first colonizers, called pioneer bacteria, and on coaggregating and growth (Kolenbrander 2000). Pioneer bacteria provide attachment substrates for subsequent colonizers and influence the succeeding stages of plaque composition. Oral streptococci, the model organism *Streptococcus sanguinis* used in these studies in particular, predominate this early plaque formation followed by increasing proportions of *Actinomyces* sp. (Li et al. 2004). Both,

streptococci and actinomyces coaggregate with certain strains of capnocytophagae, haemophili, prevotellae, propionibacteria, and veillonellae. A central role is played by *Fusobacterium nucleatum* because it may form bridges between many early colonizers and many other oral bacteria (Kolenbrander et al. 2002).

Plaque-associated diseases and prevention strategies

A frequent consumption of fermentable carbohydrates (mainly sucrose, fructose, glucose) favors an acidogenic and aciduric microflora and results in a shift of the balance towards demineralization of teeth. Dissolution of the enamel matrix by organic acids, particularly lactic acid produced by mutans streptococci, is considered to be the primary event in caries development (van Ruyven et al. 2000; Selwitz et al. 2007). There appears to be a very distinctive abundance of bacterial species, including *Streptococcus mutans*, *Lactobacillus* spp., and *Actinomyces* sp., associated with caries-active plaque. These exhibit an inverse relationship to beneficial bacterial species, such as *S. sanguinis*, *S. oralis*, *S. mitis*, *S. parasanguinis*, *Abiotrophia defectiva* (Corby et al. 2005). In advanced carious lesions the microflora was found to be much more complex, including diverse lactobacilli, prevotellae, *Selemonas*, *Dialister*, *Fusobacterium nucleatum*, *Eubacterium*, *Bifidobacterium*, *Propionibacterium* sp. (Becker et al. 2002; Munson et al. 2004; Beighton 2005; Chhour et al. 2005).

Increased plaque formation was observed on dental materials as well. It can lead to secondary or recurrent caries on adjacent tooth surfaces. Oral microorganisms invading through microgaps between the restoration and the tooth may not only induce secondary caries, but may also damage the pulp (Fontana and Gonzalez-Cabezas 2000; Mjör and Toffenetti 2000; Deligeorgi et al. 2001).

Accumulation of dental plaque, particularly along the gingival margin, may lead within a few

weeks to a reversible inflammation of the gingiva (gingivitis). If it's not reversed by elimination of the plaque, gingivitis increases in severity. The junctional epithelium loses some of its resiliency, permitting the penetration of bacteria between the tooth and the epithelium. A gingival pocket develops which provides a new, rather anaerobic ecological niche. Strictly anaerobic bacteria predominate this subgingival plaque. Some of these lesions may progress into periodontitis characterized by inflammatory infection and progressive loss of connective tissue and bony support (Ligtenberg et al. 2007).

The subgingival microflora is particularly complex; so far over 400 bacterial species have been identified from the periodontal pocket (Socransky and Haffajee 2005; Paster et al. 2006). Several putative pathogens, primarily *Actinobacillus actinomycetemcomitans*, *Prophyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* have been described based on association with diseased sites, production of virulence factors plausibly related to disease pathogenesis, a specific immune response of the host during periodontal infection, and clinical improvement after species elimination by periodontal therapy (Teles et al. 2006).

However, these attempts to identify etiologic agents of periodontitis may have been too simplistic for two obvious reasons:

a) It has become increasingly clear that host factors, particularly IL-1 genotypes, modulate decisively the clinical outcome of periodontal infection (Shapira et al. 2005; Huynh-Ba et al. 2007; Nibali et al. 2007).

b) Individual clones within bacterial species may have widely different properties including virulence (Kilian et al. 2006). Combined with the biodiversity in the oral microflora, this implies an individuality of dental plaque, far exceeding earlier expectations.

Conventional strategies to prevent dental diseases include oral hygiene (i.e. mechanical removal of the dental plaque), dietary modification (abstinence from dietary sugars, in

particular between meals), application of fluoride (e.g. in tooth paste to promote remineralization), and biocide mouthwashes. Of all available antimicrobials in dentistry chlorhexidin is still the most frequently used agent (Twetman 2004; Ribeiro et al. 2007). However long-term use is not advised because of side effects (e.g. desquamation of the mucosa). Vaccination against dental caries has been explored by both active and passive immunizations aiming at preventing or reducing oral re-colonization by mutans streptococci, so far with limited success (Russell et al. 2004; Taubman and Nash 2006). A potentially new strategy is the use of genetically modified *S. mutans* with 1000-fold reduced acid production and about 50% reduced cariogenicity in rats as effector strains in the replacement therapy of dental caries (Hillman et al. 2000; Hillman et al. 2007). Further, the selective elimination of *S. mutans* from plaque by specifically targeted antimicrobial peptides (STAMPs) could be useful as anticaries preventive and therapeutic measures (He et al. 2007). A further attempt concerns a genetic manipulation of the salivary glands to increase total salivary flow or secretion of specific antimicrobial proteins, like histatins into the oral cavity (so far in rats) (Cotrim et al. 2006; Piludu et al. 2006).

Dental materials

Patients and dentists have a variety of options when choosing materials and procedures for restoring carious lesions and for substituting missing teeth. However, there is a need for more economical, more durable and safe materials. Bacterial accumulation on marginal areas of enamel and restorative materials is a key factor for inducing secondary caries which is in turn one of the major causes for replacement of restorations (Wallmann and Krasse 1992; Fontana and Gonzalez-Cabezas 2000; Deligeorgi et al. 2001). Therefore, there is an increasing interest in producing dental materials which reduce or inhibit plaque formation. In order to prevent secondary caries formation, both the development of dental materials with distinct adhesion

or/and growth-inhibitory properties (Boeckh et al. 2002) and the coating of dental materials with antimicrobial agents could play an important role (Hetrick and Schoenfish 2006). Examples are the application of a chlorhexidin-containing varnish (Zhang et al. 2006) or material slowly releasing fluoride (Wiegand et al. 2007). Dental materials are routinely tested for mechanical properties, chemical stability and toxicity, less often with respect to adhesion and biofilm formation. To this end simple test methods would be useful.

In vitro and in vivo models to study bacterial adhesion and dental biofilm formation

In vivo and *in vitro* studies have examined bacterial colonization on human enamel and dental materials using different models and microscopic observation methods. These models differ widely in purpose and complexity (Sissons 1997; Tang et al. 2003).

In vivo models examine biofilm growth on slabs of dental materials (glass, hydroxyapatite, bovine or human enamel, a few restorative materials) mounted on teeth or appliances to the oral environment of a limited number of volunteers during several hours or days (Giertsen et al. 2000; Konishi et al. 2003; Auschill et al. 2004). These models permit the development of a “natural” plaque on the experimental surface because it originates directly from the natural microflora. The microbial composition of this plaque is very complex and can vary between different teeth in the same mouth and on the same tooth in different people. The natural variations of intra-oral conditions make it very difficult to get standardized results. In addition, ethical issues may hamper the study of new materials.

In vitro biofilm models use experimental conditions which reflect physiological conditions of the oral cavity. These models include mono-species or multi-species biofilms (varying from 5 different microorganisms to at most ten) in flow culture or batch culture systems (Marsh 1995; Kinniment et al. 1996; Guggenheim et al. 2001; Thurnheer et al. 2001; Tang et al. 2003). The microorganisms are provided with continuous or intermittent supply of nutrients.

Biofilm formation, composition and structure are analyzed using light microscopy, scanning and transmission electron microscopy and/or confocal laser scanning microscopy (CLSM). Species-specific fluorescence-labeled antibodies in conjunction with CLSM allow characterization of the spatial arrangement and interspecies associations of the microorganisms used. Because of their complexity these models are less applicable for rapid screening of a large number of dental materials.

A few adhesion models using human enamel, hydroxyapatite or glass as support, streptococci suspended in various buffers, artificial or human saliva have been described (Busscher et al. 1992; Tarsi et al. 1997; Rudney and Staikov 2002). We chose an *in vitro* flow chamber adhesion model previously used to study the effect of plaque inhibiting agents (Weiger et al. 1999) for the examination of different dental and implant materials. The model integrates some host components such as human enamel or dental materials as substrate, human saliva for suspending the bacteria and a defined flow rate of the circulating suspension simulating some share forces of the oral cavity. Adhesion was analyzed by fluorescent microscopy to determine adhered live and dead cells (Decker 2001).

As metabolic processes of bacterial cells are usually accompanied by heat production microcalorimetry has been applied in a wide range of fundamental studies and technical applications in biological research and quality control (Wadsö 2002). Microcalorimetric measurements have up to now scarcely been used in the evaluation of biofilm formation (Wentzien et al. 1994; Morgan and Beezer 1998). Recently, this method has been used for early detection of microbial growth in clinical specimens, such as blood and cerebrospinal fluid (Trampuz et al. 2007) and may be used to determine antimicrobial susceptibility (A. Trampuz personal communication). To our knowledge it has not been applied in the study of bacterial adhesion.

Aims of the thesis

The first aim of the thesis was to adapt a flow chamber adhesion model which had been used to study adhesion of *Streptococcus sanguinis* to glass and human enamel (Weiger et al. 1999; Decker et al. 2003b) to compare adhesion of several oral streptococci to restorative and implant materials. Paper I showed that the model is, indeed, suitable to quantitate adhesion of *S. sanguinis* to dental implant and restorative materials. Paper II analysed adhesion of four oral *Streptococcus* species to a group of new all-ceramics dental restorative materials.

The second aim of the thesis was to answer the question whether an isolated process like the adhesion of bacteria releases enough heat that it can be measured by microcalorimetry. Paper III describes that, indeed, adhesion of *S. sanguinis* to glass surface can be measured by isothermal microcalorimetry. Thus microcalorimetry may offer another direct approach, potentially allowing one to measure the energetics of the adhesion process.

Adhesion of *Streptococcus sanguinis* to Dental Implant and Restorative Materials *In Vitro*

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Bacterial adhesion to tooth surfaces or dental materials starts immediately upon exposure to the oral environment. The aim of this study, therefore, was to compare the adhesion of *Streptococcus sanguinis* to saliva-coated human enamel and dental materials — during a one-hour period — using an *in vitro* flow chamber system which mimicked the oral cavity. After fluorescent staining, the number of adhered cells and their vitality were recorded. The dental materials used were: titanium (Rematitan[®] M), gold (Neocast 3), ceramic (Vita Omega 900), and composite (Tetric Ceram).

The number of adherent bacterial cells was higher on titanium, gold, and ceramic surfaces and lower on composite as compared to enamel. As for the percentage of adherent vital cells, it was higher on enamel than on the restorative materials tested. These results suggested that variations in the number and vitality of the adherent pioneer oral bacteria, *S. sanguinis*, in the *in vitro* system depended on the surface characteristics of the substratum and the acquired salivary pellicle.

The *in vitro* adhesion model used herein provided a simple and reproducible approach to investigate the impact of surface-modified dental materials on bacterial adhesion and vitality.

Keywords: *Streptococcus sanguinis*, Adhesion, Implant and prosthetic materials

INTRODUCTION

Dental plaque, as an oral biofilm, is recognized as a key etiologic factor for caries and periodontal diseases in humans. Bacterial colonization of tooth surfaces or dental materials — like filling materials, dental implants, or prostheses — starts immediately upon exposure to the oral environment. In the process of plaque formation, early colonizers, including *Streptococcus sanguinis*, adhere to the salivary coating covering the tooth surfaces¹. This initial adhesion is an important step in biofilm formation as it may influence the composition of mature dental plaque².

Different strategies to investigate *in vitro* the development and structure of the biofilm on oral hard tissues and dental materials have been used as tools in dental research. Attachment and growth of various plaque bacteria on substrata has been assessed using different models^{2,3}. However, salivary pellicle-coated enamel as a substratum was found in only a few reports^{4,5}.

Presently, commonly used materials for dental restorations include metals, ceramics, and resin composites. Although these materials are safe and effective for their intended use⁶, there is also an increasing interest in dental materials which minimize plaque formation⁷.

Adhesion and vitality of *S. sanguinis* have been investigated *in vitro* in a flow chamber system using human enamel as a substratum in a salivary milieu⁵. The aim of the present study, therefore, was to compare the adhesion of *S. sanguinis* to commonly used implant and restorative materials with that to human enamel, and also determine the vitality of the initial adhered bacteria. We hypothesized that the number and vitality of adherent bacterial cells would vary with the nature of dental restoratives.

MATERIALS AND METHODS

Bacteria, culture conditions, and hydrophobicity measurement

A 10- μ l inoculum of *S. sanguinis* DSM 20068 (German Collection of Microorganisms and Tissue Culture Cells, Braunschweig, Germany) preserved in skim milk solution at -20°C was suspended in 5 ml of Schaedler broth (BBL[™], Becton Dickinson, Basel, Switzerland) and incubated aerobically at 37°C for eight hours. An inoculum was then transferred to fresh Schaedler broth (1:50) and grown at 37°C for 16 hours. This culture was sonicated for one minute (30 W, Sonifier Ultraschall-Desintegrator, Branson Sonic Power Co., Berlin, Germany), washed with physiological saline, harvested by centrifugation at

8000 g for five minutes, and resuspended in saliva to a density of 10^8 - 10^9 cells/ml.

Density of bacteria per ml in the bacteria-saliva mixtures at the beginning and end of each experiment were determined by phase contrast microscopy (Provis AX70, Olympus AG, Volketswil, Switzerland) using a standard Neubauer chamber.

Hydrophobicity measurement of *S. sanguinis* cells was done as described by Grivet *et al.*⁸ using partitioning into hexadecane (Sigma-Aldrich GmbH, Buchs, Switzerland).

Saliva

Whole saliva was collected by paraffin stimulation from a healthy volunteer (after not drinking or eating for two hours). Saliva samples were sonicated (1 minute, 30 W), filtered through a 70- μ m filter (Cell Strainer, Becton Dickinson, Basel, Switzerland), and centrifuged at 22,000 g for 60 minutes at 4°C. The supernatant was filtered through two low-protein-binding filters (pore sizes of 0.45 μ m and 0.22 μ m; Millex-HV and Millex-GV respectively, Millipore, Switzerland) connected in series. Sterilized saliva was stored at 6°C and processed within two days. Before use, the pH was adjusted to 7.1–7.3 with phosphate buffer (0.067 mol/l Na_2HPO_4 , 0.067 mol/l KH_2PO_4).

Adhesion substrata

Table 1 lists the materials tested in this study: titanium, gold, ceramic, and composite. Rectangular test specimens ($14.4 \times 14.4 \times 0.2$ mm³) were prepared and polished to a defined surface roughness. Surface roughness was measured by a Hommel tester (T 1000, Hommelwerke GmbH, VS-Schwenningen, Germany). Tooth slices were prepared as previously

described⁹ and mounted with epoxy glue on glass plates (Fig. 1). Only the enamel portion was used for analysis. All the slides were decontaminated with ethanol. Before the adhesion experiments, the slides were exposed to the sterile human saliva used in the flow chamber system at room temperature for 15 minutes.

Measurement of contact angles — as indices of hydrophobicity — was carried out using a K100 Processor Tensiometer (Krüss GmbH, Hamburg, Germany).

Adhesion of *S. sanguinis* to substrata in the flow chamber

The *in vitro* model used herein was as that previously described by Weiger *et al.*¹⁰. Briefly, the bacteria-saliva suspension circulated from a Teflon dispenser (Multimed GmbH, Kirchheim unter Teck, Germany) to the flow chamber (No. 1301, Minucells, Bad Abbach, Germany) in which the test specimens were mounted in

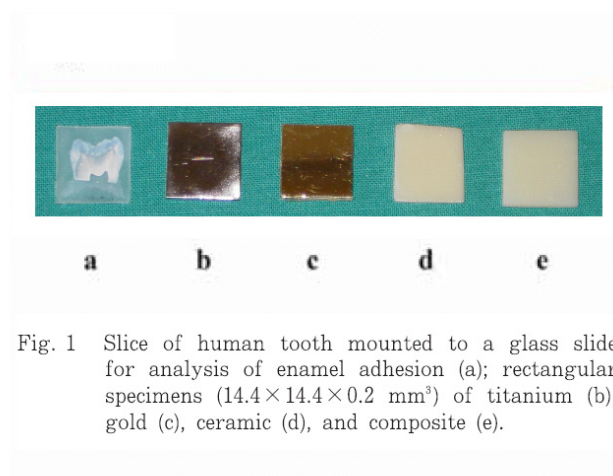


Fig. 1 Slice of human tooth mounted to a glass slide for analysis of enamel adhesion (a); rectangular specimens ($14.4 \times 14.4 \times 0.2$ mm³) of titanium (b), gold (c), ceramic (d), and composite (e).

Table 1 Restorative materials used in this study

Type of material	Chemical composition (%)	Commercial name	Manufacturer
Titanium	Ti (min. 99.5), Fe, O, H, N, C	Tritan, Rematitan® M	Dentaurum, Ispringen, Germany
Gold	Au (71.6), Pt (3.7), Ag (12.7), Cu (10.8), Zn (1.1)	Neocast 3	Cendres & Métaux SA, Biel-Bienne, Switzerland
Ceramic	*SiO ₂ (58-62), Na ₂ O (5-6), K ₂ O (9-11), Al ₂ O ₃ (14-16), BaO (3-4), MgO (0.3-0.8), CaO (1-2), ZrO ₂ (1-1.5), SnO ₂ (1-1.5), B ₂ O ₃ (0.3-0.8)	Vita Omega 900	Vita, Bad Säckingen, Germany
Composite	**Bis-GMA (8.3), UDMA (7.6), TEGDMA (4.3), BG (50.6), YbF ₃ (17), O (5), Ba-Al-FG (5), SiO ₂ (1)	Tetric Ceram	Ivoclar Vivadent AG, Schaan, Liechtenstein

*Reference: Rose, DR. Bond strength between a non-precious bonding alloy and ceramic after sol-gel-oxide coating. Thesis, FU Berlin, 2000.

**Bis-GMA: bis-A(di)glycidyl methacrylate; UDMA: urethane dimethacrylate; TEGDMA: triethyleneglycol dimethacrylate; BG: silanized barium glass; Ba-Al-FG: barium-aluminum-fluorosilicate glass

parallel. The dispenser and flow chamber were connected by tubes to a peristaltic pump (Spetec GmbH, Erding, Germany) with an integrated speed controller. Flow rate of the suspension was 0.8 ml/min, which corresponded roughly to physiological conditions of low shear in the oral cavity¹¹. The system was placed on a shaker adjusted at 260 impulses/min to maintain the homogeneity of the bacterial suspension at room temperature for one hour (Fig. 2). Thereafter, test specimens were removed and analyzed microscopically. Each material was tested in at least five independent experiments.

Determination of microbial vitality on substrata and in suspension

The vitality of adhered bacteria was evaluated by applying a dual fluorescent staining method (Live/Dead BacLight Bacterial Viability Kit, MoBiTec, Luzern, Switzerland) according to Decker¹², which allows differentiation between vital and dead bacterial cells. To stain bacteria attached to enamel and restorative dental materials, the test specimens were removed from the flow chamber, carefully dipped into distilled water to eliminate planktonic and loosely attached cells, covered with 7.5 μ l of staining solution for 15 minutes at room temperature in the dark, and subsequently placed on a slide. The cells were analyzed by epifluorescence microscopy (Provis AX70, Olympus AG, Volketswil, Switzerland) using two filters: blue excitation at 450-490 nm (FITC) and green excitation at 546 nm (rhodamine). The number of adherent vital cells and dead cells at eight randomly selected sites on

each substratum were counted and then calculated in per mm^2 (Fig. 3). Results were mean values of at least five independent experiments.

In addition, colony-forming units per ml of the bacteria-saliva suspensions were determined before and after the adhesion experiment. Appropriate dilutions of 100 μ l were plated onto Schaedler agar plates (BBLTM, Becton Dickinson, Basel, Switzerland) in duplicate and incubated anaerobically (AnaeroGenTM Compact, Oxoid AG, Basel, Switzerland) at 37°C for 48 hours.

Statistical analysis

Statistical analysis was performed using an open source programming language, R Version 1.6.1. Mann-Whitney test was used to compare data of each material with those of enamel. Level of significance was set at $\alpha=0.05$.

RESULTS

The tooth slices showed a surface roughness of $R_a=0.24 \mu\text{m}$, corresponding to the average roughness of enamel surfaces. As shown in Table 2, the surface roughness values of the materials tested were close to that of enamel.

Tooth slices showed a moderate hydrophobic surface with contact angles of 64.4° for the uncoated slide and 63.5° for the coated slide. Contact angle values of dental materials were in the range of 59.8° to 82.7° for the uncoated slides and 39.1° to 46.3° for the saliva-coated slides, thus indicating higher hydrophobicity of the uncoated metal surfaces (Table 2). In particular, the highest reduction in contact angle value by saliva coating was observed for the composite slide. Compared to the saliva-coated dental materials, the saliva-coated tooth slide was more hydrophobic.

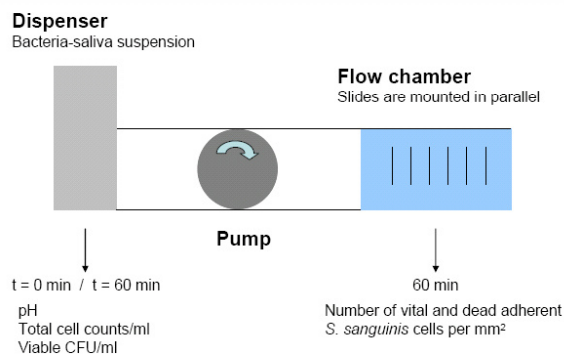


Fig. 2 Schematic diagram of study design. *S. sanguinis* saliva suspension circulated from the dispenser to the flow chamber containing the slides mounted in parallel. Total cell counts per ml, viable CFU per ml, and pH of bacteria-saliva mixture were determined at the beginning and the end of the experimental period. Analysis of slides was conducted after 60 minutes.

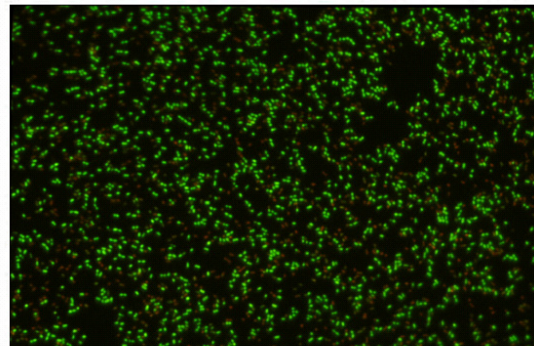


Fig. 3 Representative section of the gold surface after staining. Live attached bacteria are green, while dead ones are red.

Table 2 Surface roughness Ra (μm) and contact angles CA (degrees) of the tested substrata. Shown are means and standard deviations of Ra (n=10 for tooth slides, n=5 for dental materials), and for CA mean and quality of fit (=linear equation to experimental dataset)

	Enamel	Titanium	Gold	Ceramic	Composite
Ra (μm)	0.24 \pm 0.09	0.25 \pm 0.09	0.15 \pm 0.08	0.23 \pm 0.05	0.21 \pm 0.12
CA (degrees)					
Uncoated	64.4 (98.2%)	62.2 (99.1%)	65.3 (99.5%)	59.8 (98.4%)	82.7 (99.2%)
Saliva-coated	63.5 (99.5%)	42.8 (99.3%)	40.3 (98.9%)	46.3 (99.6%)	39.1 (99.2%)

Table 3 Parameters in the bacteria-saliva suspension at the beginning t_0 and at the end t_{60} of the experimental period. Shown are the means and standard deviations (n=10)

	t_0	t_{60}
pH	7.26 \pm 0.07	7.47 \pm 0.06
Total cell counts/ml (log)	8.99 \pm 0.09	8.89 \pm 0.09
Viable CFU/ml (log)	8.42 \pm 0.27	8.36 \pm 0.25

S. sanguinis suspended in PBS was found to be highly hydrophobic since this bacterial strain showed 89.9% partitioning to hexadecane. However, suspension of *S. sanguinis* cells in human saliva resulted in a low (<1%) partitioning to hexadecane, meaning that the bacterial cells exhibited hydrophilic behavior.

During the one-hour experimental period, bacterial density and vitality in the bacteria-saliva suspension of the flow chamber system remained fairly constant. Notwithstanding the slight increase in pH at the end of the test period, the bacteria-saliva suspension could be considered as a resting cell suspension (Table 3).

Figure 4 summarizes the results of the adhesion experiments. The number of adherent *S. sanguinis* cells per mm^2 was significantly higher on titanium, gold, and ceramic surfaces ($p < 0.001$) than on enamel, whereas significantly less bacteria adhered to the composite material ($p < 0.001$) (Fig. 4a). The percentage of vital adherent *S. sanguinis* was highest on enamel (92.5%), whereas it was significantly lower on the restorative materials tested, ranging from 41.5% to 69.1% ($p < 0.001$) (Fig. 4b).

DISCUSSION

In vitro approaches to studying plaque formation yield a two-fold benefit: they ensure strictly standardized test conditions and assure a high reproducibility.

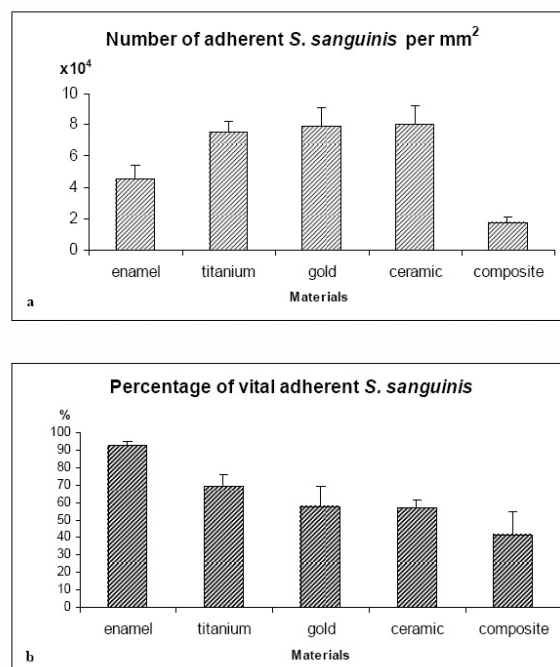


Fig. 4 *S. sanguinis* adhered to human enamel and different dental restorative materials. (a) Total number of cells per mm^2 ; (b) Percentage of vital streptococci. Shown are the means and standard deviations (n=5/10).

In the present study, the *in vitro* system used integrated host components such as enamel, saliva, and *S. sanguinis* to mimic some *in vivo* features of the oral cavity. The enamel analyzed herein represented the enamel portion of longitudinally cut tooth slices, which most likely had a different structure from the surface enamel to which bacteria adhere *in vivo*. Notwithstanding the difference, this set-up allowed comparison with other studies^{5,9}.

Results obtained with the model system revealed differences in cell adhesion and vitality of the adherent cells, thereby pointing to the different material characteristics of the substrata. It has been reported that the physicochemical properties of

a material — like surface free energy, hydrophobicity, and surface roughness, as well as material composition, affect initial bacterial adhesion¹³⁻¹⁵.

Quirynen and Bollen¹⁵ suggested that surface roughness and surface free energy were the main material-linked factors influencing bacterial adhesion. They further showed that the influence of surface roughness was stronger than that of surface free energy and surface hydrophobicity. Generally, rough surfaces promote bacterial adhesion whereas smooth surfaces minimize it¹³⁻¹⁵. According to Bollen *et al.*¹⁶, surface roughness below $Ra=0.2 \mu m$ had no further quantitative and qualitative effects on bacterial adhesion. Moreover, variations around this value had only a negligible impact on bacterial adhesion. In this study, the roughness of all test specimens was about $0.2 \mu m$ — hence differences in bacterial adhesion could not be explained in terms of surface roughness. This would mean that any observed differences in bacterial adhesion most likely resulted from other surface properties and the composition of the materials used.

Several studies reported that initial adhesion was promoted if both bacteria and the surfaces involved had similar hydrophobic properties^{8,17,18}. In this study, the *S. sanguinis* cells suspended in saliva showed a hydrophilic nature. In other words, it was expected that their hydrophilic nature would favourably facilitate their adhesion to more hydrophilic surfaces, like the pellicle-coated restorative materials used herein. Indeed, more bacterial cells adhered to three of the dental materials than to enamel — which had a moderately hydrophobic surface. These results were in agreement with other studies^{4,8,13}. However, it should also be highlighted that fewer bacteria attached to the composite, despite its hydrophilic surface property which was similar to the other materials tested.

In the oral environment, all solid surfaces are covered by an acquired salivary pellicle. The pellicle on tooth enamel and restorative materials is formed by a selective adsorption of salivary macromolecules. In this light, the physicochemical properties of a material influences microbial adhesion either directly or through adsorption of salivary proteins^{19,20}.

In the present study, salivary coating changed the surface hydrophobicity of the dental materials in a similar way. This was because no major differences in contact angle were registered between the materials. Amongst which, the composite material registered the highest reduction in contact angle value. At this juncture, it should be highlighted that pellicle composition may vary between different restorative materials. Consequently, different pellicle components would act as binding receptors for *S. sanguinis* cells, thus leading to differences in cell adhesion²¹.

Bacterial vitality during adhesion and biofilm formation is an important factor in the pathogenicity

of dental plaque²². The ability of bacteria to grow and produce acids is an essential process in dental caries development. In the present study, the percentage of adherent vital *S. sanguinis* was highest on the enamel surface and lower on restorative materials. Weiger *et al.*¹⁰ observed that during the initial events of microbial attachment, dead rather than vital *S. sanguinis* cells preferably attached to solid surfaces. Based on the data obtained, it could be said that this process was even more pronounced on the implant and restorative materials tested herein. Besides, some dental restoratives release metallic or fluoride ions into the environment — with a possible influence on the vitality of adherent bacteria²³⁻²⁵. This may augment the explanation for the lower percentage of adherent vital cells on the restorative materials used in this study.

Lowest bacterial vitality and adhesion were detected on the Tetric Ceram composite, which releases fluoride ions into the environment. Fluoride is known to have inhibitory antibacterial and anti-adherent effects²⁵. Thus, the fluoride released might have soundly contributed to the low vitality and adhesion of *S. sanguinis* cells. By way of practical application, the extent to which topically applied fluoride penetrates plaque biofilm is an important subject, since even limited fluoride penetration may serve to inhibit growth of plaque bacteria²⁶. Therefore, a constant release of fluoride ions from a substratum like composite, in addition to topically applied fluoride, might result in an improved inhibition of plaque bacteria.

The observation that the vitality of adhered *S. sanguinis* varied between dental materials suggested that different materials exerted different influences during early colonization. It was either dead cells were preferentially attracted, or that bacteria died after initial adhesion due to an antibacterial effect of the dental material, e.g., by leaching compounds. Within the detection range of this *in vitro* model, it was found that two keys factors played a pivotal role during the early stages of *S. sanguinis* colonization: physicochemical surface characteristics of the dental materials and stereo-specific interactions through the pellicle. The results confirmed that hydrophobicity, in the presence of adsorbed salivary components, significantly influenced bacterial adhesion to dental materials. In the case of the composite material, salivary pellicle might have significantly reduced the adhesion of *S. sanguinis*.

Dental plaque is a complex microbial biofilm. It involves many bacterial species which adhere to the tooth surface or restorative materials and which also interact with each other. Inevitably and predictably then, the composition and activity of dental plaque are also influenced by bacterial replication during phases of nutrient supply²⁷. *Streptococcus sanguinis* is just

one of several early colonizers, but is thought to play an important role in the initial stages of plaque formation¹⁾.

In the present study, we used an *in vitro* model to quantify bacterial adhesion and vitality to different dental materials by using fluorescence microscopy. Through this model, it was confirmed that the number and vitality of adhering bacterial cells varied with the nature of the dental restoratives. For other future studies, the parameters of the system, bacterial species, dental materials, and liquid environment can be changed and then their influences evaluated individually. In particular, the influence of surface modification of dental materials can be determined directly.

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Adhesion of oral streptococci to all-ceramics dental restorative materials *in vitro*

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Abstract

In recent years, patients have benefited from the development of better and more esthetic materials, including all-ceramics dental restorative materials. Dental plaque formation on teeth and restorative materials plays an important role in the pathogenesis of oral diseases. This study investigates initial adhesion of stationary phase streptococcal species to different all-ceramics dental restorative materials. The saliva-coated materials were incubated with the bacteria for one hour in an *in vitro* flow chamber which mimics environmental conditions in the oral cavity. Number and vitality of adhering bacteria were determined microscopically after staining. Surface roughness and the composition of the materials had no distinctive influence on bacterial adhesion. However, *S. mutans* and *S. sobrinus* adhered about tenfold less numerous to all materials than the other streptococcal species. Further, there was a correlation between bacterial vitality and materials' glass content. The results showed that early plaque formation was influenced predominantly by the presence of the salivary pellicle rather than by material dependent parameters whereas the composition of the all-ceramics appeared to have influenced the percentage of viable cells during the adhesion process. This presented *in vitro* technique may provide a useful model to study the influence of different parameters on adherence of oral streptococcal species.

1 Introduction

In the oral cavity all exposed surfaces are rapidly coated with a salivary pellicle, to which early colonizers, mostly oral streptococci, adhere (Li et al. 2004). These are the first steps in the formation of the oral biofilm, called dental plaque, the cause of caries and periodontal diseases (Rosan and Lamont 2000).

Dental plaque is present on human tooth tissues as well as on restorative materials (Teughels et al. 2006). Accumulation of bacteria on marginal areas of enamel and restorative material may lead to bacterial plaque formation and secondary caries (Wallmann and Krasse 1992). Since caries formation around existing restorations represents a primary reason for replacement there are efforts to minimize or prevent plaque formation on restorative materials (Mjör et al. 2000). Several *in vitro* and *in vivo* models exist to investigate adhesion of various oral microorganisms to dental restorations and the mechanisms involved (Bos et al. 1999; Guggenheim et al. 2004; Katsikogianni and Missirlis 2004).

The applications of all-ceramic restorations for medical and dental purposes have become very favoured owing to their high strength, biocompatibility and excellent esthetic properties (Anusavice 1995). They are a metal-free alternative to the widely used metal-ceramic structures (Lüthy et al. 2005). *In vitro* investigations on the mechanical properties as well as clinical studies have been published (Anusavice 1995; Filser et al. 2001). However, information on bacterial adherence to these materials is scarce.

The aim of this study was to investigate bacterial adhesion and vitality of two early colonizing (*S. sanguinis*, *S. oralis*) and two caries-associated (*S. mutans* and *S. sobrinus*) species of streptococci to four different all-ceramic dental materials after salivary coating in an experimental model which mimics environmental conditions in the oral cavity (Hauser-Gerspach et al. 2007). Particularly the effect of surface roughness, hydrophobicity and glass content of the materials were examined. A glass surface served as the control.

2 Materials and methods

2.1 Materials

Table 1 lists the dental ceramics tested with the corresponding glass content. Rectangular test specimens (14.4 x 14.4 x 0.2 mm³) were used as obtained from the manufacturer (Vita Zahnfabrik, Bad Säckingen, Germany). The surface roughness was measured by a Hommel tester (T 1000, Hommelwerke GmbH, VS-Schwenningen, Germany). Glass (borosilicat glass, ultrapur, Labor Vetter, Ammerbuch, Germany) was chosen as the reference because it behaves similarly to enamel with regard to microbial adhesion *in vitro* (Burgemeister et al. 2001) (and our own data). Before the adhesion experiments the slides were decontaminated with ethanol and exposed to the sterile human saliva at room temperature for 15 min. Contact angles as an index of hydrophobicity were measured using a Processor Tensiometer K100 (Krüss GmbH, Hamburg, Germany).

2.2 Bacterial adherence

The bacteria used for this study were: *Streptococcus sanguinis* DSM 20068 (German Collection of Microorganisms and Tissue Culture Cells, Braunschweig, Germany), *Streptococcus oralis* ATCC 35037 (American Type Culture Collection), *Streptococcus mutans* DSM 20523, *Streptococcus sobrinus* OMZ 176 (Oral Microbiology, Zürich, Switzerland). All species were grown aerobically at 37°C overnight until stationary phase in Schaedler broth (BBL™ Becton Dickinson, Basel, Switzerland) then harvested by centrifugation, washed with physiological saline and suspended in human saliva to a final Colony Forming Unite (CFU) of 10⁸-10⁹ ml⁻¹. Whole saliva was pooled from two healthy

volunteers and processed as described previously (Hauser-Gerspach et al. 2007).

Fig. 1 illustrates the study design. The flow rate of the suspension was 0.8 ml min^{-1} , which corresponds roughly to physiological oral conditions of low shear (Dawes et al. 1989). The system was placed on a shaker adjusted at $260 \text{ impulses min}^{-1}$ to maintain the homogeneity of the bacterial suspension. The bacteria were allowed to adhere to the surfaces during one hour at room temperature. The test specimens were removed, washed, stained by applying a dual fluorescent staining (Live/Dead BacLight Bacterial Viability Kit; MoBiTec, Luzern, Switzerland) and analyzed microscopically (Provis AX70, Olympus AG, Volketswil, Switzerland). The two fluorescent dyes allowed differentiation between vital (green) and dead (red) microorganisms (Decker 2001). Each material was tested with each streptococcus species in at least five independent experiments. In addition the optical density, CFU, and the pH of the bacteria-saliva mixture at the beginning and the end of the experiment were determined.

For measurements of hydrophobicity the streptococci were grown in Schaedler broth, washed and resuspended in PSB or human saliva. The measurements were done as described by Grivet *et al.* (Grivet et al. 2000) using partitioning into hexadecane (Sigma-Aldrich GmbH, Buchs, Switzerland).

2.3 Data analysis and statistic

A total of twelve digital images (ColorView, Olympus AG, Volketswil, Switzerland) using two filters [blue excitation at 450-490 nm (FITC) and green excitation at 546 nm (rhodamine)] were obtained for each sample and the adherent bacteria enumerated from twelve fields of view (each of 0.0239 mm^2).

The statistical analysis was performed using the open source programming language R

version 1.6.1. The Wilcoxon-test was used to compare data of each material and the corresponding bacteria with those for glass. The level of significance was set at $\alpha \leq 0.05$. Regression analysis was used to correlate percentages of vital adherent cells and materials' glass contents of the all-ceramic materials.

3 Results

During the experimental period of 1 h, bacterial density and vitality in the bacteria-saliva suspension of the flow chamber system remained nearly constant. Although the pH slightly increased at the end of the test period, the bacteria-saliva suspension can be considered as a resting cell suspension (Hauser-Gerspach et al. 2007).

3.1 Properties of the surface substrata

The different R_a values for surface roughness are presented in Table 2. Values for glass, MK, and YZ were similar. The all-ceramic ICA and ICZ yielded a 5 fold higher value.

Substratum surface hydrophobicities were evaluated by measuring water contact angles. Glass and the all-ceramic restorative materials showed a hydrophobic surface (Table 2). Coating with human saliva drastically reduced hydrophobicity of all test specimens.

3.2 Properties of cell surfaces

Bacterial surface hydrophobicities were evaluated by quantifying partitioning to hexadecane (Table 3). All four streptococci cultured in Schaedler broth and resuspended in PBS were highly hydrophobic. However, suspension of the streptococcal cells in human saliva resulted in < 1% partitioning to hexadecane, meaning that these bacterial suspensions behaved hydrophilic.

3.3 Streptococcal adherence to substrata surfaces

The results of the adhesion experiments are summarized in Fig. 2 and Table 4. *S. sanguinis* and *S. oralis* were not significantly different and revealed the greatest adherence whereas *S. mutans* and *S. sobrinus* showed significantly lower adherence (Fig. 2a) to all the materials investigated.

Any given streptococcal species adhered to the different materials in similar numbers (Fig. 2a; Table 4), although the surface roughness Ra of ICA and ICZ was fivefold higher than that of MK, YZ, and glass. This indicates little material-related or Ra-related differences in adherence.

3.4 Vitality of adhered bacteria in relation to glass content

The percentages of vital adherent cells are presented in Fig. 2b. Overall they were significantly lower on ICA, ICZ, and YZ with *S. sanguinis* and *S. oralis*. *S. mutans* and *S. sobrinus* showed no significant differences in the percentage of vital adherent cells compared to the glass surface except for YZ with *S. mutans*. The linear regressions between the percentages of vital adherent cells and materials' glass contents of the all-ceramic materials are given in Fig. 3. Positive correlations were obtained for *S. sanguinis* ($r = 0.63$), *S. oralis* ($r = 0.86$), *S. mutans* ($r = 0.79$) and *S. sobrinus* ($r = 0.19$).

4 Discussion

The *in vitro* model mimics environmental conditions in the oral cavity such as human saliva, the selected bacteria and some shear forces in the circulating medium. The all-ceramic dental materials used differ in their mechanical properties like strength, reliability, and the fracture mechanism due to their glass content (Lüthy et al. 2005). The purpose of this investigation was to investigate adhesion of four streptococcal species to these different materials with regard to number and vitality. Factors like type of the culture medium, culture conditions, and growth phase of the bacteria may influence *in vitro* the early bacterial adhesion (Sardin et al. 2004). To minimize the effects of different growth conditions all strains were prepared identically so that differences in bacterial adhesion would result from the salivary pellicle, or materials properties like hydrophobicity, roughness or glass content.

4.1 Evaluation of bacterial adhesion among the streptococcal species

The composition of the materials and their physico-chemical properties like hydrophobicity are known to modulate initial bacterial adhesion (Teughels et al. 2006). This initial unspecific adhesion was facilitated if bacteria and surfaces involved had similar hydrophobic properties (Grivet et al. 2000; Teughels et al. 2006). The initial layer deposited on the dental all-ceramic specimens and glass was human saliva. This coating reduced the contact angles measured and made all surfaces more hydrophilic which is in accordance to the findings of Quirynen and Bollen (Quirynen and Bollen 1995) who concluded that coating has a drastically effect on hydrophobicity of the substratum. Since the four streptococci species suspended in saliva showed similar hydrophilic nature similar adhesion profiles to the pellicle-coated all-ceramic slides were expected. However, the results revealed about ten fold differences in cell adhesion. Therefore, hydrophobic interactions are not the only mechanism involved in the

adherence of these streptococci to the surfaces.

A more firm adhesion can be established between a bacterium and a surface through specific interactions (Katsikogianni and Missirlis 2004). This is mediated by specific components on the surface of the adhering organism and receptor molecules of the pellicle on the substratum surface (Gong et al. 2000). The observations suggest that there were fewer binding components for *S. mutans* and *S. sobrinus* than for *S. sanguinis* and *S. oralis*, even though saliva was prepared from two volunteers. Both, hydrophobic sites of the bacterial cells and sites complementary to saliva pellicle seemed contributing to bacterial adherence to the surfaces.

The specific adhesion process to the acquired pellicle is also mediated by extracellular polysaccharides (Katsikogianni and Missirlis 2004). In the presence of sucrose *S. mutans* and *S. sobrinus* synthesize extracellular glucans via glucosyltransferases (Banas and Vickermann 2003). These glucans promote adhesion of these two streptococcal species to the salivary pellicle and to other bacterial cells. The resting cells used in these experiments had been carefully washed to remove traces of the medium. After suspension in human saliva there was no or little sucrose available for synthesizing extracellular glucans *de novo*. This aspect could also be responsible for the low binding of the two species to the surfaces and emphasize the importance of glucans during the adhesion process of mutans streptococci.

4.2 Evaluation of bacterial adherence and vitality in relation to materials' properties

The effect of surface roughness on bacterial adherence is complex. It was found both *in vivo* and *in vitro* that bacteria accumulated to a greater degree on rough surfaces than on a highly polished surface (Teughels et al. 2006). According to Bollen *et al.* (Bollen et al. 1996) $R_a \leq 0.2 \mu\text{m}$ had a negligible impact on bacterial adhesion whereas higher values correlated with

higher numbers of adhering cells. In the present study the significantly higher (5 fold) surface roughness of the dental ceramics ICA and ICZ did not result in a significantly higher number of adherent bacteria. Also no relationship was found between bacterial adherence and the glass content of the materials indicating that the composition of these materials exerted no influence on bacterial adhesion in saliva. It is conceivable that salivary proteins are adsorbed onto the surface of the materials in a similar adsorption pattern regardless of different surface roughness or glass content. The influence of the specific interactions with the bacterial surface was more important in this adhesion model than materials' properties.

We found a relationship between the percentage of vital adherent streptococci cells and the glass content of the dental ceramics. These results are in agreement with an earlier study (Hauser-Gerspach et al. 2007) where a lower proportion of vital bacteria were adhering to dental restorative materials than to enamel. The question whether dead rather than vital *S. sanguinis* cells adhere preferentially to restorative materials has not been decided. The ceramic material used, Vita Omega 900, exhibited surface properties similar to MK in this study and showed similar percentages of vital adhered cells. Indeed, the vitality of adherent bacterial cells may be influenced by the composition of restorative materials as other *in vitro* and *in vivo* studies showed (Auschill et al. 2002; Boeckh et al. 2002).

5 Conclusions

The data reported in this study showed that specific interactions between streptococci cells and saliva-coated all-ceramic substrata predominate initial adhesion in this model. The materials' properties surface roughness and glass content had only a weak influence on adhesion. This *in vitro* technique may provide a useful model to study the influence of

different parameters (materials, saliva component, interfering substances) on adherence of oral streptococcal species.

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Table 1:

List of glass and dental ceramics used

Type of material	Code	Chemical composition	Glass content (vol%)	Manufacturer
Glass		Borosilicat, ultrapure	100	Labor Vetter, Ammerbuch/D
Vita Mark II	MK	Feldspathic Ceramics	96	Vita Zahnfabrik, Bad Säckingen/D
In-Ceram Alumina	ICA	Glass-Infiltrated Alumina	25	Vita Zahnfabrik, Bad Säckingen/D
In-Ceram Zirconia	ICZ	Zirconia-Reinforced Glass-Infiltrated Alumina	19	Vita Zahnfabrik, Bad Säckingen/D
In-Ceram YZ	YZ	Tetragonal Stabilized Zirconia	0	Vita Zahnfabrik, Bad Säckingen/D

Table 2:

Surface roughness Ra (μm) and contact angles CA (degrees) of the dental ceramics and glass used. Shown are means and standard deviations for Ra ($n = 4$ for each material) and for CA ($n = 3$ for each material with and without saliva-coating)

	MK	ICA	ICZ	YZ	Glass
Ra	0.26 \pm 0.01	1.33 \pm 0.08	1.34 \pm 0.13	0.26 \pm 0.02	0.24 \pm 0.05
CA:					
uncoated	82.9 \pm 2.9	86.6 \pm 2.4	83.5 \pm 4.3	81.4 \pm 4.2	81.5 \pm 1.3
saliva-coated	44.3 \pm 3.9	44.1 \pm 3.3	46.0 \pm 4.2	44.8 \pm 1.7	43.8 \pm 1.8

Table 3:

Bacterial partitioning to hexadecane

Means and standard deviations of bacteria suspended in PBS or saliva partitioning into the hexadecane phase (n=10)

solution	<i>S. sanguinis</i>	<i>S. oralis</i>	<i>S. mutans</i>	<i>S. sobrinus</i>
PBS	90.6 % \pm 3.3	90.3 % \pm 4.3	85.1 % \pm 4.0	85.6 % \pm 5.3
Human saliva	< 1 %	< 1 %	< 1 %	< 1 %

Table 4:

Means and standard deviations of adherent streptococci on dental ceramics and glass per mm² (n=5).

	<i>S. sanguinis</i>	<i>S. oralis</i>	<i>S. mutans</i>	<i>S. sobrinus</i>
Glass	46400 ±18300	41100 ±12800	4300 ± 700	2500 ±200
MK	48600 ±17000	42600 ±18700	4100 ±1100	2900 ±700
ICA	65400 ±21600	30900 ± 8600	3800 ± 400	2800 ±300
ICZ	58000 ±16600	30100 ± 6100	3600 ±1000	2700 ±150
YZ	67500 ±18300	39900 ±12100	3800 ± 300	3100 ±300

* p=0.02

Figure legends

Fig. 1:

Study Design: Starting from the dispenser, the bacteria-saliva suspension circulated via a peristaltic pump to the flow chamber containing the test specimens mounted in parallel. The different dental ceramic and glass surfaces were analyzed after 60 min (see text for details).

Fig. 2:

Streptococci adhered to different dental ceramics and glass. Shown are means and standard deviations (n=5). Values significantly different from the respective value for glass are marked with an asterisk. a: Total number of cells per mm². b: Percentage of vital adherent streptococci.

Fig. 3:

Relationship between materials' glass content and percentage of vital streptococci. ♦ *S. sanguinis* (r = 0.63); ■ *S. oralis* (r = 0.86); ▲ *S. mutans* (r = 0.79); ● *S. sobrinus* (r = 0.19).

Fig. 1:

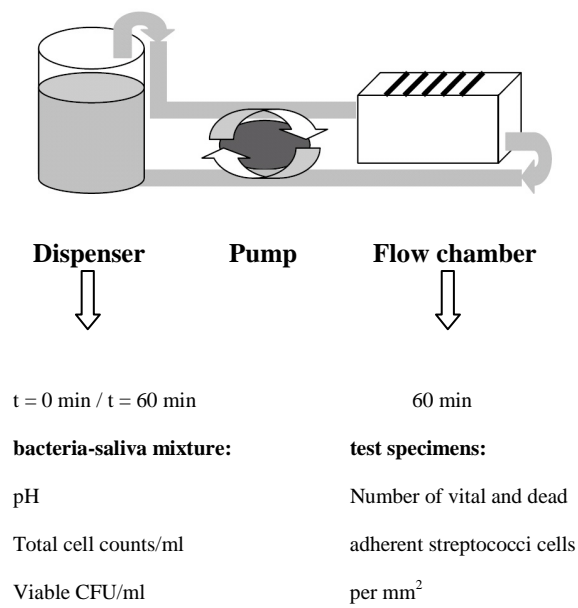


Fig. 2:

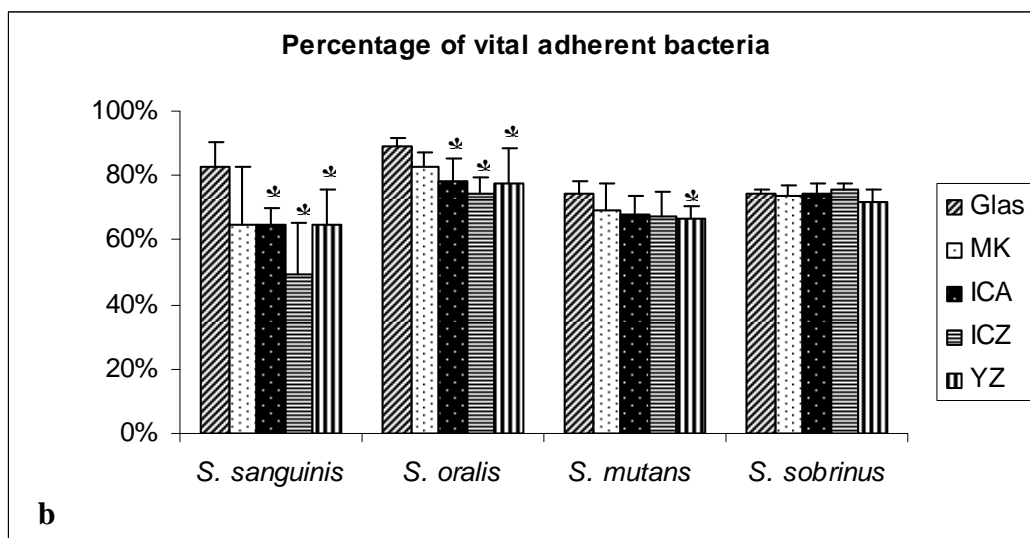
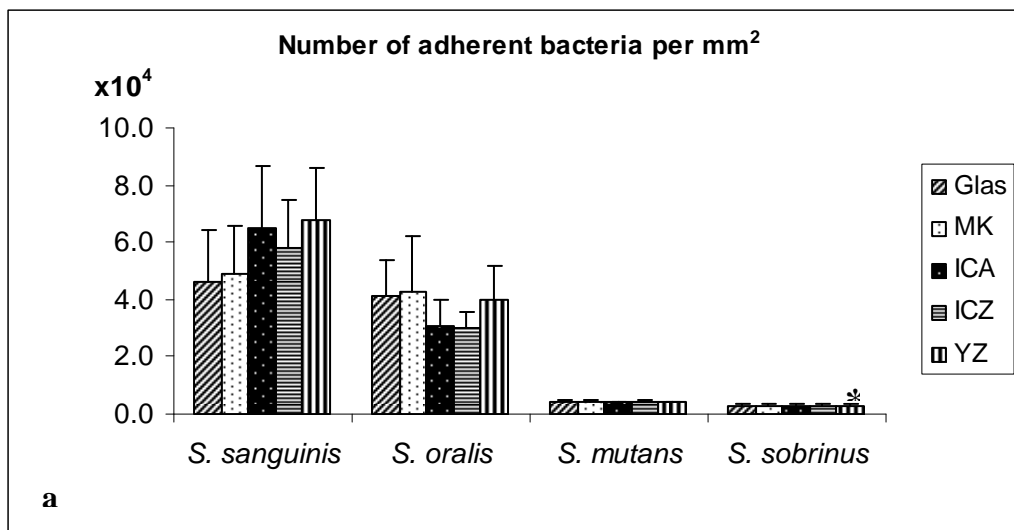
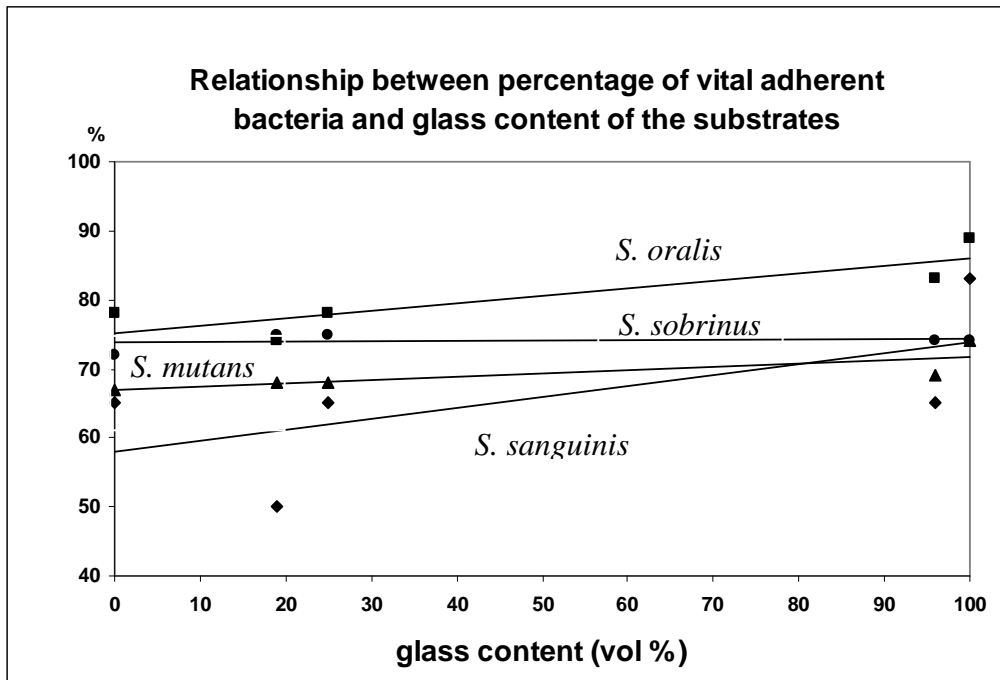
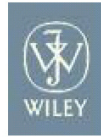


Fig. 3:





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**Adhesion of *Streptococcus sanguinis* to Glass Surfaces can be Measured
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Original Research Paper

Adhesion of *Streptococcus sanguinis* to Glass Surfaces can be Measured by Isothermal Microcalorimetry (IMC)

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Abstract:

Bacterial adhesion is the first step in the development of the oral biofilm, called dental plaque. Plaque is the cause of caries, periodontal diseases, and peri-implantitis. Investigations of dental plaque, including bacterial adhesion, employ various in vivo and in vitro models using microscopic methods. Microcalorimetry offers another direct approach.

The model organism *Streptococcus sanguinis* is one of the first colonizers adhering to the saliva-coated human tooth surfaces or dental materials within minutes after tooth cleaning. TAM III™ thermostats, equipped with microcalorimeters were used for isothermal microcalorimetric (IMC) measurements of heat production as a function of time, expressed by power-time (p-t) curves.

Continuous measurements of heat production of growing *S. sanguinis* cells showed their overall metabolic activity and were highly reproducible. For the adhesion experiments the bacteria were allowed to adhere to different amounts of glass beads.

Growing *S. sanguinis* cells produced a characteristic p-t curve with a maximum of 500 μW at 4.5h when reaching 10^9 cells ml^{-1} . The same number of stationary *S. sanguinis* cells, suspended in PBS produced only ~ 30 μW at 0.5h due to adhesion. But the amount of heat increased with available glass surface area, indicating that a portion of the heat of adhesion was measured. Similar results were obtained with stationary *S. sanguinis* cells suspended in human saliva.

This study shows that microcalorimetric evaluation of initial bacterial adhesion is indeed possible and may become a rapid, reproducible screening method to study adhesion of different bacteria to different dental materials or to modified surfaces.

Running Heads: Determination of bacterial adhesion by microcalorimetry

INTRODUCTION

Metabolic processes of bacterial cells are usually accompanied by heat production (changes in enthalpy) and can be monitored continuously using an isothermal microcalorimeter (IMC). Microcalorimetry is applied in a wide range of fundamental studies and technical applications in biological research and quality control¹. In microbiology it has been used for investigating replication rates of bacterial cells, and effects of biocides on microbial activity²⁻⁴. Also, first attempts for species identification, based on the nature of the heat flow rate curves have been reported⁵⁻⁸ as well as studies on bacterial motility and coaggregation⁹. Further, microcalorimetry was used to compare metabolism of planktonic cells vs cells in biofilms¹⁰⁻¹² and is currently being explored in diagnostics of septic infections (Daniels et al. in preparation).

In the oral cavity, hard tissue surfaces such as human enamel and restorative dental materials are substrates for dental plaque formation. Microbial colonization on such surfaces is always preceded by the adsorption of host saliva proteins, called pellicle¹³. Depending on plaque composition and activity, plaque can result in the development of dental caries and periodontal diseases¹⁴. Bacterial adhesion is an early step in the development of biofilms leading to plaque formation, and it may influence the composition of the mature plaque¹⁵. The activity of microorganisms and their physiological status in this process are complex. Reported investigations in the field of dental plaque, including bacterial adhesion, employ various in vivo and in vitro models and use microscopic observation methods¹⁶⁻¹⁹. Microcalorimetry (IMC) offers another approach, potentially allowing measurement of the energetics of the adhesion process.

Streptococci constitute a major population of the oral microbiota. *Streptococcus sanguinis* was selected as test species because it is one of the early colonizers on exposed tooth surfaces and dental materials²⁰. The early colonizers are of great importance in the successive stages of

dental plaque formation, providing sites for attachment, or modifying the environment for subsequent colonizers. Adhesion of these pioneer bacteria is initially mediated by various combinations of electrostatic charge, Van der Waals (dipole interaction) and other forces acting between the bacterial cell surface and the substratum. Subsequently, specific adhesin-receptor interactions are involved²¹.

Adhesion of *S. sanguinis* to human enamel, glass slides, and commonly-used dental restorative materials has been observed *in vitro* during one hour in a flow chamber²². The bacteria were suspended in human saliva which simulates conditions in the mouth. Also they represented a resting cell population in which nearly all metabolic activities were at a very low level or eliminated. Thus, adhesion to a surface was observed as an isolated process²².

The aim of this study was to measure directly by IMC this primary, isolated process of microbial adhesion to a glass surface. Glass beads were chosen as a substratum because glass is easily obtained as beads, and glass and enamel behave similarly with regard to microbial adhesion *in vitro*²³.

For the IMC studies conditions similar to those in the flow chamber were used, plus for comparison, bacteria suspended in PBS, a liquid with no nutrition source. In addition the heat production of growing *S. sanguinis* cells was measured continuously by IMC to demonstrate the marked difference between total metabolic activities of replicating cells, in contrast to resting cells suspended in human saliva or PBS.

MATERIALS AND METHODS

1. Bacteria and culture condition

A 10 µl inoculum of *Streptococcus sanguinis* DSM 20068 (German Collection of Microorganisms and Tissue Culture Cells, Braunschweig, Germany) preserved in skim-milk solution at -20°C was suspended in 5 ml Schaedler broth (BBL™ Becton Dickinson, Basel, Switzerland) and incubated aerobically at 37°C for 8 h. An inoculum was transferred to fresh Schaedler broth (1:50) and grown at 37°C for 16 h. This culture was sonicated for 45 s (30 Watt, 20 kHz, Vibracell™ Ultrasonic Processor, Sonics, Newtown, USA) to break bacterial chains and aggregates, washed with physiological saline, harvested by centrifugation at 8000 g for 5 min, and resuspended in PBS (GIBCO, Invitrogen, Switzerland) or human saliva to the experimental concentration needed.

The number of bacteria in the suspensions at the beginning and at the end of each experiment were determined both microscopically (Provis AX70, Olympus AG, Volketswil, Switzerland) using a standard Neubauer chamber, and by culture. Colony-forming units per ml (CFU ml⁻¹) of the bacterial suspensions were determined by plating of appropriate dilutions in duplicate onto Schaedler agar plates (BBL™ Becton Dickinson, Basel Switzerland). In addition, presence of aggregates of more than approx. 20 cells was monitored at the beginning and at the end of the experiments by phase contrast microscopy. A few aggregates were occasionally observed at the beginning in the saliva suspension but not in PBS.

2. Saliva

Whole saliva was collected by paraffin stimulation from a healthy volunteer (after not drinking or eating for 2 h). To remove debris the saliva was sonicated (45 s, 30 Watt, 20 kHz), filtered through a 70 µm filter (Cell Strainer, Becton Dickinson, Basel, Switzerland) and centrifuged at 22,000 g for 60 min at 4°C. The supernatant was filtered through two low

binding filters (pore sizes 0.45 µm and 0.22 µm; Millex-HV and Millex-GV respectively, Millipore, Switzerland) connected in series. The result was a saliva sample free of microorganisms, particulate debris, and may have lost some mucins. The processed, sterile saliva sample was stored at 6°C and used experimentally within two days. Before use, the pH of the sample was adjusted to 7.1-7.3 with phosphate buffer (0.067 mol/l Na₂HPO₄/KH₂PO₄).

3. Isothermal calorimetry

The heat flow during adhesion and/or growth of *S. sanguinis* was measured in isothermal microcalorimetry (IMC) systems (Thermometric AB, Järfälla, Sweden). Two sets of microcalorimeters were used. Each set was installed in a TAM III™ thermostat. One thermostat was equipped with 48 individual microcalorimeters (Thermometric No. 3206) and the other thermostat was equipped with 6 microcalorimeters identical to the other 48, plus 3 twin nanocalorimeters not used in this work. The TAM III™ thermostat is capable of maintaining a set temperature within ± 1.0 × 10⁻⁸ °C over a 24 hour period, with an absolute accuracy of ± 0.1 °C. The detection limit of each microcalorimeter is expressed by Thermometric AB using a 24-hour error function:

$$Error = \sqrt{\left(\frac{Drift}{2}\right)^2 + Deviation^2}$$

where

Drift = slope of the linear baseline fit

Deviation = twice the standard deviation of the baseline fit

Error = maximum error of the heat flow after 24 hours

The value given by Thermometric AB for the error for the No. 3206 microcalorimeters is ± 0.225 µW. In practice, this means that a heat flow signal of ~ 0.3 µW above baseline, or a rise in heat flow of ~ 0.3 µW, or a difference of ~ 0.3 µW between two heat signals is detectable.

Signal collection was done using the SciTech Software AB (Thermometric AB, Järfälla, Sweden) supplied with the thermostats and microcalorimeters.

4. Calorimetry of growth of *S. sanguinis*

Sterile ampoules were filled with 2.5 ml Schaedler broth (BBL™ Becton Dickinson, Basel, Switzerland) at 37°C and an inoculum of *S. sanguinis* was transferred to the liquid to final concentrations of CFU $5 \times 10^6 \text{ ml}^{-1}$ or CFU $5 \times 10^5 \text{ ml}^{-1}$. Two ampoules each were immediately sealed with a sterile cover and put in the microcalorimeters at 37°C for measurement. If bacteria are allowed to replicate in a microcalorimeter ampoule, the rate of increase in the heat signal ($\mu\text{J}/\text{sec}/\text{sec} = \mu\text{W}/\text{sec}$) at time t is proportional to the replication rate of the bacteria at that time. The heat flow rate ($\mu\text{J}/\text{sec} = \mu\text{W}$) at time t is proportional to the number of replicating bacteria present, and the total amount of heat evolved (μJ) by time t is proportional to the number of bacteria that have been produced up to time t .

Additional ampoules at both initial CFU were incubated outside the microcalorimeters in a 37° C water bath for separate, parallel quantitation of cell growth. Samples of these cultures were taken at different times to determine optical density, pH, and CFU ml^{-1} . The experiments were repeated twice resulting in six measurements.

5. Calorimetry of adhesion of *S. sanguinis* to glass

S. sanguinis cells were allowed to adhere to three configurations of glass surfaces: (a) the inner glass wall of the ampoules alone, (b) inner glass wall plus a low (0.3 g) amount of glass beads and (c) inner wall plus a high (3.0 g) amount of glass beads ($d=2.13 \text{ mm}$; Labo-Tech LTS AG, Biel-Benken, Switzerland) placed in the ampoule. Prior to use the glass ampoules and beads were cleaned with detergent, rinsed in distilled water and autoclaved.

To observe only the heat production related to attachment of the bacteria to the glass (and not

heat production related to replication) the bacteria were kept in a resting phase in which little or no metabolic activity or replication was expected. In order to achieve this goal, the cells were washed and suspended in either PBS or in human saliva.

Experiments were performed isothermally at 37° C in autoclaved duplicate glass ampoules and filled with liquid (PBS or human saliva)/glass beads as shown in Figure 1. The 4 ml ampoules, without or with a low or high amount of glass beads, were filled with 2.25 ml PBS or human saliva, and were allowed to equilibrate over night within the microcalorimeters. After obtaining baseline heat flow rate values for the solution, the ampoules were removed from their microcalorimeters and injected with 250 µl of the streptococcal suspension at 50 µl s⁻¹ (resulting in a concentration of approximately 10⁹ CFU ml⁻¹), while control ampoules were injected with 250 µl of PBS or human saliva at 37°C (Fig. 1).

The ampoules were put immediately back into the microcalorimeters. As per standard IMC practice, they were first lowered to the microcalorimeter's temperature equilibration position, kept there for 15 minutes, and then lowered to the measurement position. The heat flow was then measured during at least three hours. The experiments were repeated five times.

6. Confirmation of adhesion by methods other than microcalorimetry

6.1. Visualization of bacteria adhered to glass surface

To stain and detect bacteria attached to the inner glass wall of an ampoule, the bacterial suspension was removed, and the ampoules were washed carefully with distilled water in order to eliminate planktonic cells. The ampoules were then subjected to dual fluorescent staining (Live/Dead BacLight Bacterial Viability Kit; MoBiTec, Luzern, Switzerland). The stained cells adhering to the ampoule glass were analyzed by epifluorescence microscopy (PROVIS AX70, Olympus AG, Volketswil, Switzerland) as described²². Adhesion to the glass beads was not evaluated microscopically.

6.2. Removing viable bacteria from glass surfaces after measurements in the microcalorimeter.

At the end of the calorimetric measurements the bacterial suspension was removed and the ampoules (both those without beads and those containing beads) were washed twice with 0.9 % NaCl. Then 2.5 ml of 0.9 % NaCl solution was added. In order to remove the bacteria adhered to the glass wall or/and the glass beads, the ampoules containing the solution were first vortexed for 30 s and then sonicated with five pulses (5 s, 30 Watt, 20 kHz). Afterwards CFU ml⁻¹ was determined.

7. Blocking bacterial metabolism with uncoupler CCCP

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma-Aldrich GmbH, Buchs, Switzerland), was used as uncoupler (Khan and Macnab 1980), which abolish protonmotive force. CCCP was dissolved in methanol (Merck AG, Buchs, Switzerland) and added to Schaedler broth (BBL™ Becton Dickinson, Basel, Switzerland) at different concentrations to determine its inhibition effect on growth and replication of bacteria. CCCP was then used at a final concentration of 12 μM, which blocks metabolism but has a low killing activity (results not shown).

Parallel heat flow measurements were done isothermally at 37 °C in glass ampoules filled with *S. sanguinis* PBS/saliva suspension (10⁹ CFU ml⁻¹) without or with 3 g glass beads and added 12 μM CCCP.

8. Data analysis and statistics

The heat flow rate (in μW) was recorded at 1 min intervals. Total energy (μJ) was calculated as the area under the power-time curves. Heat flow rate (μW) signals from bacterial activity were in all cases at least 10x the microcalorimeter's detection limit ($\sim 0.3 \mu\text{W}$) and in some cases 1600x the detection limit. Heat flow from control ampoules was almost negligibly low but was nevertheless subtracted from corresponding experimental values.

The statistical analysis was performed using the open source programming language R version 1.6.1. Unpaired t-tests were used to determine whether differences existed between groups. Results were considered statistically significant when the p-value was less than 0.5.

RESULTS

1. Heat measurements during growth of *S. sanguinis*

Heat production associated with the growth of *S. sanguinis* cells in Schaedler broth at 37° C (and heat associated with adhesion to the inner glass wall of the ampoule, see below) was monitored continuously. Typical power-time curves (p-t curves) in relation to the corresponding growth parameters are shown in Fig. 2. The two concentrations of the start inoculums (5×10^6 CFU ml⁻¹ and 5×10^5 CFU ml⁻¹, respectively) led to almost identical p-t curves, with the peak heat flow for the lower start inoculum occurring about two hours later. This time shift corresponds to the time needed to increase the number of viable cells by factor of 10 or to reach the same OD. Mean total heat produced by the bacteria of the two cultures during 24 h incubation, obtained by integration of the p-t curves was essentially independent of initial bacterial concentration (109,000 μJ vs. 107,000 μJ, n = 6). This is because the ampoules are closed systems and therefore, regardless of initial concentration bacterial replication proceeds until some set of limiting conditions is reached (e.g., consumption of available oxygen or nutrients, build up of metabolic byproducts). Therefore from any initial concentration, approximately equal numbers of bacteria are produced in the closed ampoule. The data also make it evident that the calorimetric measurements are highly reproducible, and that any variations are due to variations in experimental conditions; e.g. small differences in the amount of culture medium present in the sealed ampoule.

2. Heat flow in stationary *S. sanguinis* suspensions

To detect heat flow during attachment of the bacteria to surfaces, replication and nearly all metabolic activity were avoided by using stationary *S. sanguinis* suspensions. In order to achieve this goal, *S. sanguinis* cells were washed and suspended in a liquid with either no nutrition source, PBS, or a very limited one, human saliva. Indeed, over a period of four hours

at 37°C the number of vital bacteria (CFU ml⁻¹) in PBS or human saliva remained nearly constant (Fig. 3), indicating a resting cell population. In addition, the number of bacteria (microscopic cell counts ml⁻¹) and the vitality (CFU ml⁻¹) were determined at the beginning and the end of the experimental microcalorimetric period of 3h. Vitality remained constant in PBS (log CFU ml⁻¹ 9.35±0.15 at the beginning and 9.23±0.10 at the end) as well as in human saliva (9.35±0.51 and 9.32±0.10, n = 30, respectively). No increase in the number and size of bacterial aggregates was observed during the 3h experimental period.

Heat produced by stationary *S. sanguinis* suspensions was very low in comparison to that produced by *S. sanguinis* cultures (Fig. 4). Indeed, heat flow rate in stationary bacterial suspensions was well below 50 µW during the 3h measurements, while the same number of *S. sanguinis* (10⁹ CFU ml⁻¹) in growth (reached after 5h in culture, B in Fig. 4) produced a peak heat flow rate of ~500 µW. Heat flow from control ampoules without bacteria was negligible (< 0.1 µW), but never the less was subtracted from the values of test ampoules to produce Fig. 4.

A higher resolution presentation of the same representative p-t curves of stationary *S. sanguinis* cells suspended in PBS or human saliva (without or with glass beads to increase adhesion surface) is given in Fig. 5. Heat flow rate from ampoules with bacterial suspensions in PBS (Fig. 5a) declined continuously during measurements but was higher in the ampoules with glass beads. Heat flow from stationary bacteria suspended in human saliva (Fig. 5b) also increased when the available glass surface area was increased by addition of glass beads. However, in saliva initial heat flow rates were higher with glass beads than in PBS, and with or without the glass beads, heat flow rates remained higher from 0.5-1.5 hours before beginning to decline. Table 1 compiles the mean cumulative heat production of stationary *S. sanguinis* for the 0.5-1.5 hour period identified in Figure 5. Increased surface areas, provided by 3g glass beads, were associated with significantly greater energy production in both liquids

(Table 1). Since the number of bacteria introduced was constant, and there was no bacterial replication occurring, it seems clear in the case of PBS that the increases in total heat derive primarily from some interaction of the bacteria with the glass surface. In saliva, one can ask if the increased heat might be related in part to interaction with the bacteria (e.g. adhesion) of proteins or other large molecules in the saliva rather than bacteria alone. Heat produced by interaction of those molecules with glass, was also measured in the control ampoules and was low (the values were subtracted). Evidence presented later below suggests that the extra initial heat can be interpreted as the heat of adhesion of the bacteria to the glass surfaces. Also, the increased magnitude of the heat signals in saliva compared to PBS *after* 1.5 hours suggest that there may also be a lingering increase in residual metabolic activity of either adhered or suspended bacteria after exposure to the glass in the presence of molecules in the saliva that are not present in PBS.

Another way to inhibit bacterial growth without killing the cells is by applying an uncoupler²⁴, which blocks ATP synthesis without interfering with electron transport. Heat was measured in presence of 12 μ M CCCP and in its absence. No difference was observed in the ampoules containing *S. sanguinis* suspended in human saliva or PBS in presence or absence of CCCP (data not shown). This clearly indicates that metabolic activity contributes little to the energy measured in comparison to the adhesion process.

3. Confirmation of bacterial adhesion to ampoule glass surface

Bacterial adhesion to the inner glass wall of ampoules was visualized by staining after microcalorimetric measurements. A uniform distribution was seen in experiments with *S. sanguinis* suspended in either PBS or human saliva (Fig 6). Blocking ATP synthesis with CCCP had no effect on distribution of adhered bacterial cells (data not shown).

In addition, bacterial adhesion was quantitated after microcalorimetric measurements by using

sonication to remove the adhered cells for quantitation. The number of viable bacteria which the sonication released from ampoules with 3 g glass beads was 5.5 fold and 8.5 fold higher than from ampoules without glass beads, depending on whether the bacteria had been suspended in PBS or in human saliva. These data support adhesion of *S. sanguinis* to the ampoules and the glass beads as the source of the higher heat signals measured when the glass surface area was increased with beads.

DISCUSSION

Since bacterial metabolism under growth conditions is associated with heat production, microcalorimetry has been used as a convenient method to study growth and replication of cells in a wide array of environmental conditions. Characteristic power-time (p-t) curves have been observed, which were specific for the bacterial strain and the particular growth conditions used^{2,3,8}. The p-t curve shown here for *S. sanguinis* in Schaedler broth shares similarities but is clearly different from those published for other bacterial species in other growth media. This is no surprise because the shapes of the curves are imposed by environmental conditions (e.g., medium composition, temperature, pH, available oxygen, start inoculum) and the type of organism⁸. The data presented also document the sensitivity and reproducibility of the microcalorimetric approach.

Heat production from bacterial growth in complex media, such as that from replication of *S. sanguinis* in Schaedler broth, results from an overlap of metabolic steps, random transport, aggregation, and adhesion and produces p-t curves which reach high (hundreds of μW) levels of heat production, as demonstrated in Figure 4. These curves record the aggregate effect of different types of heat production from replicating cells and from the same number of bacteria under non-replicating conditions. Under replication conditions, it is not possible to isolate heat of adhesion from the other phenomena that produce heat.

Adhesion of early colonizers like *S. sanguinis* to teeth in the oral cavity starts immediately within minutes after exposure of clean surfaces to the oral environment and later proceeds into a complex process of biofilm formation. This adhesion process has been studied in vitro by microscopic methods²⁵⁻²⁸ under conditions comparable to those used here. Indeed, it also occurred as expected in the ampoules used for measurements. However, for technical reasons (e.g., lowering the ampoules into the measurement position itself constitutes a thermal event) microcalorimetric measurements were reliable only 30 minutes after the samples arrived in

measuring position (i.e. 45 minutes after addition of the bacteria). After 45 minutes, protein adsorption and bacterial adhesion likely had already started and even passed the maximal rate. This is reflected in the data shown in Figure 5. It means that total adhesion quite likely produced considerably more heat than measured for the one hour observation interval (Table 1). The heat of bacterial adhesion from earlier stages probably could be captured by isothermal titration calorimetry (ITC). This is a version of IMC in which sequential μL aliquots of a substance of interest (e.g. bacteria in suspension) are introduced via a capillary tube into an ampoule already equilibrated in a calorimeter chamber, and resultant heat flows are measured. The sealed ampoule IMC method was used instead in these exploratory experiments for two reasons. First, the method is much simpler. In addition, the availability of instruments with as many as 48 microcalorimeters which accept sealed ampoules also means that the sealed ampoule method was (and will be) more useful for rapid comparison of the effects of various variables on heat of adhesion. Thermostats equipped for ITC are typically available with only four calorimeters.

Could the heat measured originate from processes other than bacterial adhesion? Constant CFU values during the experimental period indicate stationary and not growing cells. Residual metabolism might contribute, but this does not seem to have been the case since heat production was not reduced in ampoules in which bacterial metabolism had been blocked by CCCP. Bacterial aggregation could represent another process. Indeed, coaggregation of the bacteria pair *Actinomyces naeslundii* 147 and *Streptococcus oralis* J22 has been shown to produce measurable heat flow with a coaggregation enthalpy of -0.015×10^{-6} mJ/bound streptococcus⁹. However, the *S. sanguinis* strain studied here showed very little aggregation in saliva and none in PBS at the beginning of the experiments and no increase during the experimental conditions used.

The increased heat signals in saliva compared to PBS could indicate that interactions of large

biomolecules such as proteins with the glass surfaces could provide additional adhesion sites for *S. sanguinis* and thus contribute to heat production. However, bacteria suspended in either human saliva or PBS had been carefully washed before to remove traces of the medium, including large biomolecules, used to grow the cells to stationary phase. It is known that salivary proteins of the pellicle influence the adherence and plaque building of oral bacterial cells. In vitro studies have identified and characterized salivary and bacterial proteins involved in different ways in this process, including effects on cell viability or metabolic activity, bacterial aggregation, and adherence to enamel pellicle²⁹⁻³¹. The detected difference in heat production for *S. sanguinis* suspended in PBS versus human saliva were small but consistently different, thus demonstrating the highly sensitive nature of the IMC technique developed in this study. Investigations with microcalorimetric measurements of human saliva probes of individuals with different levels of oral disease may offer the opportunity for useful contribution on the effect of salivary proteins in oral health.

Increased surface areas, provided by the glass beads, were associated with higher energy release in both liquids. The increase in surface area for the ampoules alone vs ampoules with 0.3 or 3 g of beads was not determined. However, increasing the amount of same-size beads by a factor of 10 (0.3 g vs 3 g) did not result in a 10-fold increase heat production over the time interval evaluated. The increase was ~14.6 % in PBS and ~13.1 % in saliva. One can argue that this could be due to geometric effects - i.e. with 3 g of beads in a static environment, the bacteria would have to negotiate longer, more tortuous paths to reach theoretically available glass surfaces. Also, planktonic bacteria found after the IMC experiments with 3 g of beads show that not all available bacteria found their way to surfaces. The IMC method developed is highly reproducible, sensitive and rapid in the study of bacterial adhesion. It could be useful to identify bacterial surface molecules involved in adhesion, by comparing suitable bacterial mutant strains. It also could be developed into a

convenient screening method to compare different dental materials or materials subjected to different surface treatments with the aim of reducing adhesion. Further, it could provide valuable information on interfering substances to be applied in oral prophylaxis.

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Table I.

Mean cumulative heat production (μJ) during 1 h adhesion (0.5 - 1.5 h period) of *S. sanguinis* (n=10; mean \pm sd). Values marked with the same letter show a statistically significant difference ($p < 0.05$).

Solution	Glass ampoule only	Glass ampoule + 0.3 g glass beads	Glass ampoule + 3 g glass beads
PBS	74,800 \pm 9,360 A	79,500 \pm 6,980 B	91,000 \pm 10,300 A/B
Human saliva	84,400 \pm 8,630 C	94,600 \pm 20,200	107,000 \pm 19,800 C

FIGURE LEGENDS

Figure 1.

Preparation of duplicate samples and sequence of steps for microcalorimetric measurements of bacterial adhesion.

Figure 2.

Examples of p-t curves of *S. sanguinis* growth and total energy produced. For the two different bacterial start concentrations: — p-t curve (μW) at 5×10^6 CFU ml⁻¹, --- p-t curve (μW) at 5×10^5 CFU ml⁻¹, — total energy produced (μJ) at 5×10^6 CFU ml⁻¹, --- total energy produced (μJ) at 5×10^5 CFU ml⁻¹. Fig a) overview b) detail. c) Corresponding growth parameters (log CFU ml⁻¹, pH and OD₆₀₀) for the two different concentrations: --■-- log CFU ml⁻¹ curve at 5×10^6 CFU ml⁻¹, --□-- log CFU ml⁻¹ curve at 5×10^5 CFU ml⁻¹, ◆ pH at 5×10^6 CFU ml⁻¹, ◇ pH at 5×10^5 CFU ml⁻¹, — OD₆₀₀ curve at 5×10^6 CFU ml⁻¹, OD₆₀₀ curve at 5×10^5 CFU ml⁻¹.

Figure 3.

Demonstration of stationary conditions of *S. sanguinis*: Vitality (log CFU ml⁻¹) of *S. sanguinis* suspension in PBS (□--□) or human saliva (●—●) over a period of four hours. Parallel measurements of vitality of *S. sanguinis* suspension in PBS (△) or human saliva (▲) from the ampoules used for microcalorimetry at the beginning (0 h) and the end (3 h) of the experiment.

Figure 4.

Comparison of the p-t curve of bacterial growth in Schaedler broth (—) to the p-t curve of bacterial adhesion in human saliva (—) and control ampoules without bacteria (---). Data measured at time A were transferred to position B to compare heat flow at equal cell number (see text).

Figure 5.

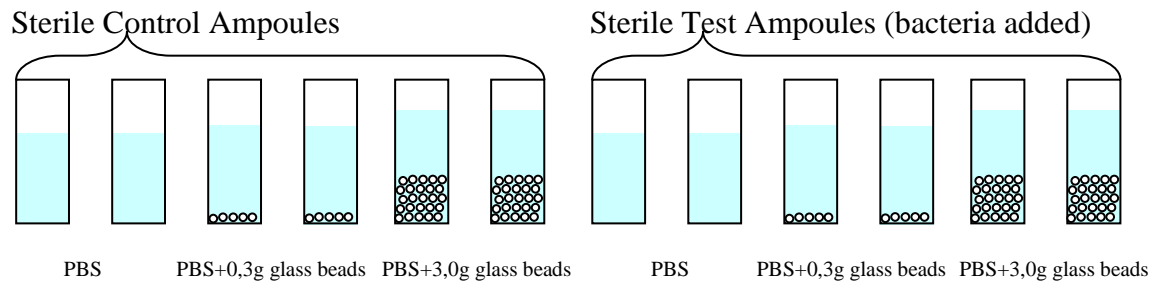
Examples of p-t curves of stationary *S. sanguinis* cells suspended in PBS (a) or human saliva (b) of ampoules with no glass beads (—), ampoules with 0.3 g glass beads (•••), and ampoules with 3 g glass beads (---). Also indicated is the number of viable *S. sanguinis* (log CFU ml⁻¹).

Figure 6.

Representative sector of the inner glass wall of an ampoule after 3h microcalorimetric measurement of adhesion starting with bacteria suspended in PBS. Most bacteria attached stained green because they were still alive, a few stained pale red indicating that they were dead at the time of staining. Due to the concave ampoule wall only part of the picture is in focus.

FIGURES

Figure 1.



Equilibration over night in the microcalorimeter at 37°C



Take out for injection of 250 µl of

either **PBS alone** ***S. sanguinis* suspension in PBS**
or **human saliva alone** ***S. sanguinis* suspension in human saliva**



leave 15 min in the equilibration position before lowering to the measurement position



Calorimetric measurements

Figure 2.

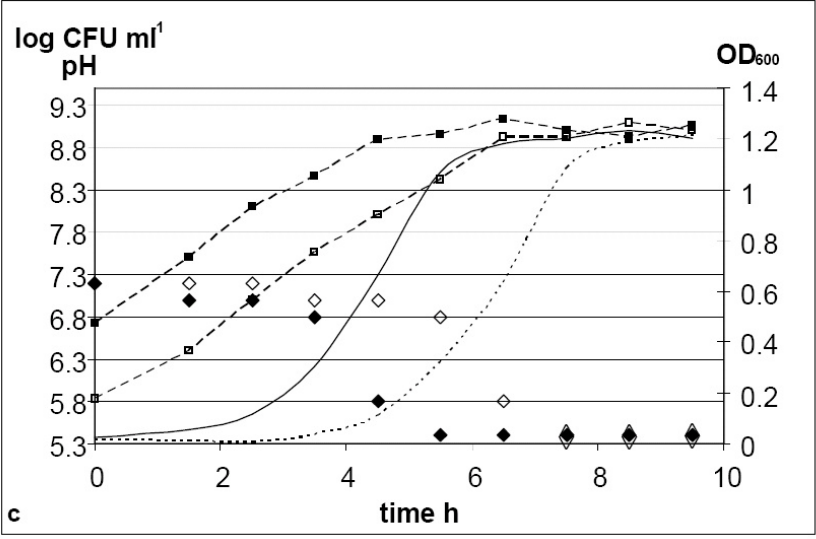
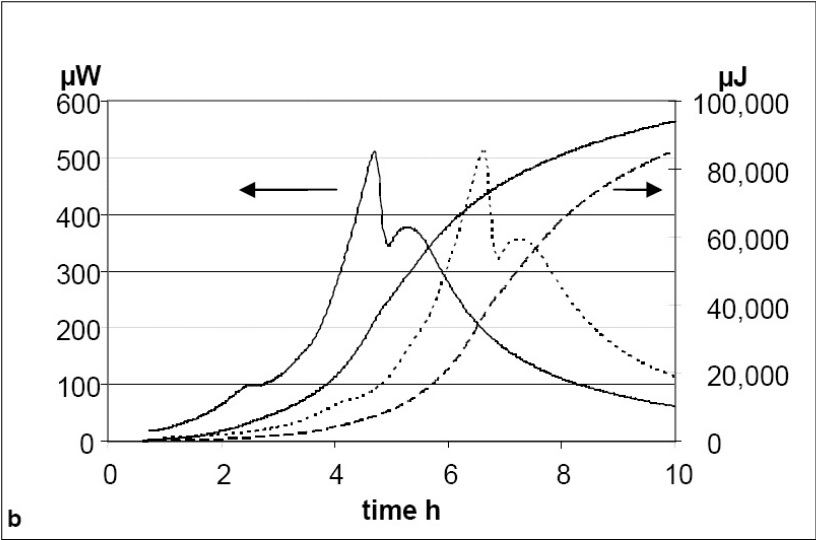
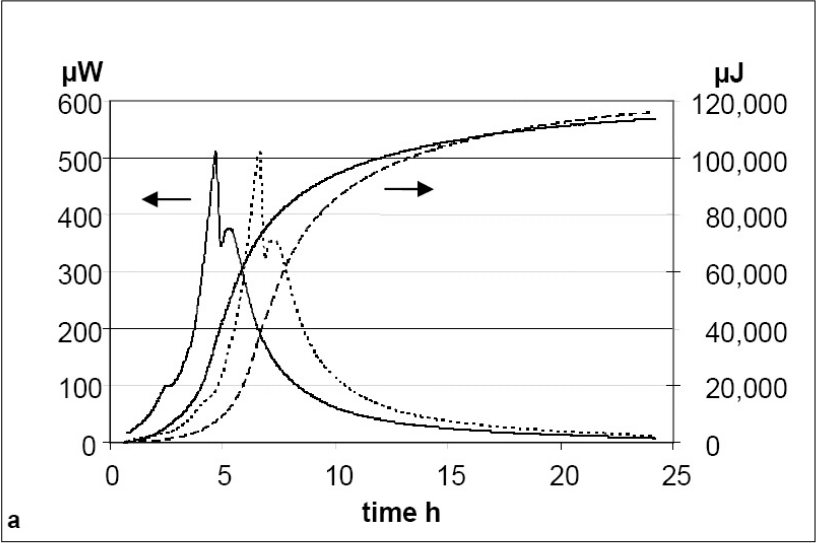


Figure 3.

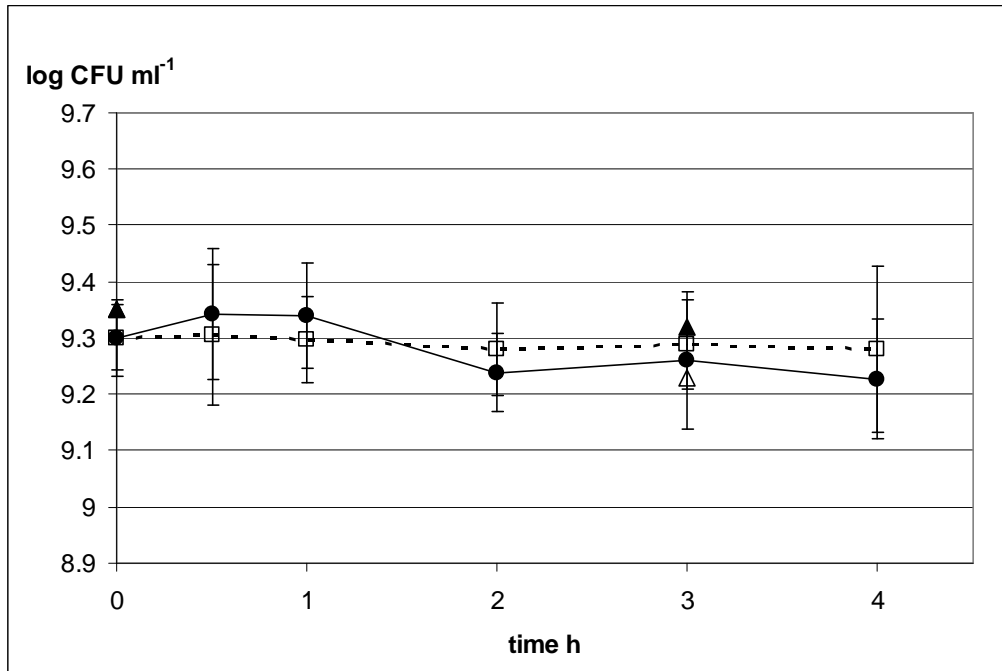


Figure 4.

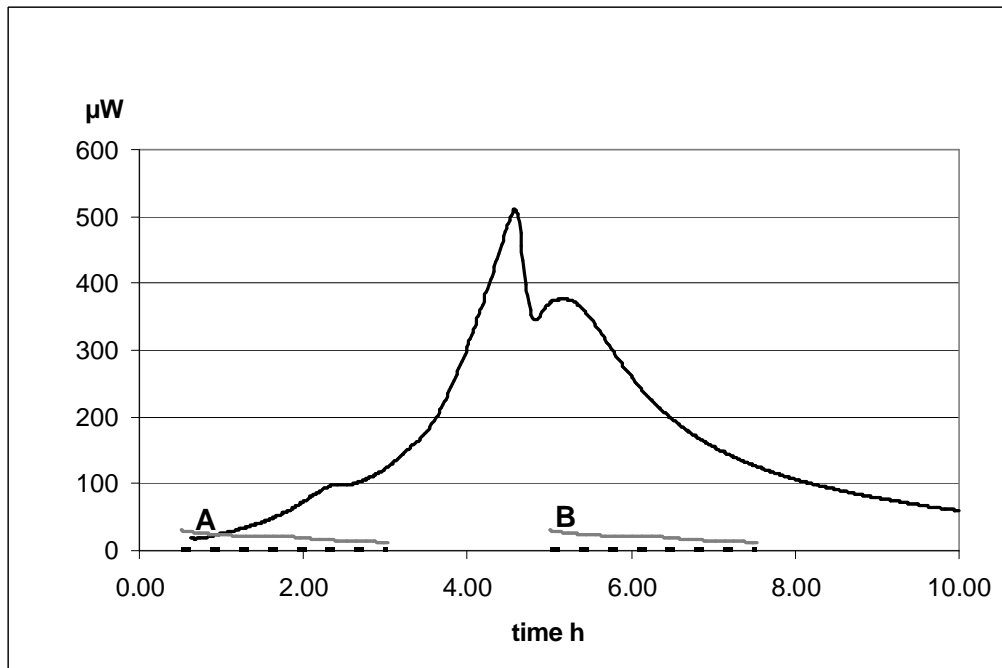


Figure 5.

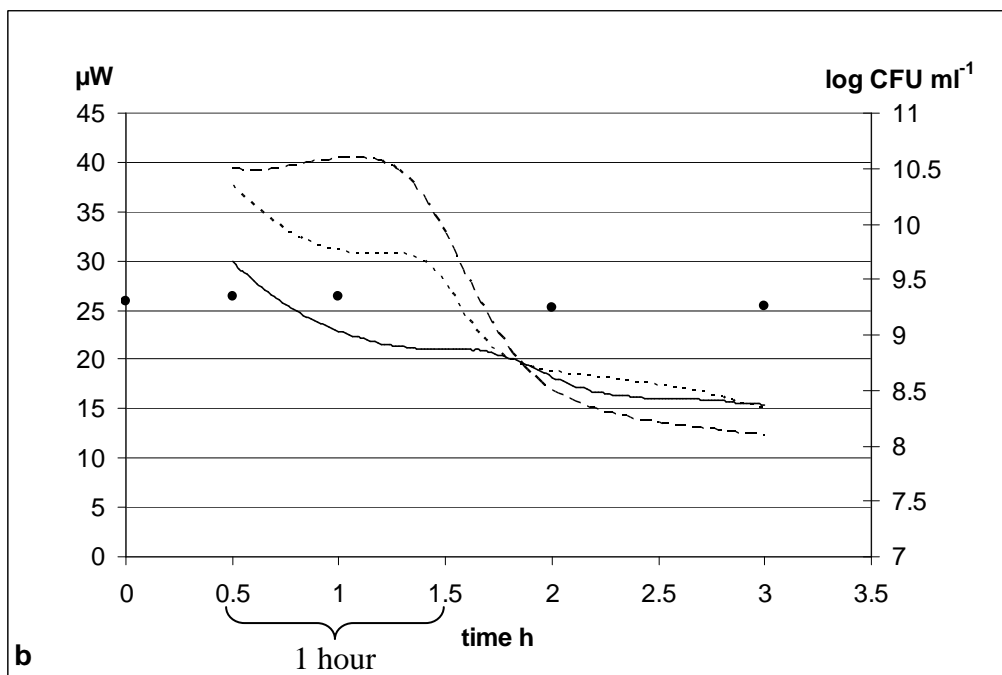
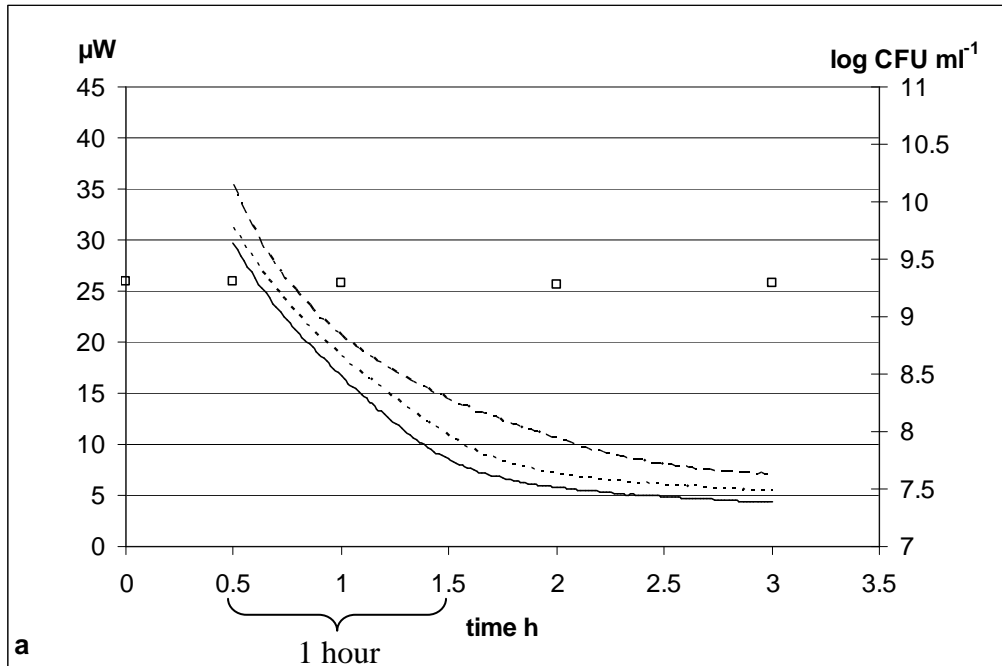
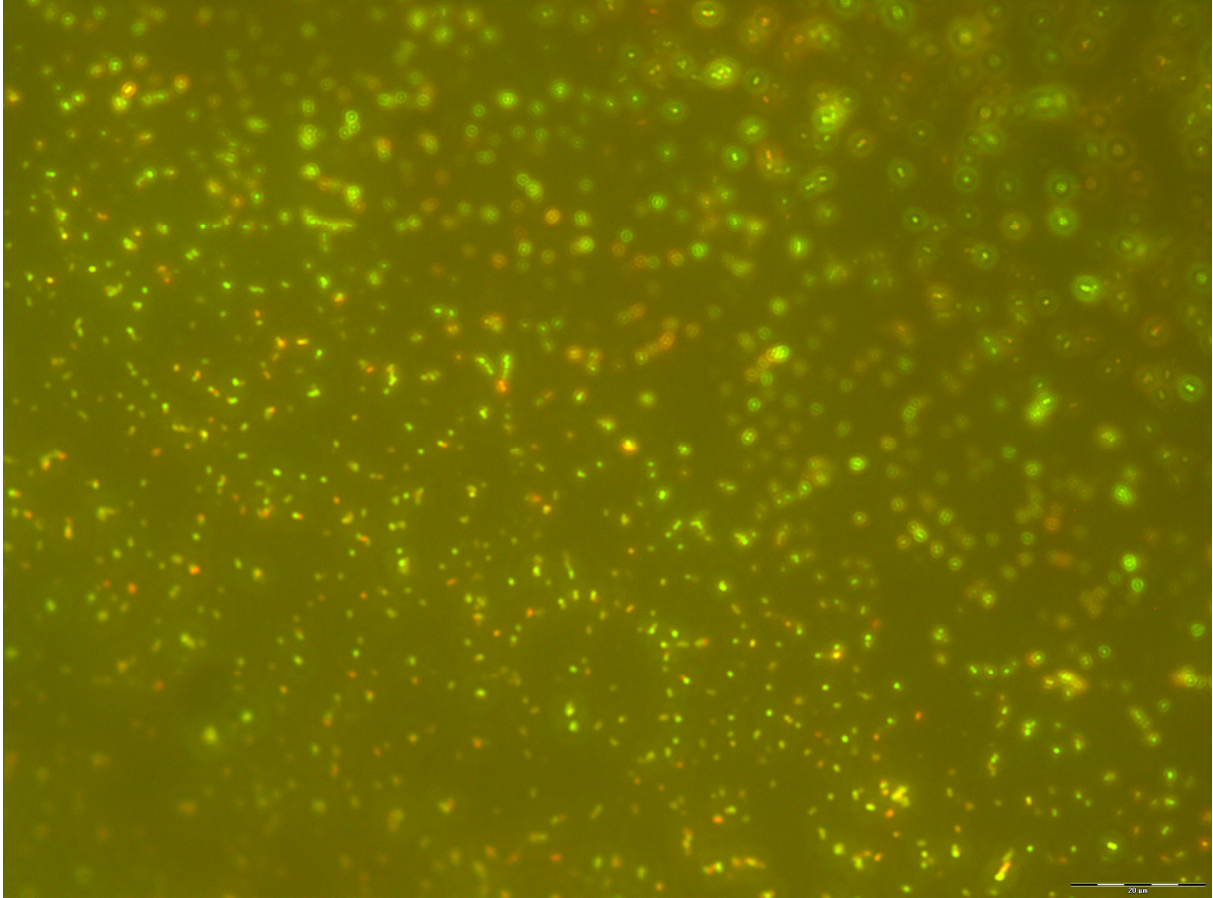


Figure 6.



Discussion

The oral cavity provides many different niches that can be colonized by diverse microorganisms as well as periodic nutrient supply resulting in a numerous complex microflora. On the tooth surface a microbial biofilm, called dental plaque is formed which may lead to dental caries and periodontal diseases. Microbial adhesion on tooth surfaces is always preceded by the adsorption of a salivary pellicle. After adhering the early colonizers provide attachment substrates for the subsequent colonizers and ultimately influence the succeeding stages of biofilm formation and the composition of the mature plaque (ten Cate 2006).

Although a wide range of materials is available for restoring or reconstructing teeth, new materials with improved biocompatibility and/or mechanical properties are being developed, some of which designed to reduce microbial adhesion. As these aspects have not been routinely tested the first aim in this work was to adapt an *in vitro* model for screening dental materials with respect to their adhesion properties. Furthermore the vitality of adhered bacteria was analyzed. As the second aim microcalorimetry was explored as an alternative technique to investigate the adhesion process.

Based on the results of paper I-III, both methods used appear to be useful to study bacterial adhesion to new dental restorative or implant materials. Materials to be tested in the flow chamber system have to be prepared as slides, which were made by a dental laboratory or the manufacturers without problems. Also, materials' properties like surface roughness and hydrophobicity influencing bacterial adhesion can be measured and evaluated reproducibly. Interestingly, human enamel as a reference has so far been scarcely used in *in vitro* models of bacterial adhesion reported in literature (Takatsuka et al. 2000; Decker et al. 2003a). Such comparisons appear necessary for evaluating dental plaque formation on new materials. With the microscope set up used, live and dead bacteria can be conveniently counted by the help of

the appropriate software, unless the surface roughness exceeds 2.0 μm . In that situation several pictures at different focal plains have to be taken and analyzed separately which is time consuming. IMC proved sensitive enough to detect bacterial adhesion to glass which has adhesion properties similar to human enamel (Burgemeister et al. 2001) (and our own data). One may expect that adhesion of *Streptococcus sanguinis* to titanium, gold, ceramic, and composite result in similar heat values because it showed similar adhesion values to glass, enamel, and material in the flow chamber model (see paper I). One difficulty might be that the materials would have to be produced as beads or barrels with similar diameter and surface roughness which is probably more difficult to achieve. To measure surface roughness (Grossman et al. 2004; Bagheri et al. 2007) and hydrophobicity (Tsuneda et al. 2003; Black et al. 2004) will work without difficulties for all shapes. A large surface of the test material is required because otherwise bacterial adhesion to the inner wall of the glass ampoules may interfere. Small beads or barrels with larger surface area are expected to result in higher heat flow from adhesion, however may hamper diffusion of bacteria to potential adhesion sites. We suspected in the study that not all available bacteria could find their way to the glass beads' surfaces. Thus, the geometry of the materials will have to be optimized in future experiments.

Many factors have to be taken in consideration to create *in vitro* systems simulating the complexity of oral conditions (Marsh 1995; Sissons 1997; Tang et al. 2003). A parameter which can be standardized in *in vitro* experiments is the fluid in which adhesion is measured. PBS or other salt solutions can be easily obtained in large quantities and standard quality and are thus very convenient. However, they don't reflect the oral cavity. Due to the particular characteristics of human saliva which is a complex mixture of fluids secreted by several salivary glands, (see Introduction chapter on saliva) it is almost impossible to duplicate. Due to the inconsistent and unstable nature of natural human saliva it has to be used fresh.

Freezing protocols proved unrealizable but would facilitate collection of large quantities. Attempts to define an artificial saliva are numerous (Gal et al. 2001). In fact, 60 different formulae have been described in literature ranging from inorganic solutions of Na, K, Ca, Cl, PO₄ ions, to more complex recipes containing several proteins and other organic compounds (urea, organic acids). Some have only been used in laboratory experiments while others have been designed to substitute in patients suffering hyposalivation and are available in pharmacy. However, application of these artificial saliva solution in comparison to natural saliva are scarcely reported in literature (Leung and Darvell 1997; Duffo and Quezada Castillo 2004). The composition used depends on the process studied. For dental material corrosion studies in artificial saliva another formula was employed than for investigations of tooth enamel fluoride uptake or of the caries or the remineralization process.

For the study of bacterial adhesion to dental materials we decided to use natural saliva containing a range of proteins which could mimic pellicle formation. As others before, we used natural saliva obtained under standard conditions (see paper I). The volunteer had to be in good health, follow good oral hygiene, be caries-free, does not smoke, and does not receive any medication. Saliva collection was made 2 h after not eating and drinking and at the same time of the day (e.g. in the morning). For practical reasons human saliva was obtained from one or two persons. Other investigators have used saliva pooled of several volunteers to average interindividual variations (Carlén et al. 1998a; Steinberg et al. 1999). Our results over the experimental time of four years were highly reproducible and documented reliable standardization.

Inevitably saliva may change surface properties of the materials as well as of the bacteria. Quirynen and Bollen (1995) suggested that saliva coating of oral hard surfaces converge the hydrophobicity values of all surfaces. Indeed paper I and II of the present work showed that conditioning with human saliva decreased hydrophobicity of the dental materials and of the

oral streptococci. In spite of this, the number and vitality of adhered bacteria varied between the materials. These results indicate that in addition to hydrophobicity, other surface characteristics of the substratum and/or the quality of the acquired pellicle modulate adhesion of *S. sanguinis* in the *in vitro* system. In fact, several authors described important pellicle binding sites for oral streptococci (Carlén et al. 1996a; Carlén et al. 1996b; Edgerton et al. 1996; Carlén et al. 1998b; Gong et al. 2000). They argued that the influence of the salivary pellicle may be more important with respect to the retention of adhering bacteria than the surface properties of the substrata themselves. The difference in heat production detected in the microcalorimetric studies of *S. sanguinis* suspended in PBS versus human saliva may indicate additional bacterial adhesion site provided by saliva.

The flow chamber established a dynamic flow system with a high degree of vitality of microorganisms and a stable environment. It has the advantage of creating shear forces during interaction of the bacteria with the surface therefore simulated conditions of the oral cavity. The mass transport was controlled and generally fast as it was independent of diffusion. The substrata were positioned vertical to the flow chamber's ground which reflects the position of some but not all tooth surfaces. Compared to the flow chamber system, the microcalorimetric technique represents a batch system where bacteria reach the surface by diffusion. In addition heat flow measurements were reliable for technical reasons only 30 minutes after the samples arrived in the measuring position. At this time bacterial adhesion most likely had started already and may have even passed the maximal rate. Thus this technique will not reflect adhesion dynamics entirely.

A modification of the technique, the isothermal titration calorimetry (ITC) system, was used to study the coaggregating bacteria pair *Actinomyces naeslundii* 147 and *Streptococcus oralis* J22 (Postollec et al. 2003) and allowed to quantitate continuously. It may be applicable for investigating bacterial adhesion. But the ITC system is less useful as a rapid screening

technique because the application is more complex and thermostats equipped for ITC are typically available with only four calorimeters. In contrast IMC allow parallel measurements in 48 channels. On the other hand there are *in vitro* systems described in literature investigating bacterial adhesion in batch cultures (Grivet et al. 2000; Takatsuka et al. 2000; Steinberg and Eyal 2002; Montanaro et al. 2004; Sardin et al. 2004).

The different heat flows in PBS as compared to human saliva showed the highly sensitive nature of the IMC technique. It provide useful in the study of the functions of specific saliva proteins (high and low aggregation, killing, inhibition of adherence) which in the future might be included in preventive strategies.

With respect to a full evaluation of the two techniques as screening models the results of paper I-III represents a mosaic. The following gaps will have to be closed:

- 1) In the flow chamber system, the spectrum of early colonizers should be extended to include at least other oral streptococci and *Actinomyces* sp. (Whittaker et al. 1996; Li et al. 2004). Periodontopathogenic bacteria could also be included (Steinberg et al. 1998).
- 2) In the microcalorimetric model different dental materials and microorganisms should be investigated.

An *in vitro* model for characterizing of an antiadhesive and/or antimicrobial substrate is only partially predictive of the efficacy against the natural complex microbial community in the oral cavity. But it may be valuable for screening a large number of new or modified dental materials. Such studies are comparatively cheap, rapid and reproducible whereas *in situ* studies are more expensive, time-consuming, and difficult to standardize. Therefore an *in vitro* model may lead to the selection of promising materials that warrant detailed tests *in situ*. The choice for one of the presented technique will depend on the questions being addressed, the resources that are available (mainly shape and surface of the dental materials), and the stage of development. The flow chamber model is versatile but is more laborious than the

microcalorimetric system. IMC has the advantage of providing rapid, accurate, sensitive and reproducible measurement of heat and will find further application in the research of dental plaque formation.

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Adhesion of *Streptococcus sanguinis* to Dental Implant and Restorative Materials *In Vitro*

Irmgard HAUSER-GERSPACH, Eva M. KULIK, Roland WEIGER, Eva-Maria DECKER, Christiane VON OHLE, Jürg MEYER

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Adhesion of *Streptococcus sanguinis* to Glass Surfaces can be Measured by Isothermal Microcalorimetry (IMC)

Irmgard HAUSER-GERSPACH, Patricia SCANDIUCCI DE FREITAS, A.U. Dan DANIELS, Jürg MEYER

J Biomed Mater Res, in press

Adhesion of oral streptococci to all-ceramics dental restorative materials *in vitro*

Roland MEIER *, Irmgard HAUSER-GERSPACH*, Heinz LÜTHY, Jürg MEYER

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Immediate antimicrobial effect of gaseous ozone on the micro-organisms of the cavitated occlusal carious lesions in children *in vivo*

Victoria PFÄFFLI-SAWCHENKO, Irmgard HAUSER-GERSPACH, Jan Eric DÄHNHARDT, Jürg MEYER, Adrian LUSSI

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