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The effect of titanium implant surface modification on the dynamic process of initial microbial adhesion and biofilm formation

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ABSTRACT

Purpose: The aim of the study was to investigate the dynamic process of biofilm adhesion on titanium implant with two surface treatments, either pickled (PT) or moderately roughened by sandblasting with large grits and acid-etched (SLA).
Materials and methods: Two types of titanium disks with various surface treatments, *i.e.* PT and SLA with respective surface roughness (S_a) of 0.3 μm and 1.4 μm, were used as substrata. Three types of biofilms, *Streptococcus mutans*, *Streptococcus sanguinis* and polymicrobial biofilms (Microcosm), were grown on the two respective types of titanium disks for 2 h, 1 day, and 7 days. The formation of the biofilms was quantified by colony forming unit (CFU) count, and the structure of the biofilms on the titanium disks was observed by scanning electron microscope (SEM).
Results: At 2 h, the number of bacterial cells adhered to SLA surfaces was significantly higher than those to PT surfaces for all tested microorganisms. On day 1 and 7, no differences in biofilm CFU counts were observed between SLA and PT surfaces for *S. mutans* and Microcosm biofilms, while the *S. sanguinis* biofilm formation on SLA surfaces was significantly higher than the biofilm on PT surfaces throughout the whole test period. SEM images showed the increasing biofilm formation in time for all types of biofilms. Microcosm biofilms displayed different morphology from the other two single-species biofilms.
Conclusions: The higher roughness of a titanium surface would favor the early bacterial adhesion of *S. mutans*, *S. sanguinis* and Microcosm. However, as the biofilm became mature, the influence of surface roughness was diminished in a bacterial species dependent manner. Our results underline the importance of dynamic biofilm formation process in the implant study.

1. Introduction

The microbial infections around implanted medical devices are biofilm-related infections. Biofilm is a microbial derived sessile community characterized by bacterial cells that are irreversibly attached to a substratum or interface to each other, embedded in a matrix of extracellular polymeric substances that they have produced [1]. These device associated biofilm infections are extremely difficult to treat due to the biofilm resistance to immune defense mechanisms and antimicrobials [2]. Consequently, these infections lead to chronic inflammation and eventually the removal of the infected device.

The properties of biomaterial surface have been considered as a key factor in minimizing biofilm infection on a medical device. So far considerable research efforts have been directed towards modification of biomaterial surfaces, in order to reduce, if not eliminate, the biofilm formation on medical devices [2]. Dental implants, one type of implanted medical devices, are inert, alloplastic materials embedded in the jaw bone for management of tooth loss. Since the oral cavity harbors more than 700 different bacterial species, dental implants are likely to be in contact with oral bacteria. An ideal surface of a dental implant should facilitate optimum osseointegration (bone to implant contact) without

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attracting bacterial cells. The roughness of the implant surface is believed to be the crucial parameter in achieving the ideal surface. To obtain optimum osseointegration, a considerable amount of evidence has suggested that a moderate rough surface with roughness value R_a of 1–2 μm is required [3]. However, the ideal roughness for minimizing biofilm formation on the surfaces is still in debate [4].

Some studies have shown that bacterial adhesion to the implant surface was strongly affected by the roughness of the surface. With the increase of the surface roughness, there was an exponential increase in the amount of adhered bacterial cells [3,5]. A roughness (R_a) value of 0.2 μm was suggested to be the threshold surface roughness below which bacterial adhesion cannot be further reduced [6]. Meanwhile, other studies demonstrated that the biofilm formation was similar on all implant surfaces, irrespective of the roughness [7–10]. The reasons for such discrepant results on the relationship between surface roughness and biofilm formation are not clear. The discrepancy is unlikely due to the bias of the model (*i.e. in vitro* and *in vivo*) or the choice of microorganism (*i.e.* single-species, multi-species or Microcosm's biofilms) used, since all these factors have been included in all these studies. Unfortunately, most studies (except Al-Ahmad et al. [8] which examined the biofilm formation on implant surfaces after 3 and 5 days) have evaluated the duration of biofilm formation by only picking randomly (or intentionally) one time point between 2 h and 7 days. As a consequence, these studies might derive subjective or even erroneous conclusions.

In fact, the formation of a biofilm on a surface is a dynamic complex process which usually takes days before the biofilm is mature. Upon implantation of a medical device, there is a competition between the integration of the material into the surrounding tissue and the adhesion of bacteria to the implant surface, followed by biofilm formation [11]. For a successful implant, tissue integration should occur prior to appreciable bacterial adhesion, thereby preventing colonization at the implant. Host defenses often cannot prevent further colonization once bacterial adhesion occurs before tissue integration [11]. A post-implantation “decisive period” has been suggested to be the critical period for implantation [12,13]. During this period, an implant is particularly susceptible to microbial colonization and infection. Examining the speed of biofilm formation may be helpful to identify this critical period and provide new information for implant design and clinical practice.

The aims of the current study are to examine the dynamic process of biofilm formation on titanium implant surfaces with similar surface chemistry but with various roughness. The biofilm formation on an implant surface was examined after 2 h, 1 day and 7 days. *Streptococcus sanguinis*, *Streptococcus mutans* and Microcosm were used as the bacterial sources. The hypothesis was the surface roughness did not significantly affect the various biofilm species under the stated time period.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacteria strains used in this study were *S. sanguinis* ATCC10566, *S. mutans* UA159. They were routinely grown under anaerobic condition (80% N_2, 10% H_2, and 10% CO_2) at 37 °C. Brain Heart Infusion (BHI) agar was used for maintenance and viable counts of both *S. sanguinis* and *S. mutans*. The biofilms were grown in modified semi-defined biofilm medium (BM) containing 0.2% sucrose (BMS), which contains 76 mmol/L K_2HPO_4, 15 mmol/L KH_2PO_4, 10 mmol/L $(NH_4)_2SO_4$, 35 mmol/L NaCl, 2 mmol/L $MgSO_4 \cdot 7H_2O$ and was supplemented with filter-sterilized vitamins (0.04 mmol/L nicotinic acid, 0.1 mmol/L pyridoxine HCl, 0.01 mmol/L pantothenic acid, 1 μmol/L riboflavin, 0.3 μmol/L thiamine HCl, and 0.05 μmol/L D-biotin), amino acids (4 mmol/L L-glutamic acid, 1 mmol/L L-arginine HCl, 1.3 mmol/L L-cysteine HCl, and 0.1 mmol/L L-tryptophan), and 0.3% (wt/vol) yeast extract, glucose (0.2%), or sucrose (0.2%) was added where indicated. The pH of this medium was adjusted to 7.0 [14].

2.2. Saliva collection

Human saliva was collected to initiate Microcosm biofilm formation. In brief, the unstimulated saliva was collected from three volunteers and pooled. The volunteers were asked not to brush their teeth 24 h before the collection and to refrain from food or drinks at least 2 h before the collection. The collected saliva was diluted 2-fold with 60% glycerol and store at −80 °C before use.

2.3. Substrata

Two types of titanium discs were used for biofilm growth: one type with a pickled (PT) surface on both sides of the disc and the other type with a PT surface on one side and a sandblasted and acid-etched (SLA) surface on the other side. All the titanium discs were gamma-sterilized and provided by the Straumann (Basel, Switzerland) as standard circular discs (5 mm in diameter and 1 mm in thickness). Surface roughness (S_a) was analyzed by atomic force microscope (AFM) (Dimension EDGE, BRUKER, Germany). Three areas were taken randomly for each sample to calculate S_a by AFM. The manufacturer has claimed the surface properties of the provided PT and SLA titanium discs, *i.e.* hydrophobicity of both surfaces (as indicated by the contact angle values), are the same. This has been tested and confirmed in another study [9]. Another study has also shown and further confirmed the surface chemistry of both surfaces in terms of titanium (Ti2p), oxygen (O1s), nitrogen (N1s) and carbon (C1s) were similar [15].

2.4. Biofilm formation

All biofilms were grown on the titanium disc surfaces in 48-well microtitre plates (Corning, USA). Since only one side of the titanium discs has an SLA surface, all the discs were placed at the bottom of the well with the tested surface (SLA surface) upwards to allow biofilm growth only on this side of the titanium disc.

To grow *S. sanguinis* or *S. mutans* biofilms, the overnight full-grown culture was diluted to a final OD_{600} of 0.01 in BMS and dispensed into a sterile 48-well plate containing a titanium disc in each well (0.5 ml/well). The growth medium was refreshed every 8 h and 16 h. The medium refreshing was achieved by transferring all the discs into a new 48-well plate containing fresh BMS. The adhesion of bacterial cells or the biofilm formation was evaluated 2 h, 1 day and 7 days after inoculation. To grow Microcosm biofilms, the stored saliva stock was thawed at room temperature and diluted at the ratio of 1:20 in fresh BMBG (BM containing 10% fetal bovine serum and 0.1% glucose). The dilution was dispensed into the sterile 48-well plate containing titanium discs in each well (0.5 ml/well). The growth medium (BMBG) was refreshed every 8 h and 16 h. The adhesion of bacterial cells or the biofilm formation was evaluated 2 h, 1 day and 7 days after inoculation.

In each test, all test groups has three samples, and the experiment was repeated for 3 times.

2.5. Quantification of biofilm formation

At designated biofilm collection time point, the titanium discs with biofilms were rinsed once in phosphate buffered saline solution (PBS, composed of 8.0 g NaCl, 0.2 g KCl, 1.0 g Na_2HPO_4, and 0.2 g KH_2PO_4 per litre, adjusted to pH 7.4) and transferred into