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Biofilm Formation on Dental Implant Biomaterials

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ABSTRACT

Most nosocomial infections reported so far have been associated with medical device based infections. A combination of opportunistic pathogens is responsible for causing implant infections. The severe form of implant infections – biofilm, has caused problems in identifying appropriate treatments because biofilm possess undeniable ability to sustain shear force and shows antimicrobial resistance. The biofilm formation is dependent on cell attachment over implant surface which can be made from variety of biomaterials. The presence of biomaterial choices for implant production can be limited down to their ability to attach cells during infection. Thus, the present study evaluates metal, ceramic, polymer and carbon based biomaterials for susceptibility towards biofilm formation. The results have shown that polymer based implants are most susceptible to biofilm formation than other metal implants and mixed implants. These results can be correlated with other in vivo analysis such as cytotoxicity to select most appropriate biomaterial for implant preparation to reduce biofilm infection and improve life of the implant.

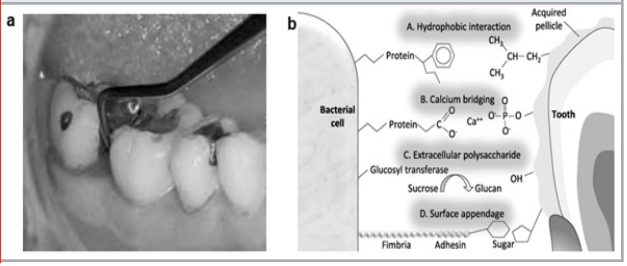
KEY WORDS: BIOFILM, BIOMATERIALS, CELL ADHESION, DENTAL IMPLANT, POLYMER IMPLANT.

INTRODUCTION

Mouth cavity has been the most convenient niche for microorganisms by providing different forms of ideal adhesive surfaces. Dental plaque is the most common disease exhibiting deposition of microorganisms on tooth surface. The tooth surface favors different interactions benefiting diverse community of microbial cells. These interactions strengthen with time and form more resistant forms of microbial deposition which becomes difficult to treat. One such form of microbial aggregation on tooth is dental biofilm. Biofilm is defined as unidentified microbial community adhering to the tooth surface or any other non –shedding material encased within a matrix of extracellular bimolecular. The formation of dental biofilm is a multistep process.

Briefly, microbial load in saliva or overall mouth identifies different adhesion proteins on the tooth surface. The interaction between cell receptors and adhesion proteins initiate deposition of a layer of cells (Figure 1). These cells secrete extracellular matrix (ECM) to stabilize their existence and prepare surface for other cells. The cellular communication in the form of quorum sensing attracts other cells to accumulate and stick together to form an aggregated network of microbial cells which is extremely resistant to shear stress, antimicrobial compounds and other tooth cleaners. The biofilm deposition appears unusual in the form of yellow, dark brown appearance on the tooth surface.

Figure 1: Biofilm formation on tooth surface. a) Dark plaque deposition on the surface and core of teeth representing biofilm formation (http://www.thieme.com/media/samples/pubid1114459271.pdf. et. al.). b) Molecular interactions responsible for bacterial cell adhesion on tooth surface.(J. Chandra et. al. (2001)



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Biofilms are ubiquitous, superficial form of cells deposition that can form on any surface existing in natural aqueous environment. The most common surfaces for biofilm formation include tap openings, living tissue, implanted devices, tooth surface, dental implant or any other abiotic surface (J.W. Costerton et. al. (2005)). Biofilm mediated dental infections mostly observed on heart valves implant, catheters, vascular prosthesis, breast implants, intraocular lenses and dental implants. Infections caused by implants result from interactions between pathogens, implant biomaterial and host's immune response towards both pathogens and implant. The presence of foreign body (implant) creates critical spots exhibiting pathogen exposure to mouth tissue including cheeks and gum leading to infection progression.

Other opportunistic pathogens arriving in the mouth are usually cleared by body's immune system. The constant pathogen exposure through implants, granulation (foreign body reaction)and fibrous encapsulationcause immune depression(V. Menkin et. al. (1931). The initial cell attachment over implant greatly depends on biomaterial used to prepare the implant. The biomaterial dictates several interactions and cell stabilization properties leading to biofilm maturation. Despite a decade of research on biofilm, biofilm formation on different surfaces has not been studied in detail. Thus the present study evaluates susceptibility of biofilm formation on different surfaces of dental implants.

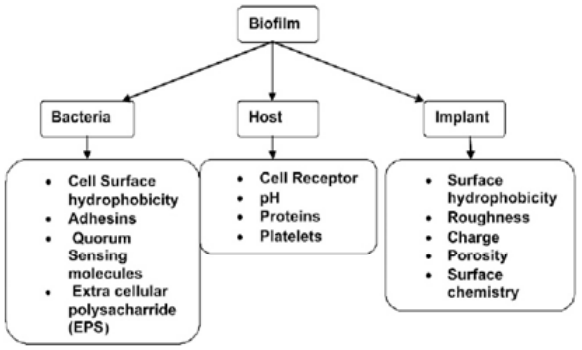
Literature Review: Dental implants have been prevalent in case of edentulous patients or in case of partial edentulous. Among 1 million dental implants placed annually, the success rate has been close to 95% despite of bacterial colonization at later stages. Implant mucositis is has been reported more than 60% (S. Renvert et. al. (2009)). The various parts of an implant contacting mouth tissue include implant post, abutment along with crown. The implants can be classified as endosteal (root/plate form implant), subperiosteal (implant placed on or around bone), transosteal (implant inserted through chin and supported by plate) and temporary implant (for temporary structuring of bones and alignment of teeth) (S. M. Balaji et. al. (2007)).

According to a study, it has been reported that plaque in peri-implantitis is caused by spirochetes and cells of coccoids, usually depicting accumulation of highly pathogenic Enterobacteria, Staphylococcus aureus, Pseudomonas sp. present in salivary fluid(A. Heimdahl et. al. (1983)). It has been known that titanium based implants are usually contaminated with gram negative anaerobes which develop a niche in depths of the implant. Another study conducted by Mombelli et al. suggested that gram negative anaerobes are mostly found in the failed implants whereas even successful implants depict very low incidence of gram positive bacteria(A. Mombelli et. al. (1987)).

Initial bacterial attachment over an implant surface or an abiotic surface occurs mostly by non specific forces such as van der walls, Lewis acid-base or electrostatic interactions. The bacterial mobility components such as appendages or pilli interact with surface proteins of the implant's biomaterial and initiate cell attachment. The factors of the implant biomaterial affecting biofilm formation include surface hydrophobicity, roughness, porosity, charge and overall surface chemistry. The presence of biofilm on implant surface leads to infection progression in other parts of the body.

Mechanical cleaning, dental chemical based cleaners and other antimicrobial compounds exhibit limitations in terms of relapse and bacterial resistance(H.J. Busscher et. al. (2010)). Thus, there is a need to understand bacterial adhesion over different implant surfaces to provide a selective choice of biomaterial eliminating future complications. The present study has been conducted on clinically used implants including metallic, ceramic, polymer and carbon implants. The bacterial adhesion on different surfaces has been studied to depict adhesion capabilities in different implant biomaterials (Figure 2).

Figure 2: Factors of pathogen (bacteria), host and dental implant contributing to biofilm formation(V. Nandakumar et. al. 2013).



Research Questions: What is the probability of biofilm formation on different dental implants based on biomaterials used for manufacturing?

MATERIAL AND METHODS

Sample collection: Clinical samples of used and replaced dental implants were collected from a dental hospital. The implants were procured on the basis of categories as metallic, ceramic, polymer and carbon implants. Most of these implants were used as tooth supporting screws placed in jaw tissue and used for more than 12 months before being removed. These implants have been diagnosed for possessing bacterial infections which have reached a sever stage causing implant replacement. The implant samples were stored at 4°C for future experiments. Alternatively, fresh samples of implants were also purchased from commercial vendors to study susceptibility of implants to bacterial adhesion (Table 1).

Assessment of clinical biofilm: The presence of biofilm on all the clinical samples was analyzed by crystal violet staining method as described by O'Toole (G.A. O.'Toole et. al. (2010)). Briefly, implants were washed with phosphate buffer saline (PBS) to remove unattached cells and subsequently dried. Each implant was dipped in

crystal violet stain (1% w/v) and incubated for 10 minutes. Further, the implants were washed twice with PBS and then incubated in 5ml acetic acid solution (30% v/v). The spent crystal violet dissolved acetic acid solution was quantified by measuring absorbance at 560 nm.

Table 1. Different categories of implant biomaterials tested for biofilm formation.

Type of implant	Pictorial description	Biomaterial
Metallic implant		Titanium
Ceramic implant		Zirconia
Polymer implant		Polymethylmethacrylate (PMMA) and Polytetrafluoroethyle (PTFE) implant
Carbon implant (also a metallic implant)		Stainless steel

In-vitro cell adhesion assay: In order to study susceptibility of cell adhesion on different biomaterials of the implant, fresh implants were inoculated with salivary microbes and percentage cell adhesion was analyzed. Fresh implants were washed with PBS to remove unattached biomass from the implant surface. The implant was placed in petri dish filled with Trypton Soy Broth (TSB) media which was inoculated with saliva sample under sterile conditions. The petri dish was sealed with parafilm and incubated at 370 C for 48 hours, under static conditions (without mixing). After 72 hours, the samples were washed twice with PBS and attached microbial cells were identified by crystal violet staining mentioned previously.

Biofilm aggregation assay: In order to confirm biofilm formation, congo red dye based assay was performed to study aggregation of extracellular proteins which are characteristic feature of biofilm stabilization. Congo red solution was prepared as described by Singh et al., and used to stain the biofilm attached on the implant surface. For convenience, biofilm grown implants were lyophilized and biofilm was scratched from the surface to obtain separate, solid powder form of the attached biofilm. The biofilm was equally weighed and stained with congo red and dye attachment in proportion to aggregation was quantified by recording congo red spectra.

Removal of implant biofilm: To evaluate robustness of biofilm formed on the implant surface, biofilm containing samples were washed with common dentifrice having antimicrobial properties along with sonication treatment for removal of attached cells. The samples obtained post sonication and cleansing were further placed in media and incubated again at 370 C for 72 hours to study the biofilm relapse. The biofilm remaining after cleaning as well as relapsed biofilm was evaluated by crystal violet staining as mentioned previously.

RESULTS AND DISCUSSION

Biofilm formation on clinical implant samples: Implant samples replaced and thrown after microbial infections, obtained from clinical settings were subjected to crystal violet assay to analyse amount of biofilm formed on the surface. Broadly, nickel, zirconium and stainless steel (Ni, Zr and SS) belong to metal based implant however some of their surface properties are different due to mixing with other elements. Zirconium is also called ceramic metal which is used in making dental crown and screws. According to data obtained from crystal violet assay, zirconium has shown more biofilm attachment as compared to Ni and SS, however, the difference is insignificant (Figure 3). Polymer based implants have shown maximum biofilm attachment in comparison to all the implant categories. The results obtained may be due to more amount of salivary proteins attachment over polymer based implants than other metal based implants. Overall, results suggest that polymer based implants are more susceptible to biofilm formation than other types of implants.

Figure 3: Biofilm attachment on discarded (used) implants with infections obtained from clinical settings. (*p< 0.05, ns – non significant)

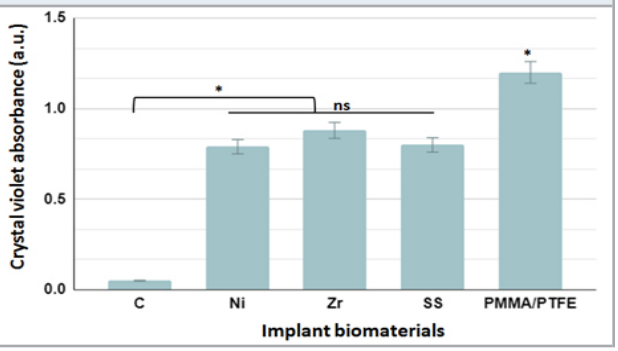
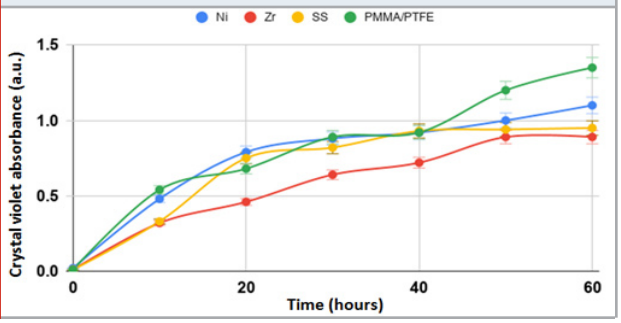


Figure 4: Time based biofilm formation on different implant biomaterials. Ni, Zr, SS and PMMA/PTFE represents Nickel, Zirconium, Stainless steel and polymer Polymethylmethacrylate and Polytetrafluoroethyle implants.



In-vitro cell adhesion on implants: The aim of forming biofilm over implants in vitro was to estimate time based cell attachment over different implant biomaterials. Several implants (replicates) were subjected to biofilm formation for 48 hours and one of them was taken out at specific time point to evaluate amount of bacteria attached on the implant surface. It was observed that cell attachment increased at logarithmic rate for 20 hours which could be attributed to the log-phase and biofilm structuring phase of the biofilm. Later from 20 hours to 40 hours, the bacterial growth rate observed to be stabilized, progressing towards a plateau stage, which may be correlated with phase of extracellular matrix secretion where biofilm attains stability against shear force.

Beyond 40 hours, biofilm attachment on most of the biomaterials was stable at a constant range (Figure 4). Overall, PMMA/PTFE showed maximum biofilm attachment in comparison with other biomaterials. Ni, Zr and SS showed similar pattern of biofilm formation. Thus the obtained result was in coordination with the result obtained from clinical implant samples. Therefore, it can be concluded that polymer based implants show more attachment to microbial cells than other types of implants. Dental implants are quite susceptible to biofilm formation both in vitro as well as in vivo scenario.

Figure 5: Congo red based aggregation assay on implant biomaterials.

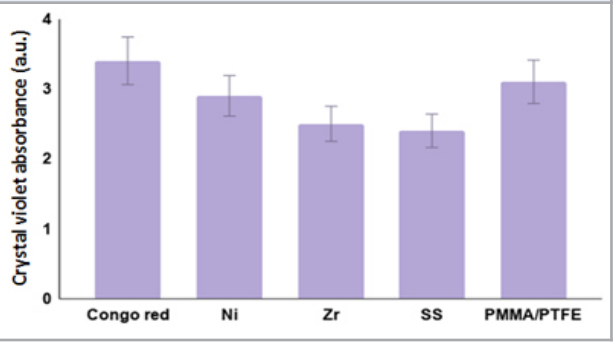
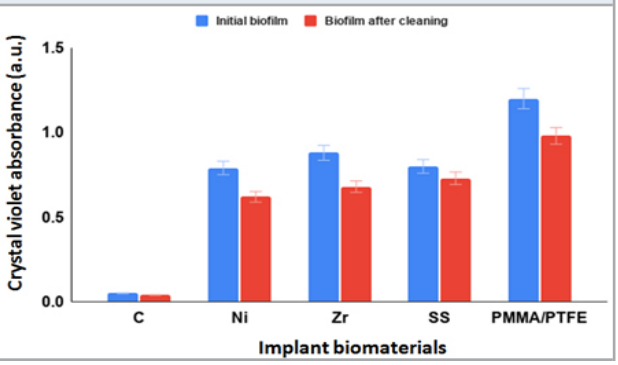


Figure 6: Comparative analysis of biofilm attached to implants before and after cleaning procedures.



Aggregation and biofilm formation on implants: Till now it has been depicted that dental implants are susceptible to biofilm attachment as well as cell attachment. In order to confirm the cell attachment from biofilm perspective, aggregation assessment was done to investigate presence of protein aggregates in the attached biofilm. The presence of protein aggregates in biofilm has been accounted for biofilm robustness and ability to resist shear force. The secondary structures of protein aggregates are very stable to surrounding changes. Congo red is a specific dye that binds to secondary structures (beta sheets) of protein units which is structural characteristic of protein aggregates. The dye molecules form interaction with these structures which is usually quantified by measuring absorbance spectra of the dye in presence of protein aggregates.

When congo red was subjected to lyophilized biofilm from implants, peak shift was observed which can be marked to the presence of protein aggregates. The absorbance peak was observed in control (aggregated Aβ peptide of Alzheimer's disease). Similarly, comparative analysis of congo red absorbance in presence of lyophilized biofilm from implants revealed the presence of aggregates in implant biomaterials (Figure 5). In accordance with the previous results, highest amount of aggregates was observed in polymeric implants. Overall, the results suggest that in vitro biomass attached on the implant surface is corresponding to robust biofilm formation.

Biofilm robustness in dental implants: The biofilm robustness depends on ability to resist common cleaning procedures involving shear force. In this study, brush cleaning procedure along with dentifrice was used for cleaning of dental implants forming biofilm in vitro. After applying rigorous shear force for cleaning implants, remaining biofilm was quantified and compared with the initial biofilm. It was found that there was not much difference in biofilm remaining on the implants after cleaning procedures (Figure 6). The biomaterials showing presence of aggregates resulted in most resistance towards cleaning procedures. Thus, it can be concluded that salivary microbes resulted into robust biofilm formation over implant materials.

CONCLUSION

Several biomaterials have been known for their applications in implants such as dental implants, heart valves, catheters etc. These implants posses certain life after which they are replaced and/or removed depending on the disease condition. With the growing incidence of microbial infections of nosocomial origin, the life of the implants has reduced causing monetary loss and poor quality of life for patients. Dental implants are most susceptible to microbial infections in the form of plaques, gingivitis which ultimately develop into most severe and resistant form - biofilm. The initiation of biofilm formation is highly dependent on cell adhesion which can vary according to different biomaterials used for preparation of implant.

The present study evaluates susceptibility of biofilm formation on metal, ceramic, polymer and carbon based implants through analysis of used or discarded implant samples with infections, obtained from clinical settings. The study was further strengthened by studying in vitro biofilm formation on fresh implants to analyze cell attachment

on different biomaterials. Overall, the study suggests that polymer based biomaterials are most susceptible to biofilm formation, may be due to ability to form better interactions with cell surfaces as polymers display several reactive groups. Thus, based on biofilm susceptibility and host toxicity choice between different biomaterials can be made in future. Further, the present study is limited to having an idea of biofilm formation over implants which can be further narrowed down to identification of microbial species responsible for biofilm formation on dental implants.

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