Surface Characterization of Ti Implants following Contamination with Biological Fluids and Effects of Various Chemical Reagents on Implant Surface Contamination

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Abstract

Ti-implants can get easily contaminated with saliva during surgery or after placement. This might alter its surface properties and interfere with the process of ossteointegration, ultimately leading to pre-implantitis and implant loss. Though several chemical agents are routinely used for implant decontamination, their exact effect is not well known and hence identifying their effect on Ti and oral contaminants is critical for developing better treatments for decontamination of Ti dental implants. Thesis objective manuscript-base was to characterize the physical properties of saliva-contaminated titanium surfaces and further assess the different chemicals that could be used for osteointegration.

In the first manuscript, we evaluated the efficacy of 6 different solutions that are commonly used to manage peri-implantitis (Listerine, 0.2% Chlorhexidine, 50% citric acid, 0.9% saline, PBS and 35% phosphoric acid) on saliva-contaminated implant surfaces. We used x-ray photoelectron spectroscopy (XPS) to assess the elemental composition of the surfaces and fluorescence microscopy to assess the bacterial load. XPS analysis revealed that amongst all the solutions assessed, citric acid and saline were the most effective in decontaminating Ti and partially restoring the original implant surface chemistry. Although none of the solutions was able to fully recuperate the original surface chemistry. All of them except saline and Listerine were effective in reducing the microbial load. These results indicate that amongst the solutions tested, citric acid and saline could be the best option for clinical application.

In second manuscript of this thesis, we assessed how saliva interacts with Ti-surfaces and the subsequent implication on Ti-blood interaction. We used contact angle measurements (CAM), x-ray photoelectron spectroscopy (XPS) and fluorescence microscopy to

characterize Ti samples before and after exposure to human saliva. The effect of saliva contamination on blood-implant interaction was further investigated. Our analysis revealed that on the Ti surfaces saliva formed a bacterial-rich hydrophobic organic layer that interfered with Ti-Blood interaction. After revealing the hydrophobic nature of saliva surface contaminants, we explored the use of solvents (acetic acid and acetone) and detergents (tween20) for Ti surface decontamination. Indeed, our analysis demonstrated that acetic acid and tween-20 achieved substantial elemental as well as microbial decontamination, suggesting that they can be potentially useful for Ti-implant decontamination. This part of this study therefore demonstrates that saliva interacts with Ti-implants interfering with blood Ti interaction but this saliva contamination can be managed with the use of acetic acid and tween-20 for Ti decontamination. Therefore saliva interferes with the interaction of Ti-implants with blood by creating a hydrophobic layer that it is rich in bacteria. However this layer can be easily remove with solvents, detergents or calcium chelators.

Résumé

Les implants en titane sont facilement contaminés par la salive durant l'intervention chirurgicale ou après la mise en place. Cette contamination risque de modifier les caractéristiques de la surface et d'avoir une incidence sur le processus d'ostéointégration, ce qui peut mener à une péri-implantite et à la perte de l'implant. Même si plusieurs substances chimiques sont souvent utilisées pour la décontamination des implants, leur effet exact reste mal connu. La détermination de leurs effets sur les contaminants du titane et de la bouche est donc cruciale lorsqu'il s'agit de développer des traitements améliorés de décontamination des implants dentaires en titane. L'objectif de la thèse est de décrire les caractéristiques physiques des surfaces de titane contaminées par de la salive et de mieux évaluer les différentes substances qui peuvent favoriser l'ostéointégration.

Dans le premier document, nous avons évalué l'efficacité de six solutions couramment utilisées pour la prise en charge de la péri-implantite (Listerine, chlorhexidine 0,2 %, acide citrique 50 %, solution saline 0,9 %, STP et acide phosphorique 35 %) sur des surfaces d'implant contaminées par de la salive. Nous avons utilisé la spectroscopie de photoélectrons XPS pour évaluer la composition en éléments des surfaces et la microscopie par fluorescence pour évaluer la charge bactérienne. L'analyse par XPS a montré que parmi toutes les solutions évaluées, l'acide citrique et la solution saline étaient les agents les plus efficaces pour la décontamination du titane et pour la restauration partielle des caractéristiques chimiques de départ à la surface de l'implant. Cependant, aucune des solutions évaluées n'était en mesure de restaurer complètement les caractéristiques chimiques de départ de la surface. Toutes, sauf la solution saline et

Listerine, étaient efficaces en ce qui a trait à la réduction de la charge bactérienne. Ces résultats laissent penser que parmi les solutions mises à l'essai, l'acide citrique et la solution saline sont peut-être la meilleure solution pour l'usage clinique.

Dans le deuxième document, nous avons évalué la manière dont la salive interagit avec les surfaces de titane, et ce que cela signifie du point de vue de l'interaction titane-sang. Nous avons utilisé la mesure des angles de contact, la spectroscopie de photoélectrons XPS et la microscopie par fluorescence afin d'observer les caractéristiques d'échantillons de titane avant et après une exposition à de la salive humaine. La recherche portait également sur l'effet de la contamination par la salive sur l'interaction sang-implant. Notre analyse a montré que sur les surfaces de titane, la salive formait une couche organique hydrophobe riche en bactéries qui a une incidence sur l'interaction sang-titane. Après avoir montré la nature hydrophobe des contaminants de surface liés à la salive, nous avons examiné l'utilisation de solvants (acide acétique et acétone) et de détergents (Tween 20) pour la décontamination des surfaces de titane. De fait, notre analyse montre que l'acide acétique et Tween 20 permettent d'obtenir une décontamination en éléments et microbienne importante, ce qui laisse penser que ces deux substances pourraient être utiles lorsqu'il s'agit de décontaminer des implants en titane. Cette partie de l'étude montre donc que la salive interagit avec les implants en titane d'une façon qui a une incidence sur l'interaction sang-titane, mais qu'il est possible de contrecarrer cette contamination par la salive au moyen d'acide acétique et de Tween 20 en tant qu'agents de décontamination du titane. La salive a donc une incidence sur l'interaction sang-implant en titane par la mise en place d'une couche hydrophobe riche en bactéries. Il est

cependant facile d'enlever cette couche avec des solvants, des détergents ou des chélateurs du calcium.

Chapter 1: Background and Literature Review

1.1 Dental Implants

The history on use of dental implants can be tracked back to 4000 year old Chinese archeological remains that revealed earlier humans using carved bamboo pegs taped into the bone to replace missing teeth's [1-3]. Metallic implants and fake teeth have also been found in the tomb of Iron Age women who died more than 2300 years ago [4]. Similarly, 2000 year-old ancient Egyptian mummies have been found with artificially carved ivory teeth and with transplanted human teeth [1-3]. Though these discoveries do not shed much light on the success of ancient dental implants, they do provide enough knowledge about the historic practical and social need for humans to replace their missing teeth.

The science of dental implant have evolved quite significantly with various research efforts focused towards greater understanding of the precise physiological processes involved in implant-human interaction, new implant materials and different ways to modify implant surfaces prior to implantation [5-12]. But the major break through in this area came from the pioneering research of Dr. PI Brånemark, who for the first time defined the process of osseointegration and is credited for developing the modern titanium based implants. His work demonstrated the osseointegration of titanium implants into rabbit and dog bones [8, 13]. On the other hand, Dr. Leonard Linkow, considered by some as the "father of implantology", is debated to pioneer the concept of using modern dental implants to avoid removable prostheses in the 1960s. Though he did not publish his clinical work, it is believed that he initiated titanium and other metal implants to hold prosthetic teeth as early as 1950s [14] . The first science based clinical trial of titanium dental implants eventually began in 1965 [8].

Groundbreaking work by these and many more researchers has established and highlighted the immense utility of endosseous dental implants as modern medical devices for retaining and supporting the dental prosthesis [15-17]. Therefore, optimizing clinical processes and utilizing evidence-based procedures for accomplishing efficient osseointegration can minimize the risk of dental implant failure to a significant level [18].

1.2 Osseointegration

Modern dentistry aims to restore patients oral health in a predictable fashion [1]. Its now known that success of dental implants is largely dependent on a physiological process called osseointegration, wherein implant materials such as Ti forms a biological connection with the host tissue/bone [9].

Per-Ingvar Branemark first coined the term osseointegration in 1969, describing it as a direct structural and functional connection between the living bone and the implant surface [19]. Ever since Branemark's initial observations, the concept of osseointegration has been described and defined by different groups at multiple levels. The American Academy of Implant Dentistry (1986) described osseointegration as "the contact established without interposition of non-bone tissue between normal remodeled bone and an implant entailing a sustained transfer and distribution of load from the implant to and within the bone tissue" [20]. Zarb and Albrektsson defined it as the time dependent healing phenomenon, wherein a rigid fixation of alloplastic implant materials is achieved and sustained in a clinically asymptomatic manner during the functional loading [21, 22]. Currently, a successful osseointegration is considered when there is no progressive relative movement between the implant and the adjacent bone to which it is in contact. Thus, the process of osseointegration is nothing but an anchorage mechanism by which

implant materials can be reliably incorporated and sustained into the living bone under all optimal conditions of loading [13, 23].

Stages of Osseointegration

Osseointegration is activated when pre-existing lesion in bone matrix exposes the matrix to extracellular fluids, facilitating the release of various non-collagenous proteins and growth factors, which ultimately activate the bone repair pathways [24]. Once activated, osseointegration follows into 3 distinct stages:

1. Tissue response to lesion/ implantation.
2. Peri-implant Osteogenesis
3. Bone remodeling

Stage 1: Tissue Response

Osseointegration involves a chain of cellular and extra-cellular events occurring at the implant-bone interface, until the implant surface is completely fused with the newly formed bone [25]. The initial host response to this process resembles the actual bone healing process characterized by the initial activation of various osteogenetic events [26, 27]. These biological events are orchestrated by the activated growth and differentiation factors set free by the activate blood cells at the implant-bone interface [28].

The initial host response following implantation is altered by the implant characteristics, the stability of fixation and intra-operative procedures [25-28]. Some of the important events during this phase of osseointegration include the formation of blood clot and mesenchymal tissue development. Blood cells including the RBCs, platelets,

granulocytes and monocytes are the first biological effectors that come in contact with the implant surface. These blood components are known to migrate into the tissue area surrounding the implant surface, wherein they are activated to release various cytokines and growth factors [28]. This initial interaction of blood cells with the implant surface therefore influences the clot formation. In this process, the foreign implant surface induces morphological and biochemical changes in platelets, which further facilitates the formation of a fibrin matrix. This fibrin matrix then acts as a scaffold for the recruitment of osteogenic cells and osteoinduction of these cells in the healing compartment [12, 28, 29].

Stage 2: Peri-implant osteogenesis

Peri-implant osteogenesis involves early deposition of new calcified matrix onto the implant surface. When a newly formed bone trabeculae develops from the host bone cavity towards the implant surface, it is known as distance osteogenesis. In contrast, contact osteogenesis refers to a newly formed peri-implant bone developing from the implant to the healing bone [30]. In both types of osteointegration, the newly formed bone ensures biological fusion of the implant surface to the bone and surrounds spaces containing mesenchymal cells and blood vessels. Ultimately, osteoblasts directly deposit a thin layer of calcified and osteoid tissue onto the implant surface whereas the mesenchymal cells and blood vessels fill in the space devoid of calcified tissue [28, 31, 32].

Following this initial implant surface calcification event, the woven and trabecular bone seal the gap at the implant-bone interface. These bones then arrange in a specific three-

dimensional conformation offering a high resistance to early implant loading and providing a very active biological scaffold for cell attachment and bone deposition [31, 33]. The woven bone is ultimately remodelled into lamellar bone that attains higher degree of mineralization.

Stage 3: Peri-implant bone remodeling

As an adaptation to stress and mechanical loading, the initially formed woven and lamellar bones contacting the implant surface undergoes morphological remodelling. The overall turnover of mature bone in osseointegrated implants is characterized by the presence of medullary spaces containing osteocytes, osteoclasts, vasculature and mesenchymal cells adjacent to the implant surface [31, 34].

1.3 Titanium

Titanium (Ti) is one of the most abundant elements in the earth's crust. Out of the total Ti mined, only 5%-10% is used in its metallic form while a significant part is converted into titanium dioxide pigments for use in the paint and dye industry [35]. Ti is less dense than steel but is much stronger than aluminum. Its coefficient of thermal expansion is lower than that of steel and aluminum. It is a rather soft and non-magnetic metal, which possesses good heat conductive properties. Most of the commercially available or surgically pure Ti products are in fact metal alloys with different elements such as oxygen, carbon, nitrogen and iron. The concentration of these elements plays an important role in determining physic-chemical and mechanical properties of pure Ti-based materials [36]. Ti is known to be non-toxic in nature and its excellent resistance to body fluids coupled with its high strength and low modulus makes it one of the most

biocompatible metals to be used in the human body [7, 37]. Ti is thought to be naturally compatible with the human host, with a normal human body containing 0.01g Ti/ 70 kg body weight. Moreover, Ti-oxides have been safely used as a food supplement and dye for several years without any demonstrated health hazards [38].

Apart from excellent biocompatibility, Ti also possesses outstanding corrosion resistant properties as a result of formation of passive, regenerative oxide layer on the Ti surface. The formation and maintenance of this Ti-oxide layer depends on the environment to which the metal is exposed. For example, oxidizing, neutral or naturally occurring conditions the favors formation of Ti-oxide layer, rendering it more corrosion resistant. However, interaction with strongly reducing environment restricts the formation of the oxide layer, making it more amenable to corrosion. Apart from these, Ti behavior also depends on the presence of metal ion inhibitors, alloying elements, temperature and many other variables [39]. The protective Ti-oxide film is believed to form instantaneously upon exposure to air with an initial thickness of 12-16 Å. Under similar conditions, this thickness is known to increase with time, reaching as high as 250 Å after 4 years [40, 41]. Temperature and oxidative conditions play an important role in regulating the thickness of this protective layer. Moreover, the composition of this layer varies across its thickness with TiO near the metal surface, TiO_2 at the outer surface and Ti_2O_3 between the two [42]. This surface Ti-oxide layer also affects the surface heat transfer properties as the thermal conductivity of Ti is greater than that of its oxides.

1.3.1 Titanium Implants

The combination of high tensile strength, low density, excellent biocompatibility and

outstanding corrosion resistance of Ti alloys have facilitated wide and diverse range of successful applications in various industries including aerospace, automotive, chemical, power generation, oil extraction and many others. One of the most important applications of Ti and its alloys has been its utility as a near-perfect biomaterial in medical, surgical and dental devices. Branemark et al. first put Ti to practical use as implant material in the early 1960s [8] and since then thousands of studies have explored various possibilities to optimize the utility of Ti-based implants in medical and dental applications.

Some of the pre-requisites for an ideal biomaterial include characteristics such as: corrosion resistance; biocompatibility, bioadhesion, biofunctionality (mechanical properties), process ability and surplus availability [43]. Ti scores high in almost all of these criteria with its outstanding resistance to corrosion by body fluids, excellent biocompatibility, high tensile strength, low modulus of elasticity, low density and most importantly, its ability to osseointegrate with adjoining host bone. All these properties make Ti, one of the best available implant materials today [44, 45]. Moreover, Ti-alloys, used to manufacture medical/commercial grade Ti are known to possess even better mechanical properties as compared to pure Ti [44, 45]. These alloys are also known to possess higher strength-to-weight ratio as compared to stainless steel based materials. Furthermore, increasing availability of different Ti-alloys has facilitated wide arrays of selection options suitable for a specific application. Surface engineering of these implant materials have further extended their performance and application window, adding to the widespread utility of this important metal.

For the reasons mentioned above Ti and its alloys have been seen as a major replacement in health care application to conventional stainless steel, cobalt-based alloys or any other

type of metallic alloys. Commercially pure Ti-alloys have therefore found wide applications in the bone and joint replacement, orthopedic treatment, dentistry, cardiovascular devices, external prostheses and surgical instruments [5].

For orthopedic replacements, biocompatibility of Ti coupled with roughened bioactive surfaces is used to induce osseointegration and enhance implant success. Ti and its alloys are also used in spinal fusion devices, pins, bone-plates, screws, intramedullary nails and external fixators in bone fracture fixation [5]. Ti based materials are also used in cardiovascular devices such as pacemakers and defibrillators, intra-vascular stents and supports for replacement valves [5].

In dentistry, Ti and its alloys are used for fabricating orthodontic wires, restorations and manufacturing of dental implants including crowns, bridges, dentures and tooth roots. These implants are mainly installed into the jawbone, which eventually results in the formation of the superstructure of the tooth, following successful osseo-integration.

1.4. Failure of Ti implants

Even though treatment with titanium implants is generally known to be a successful intervention, there are many factors that might eventually lead to implant failure. The major causes of implant failures can be attributed to 4 different categories [10] as listed in table:

Table 1: Summary of the major causes of implant failure

Category	Cause of Implant Failure
Biological	Due to inability to establish osseointegration (early failure) or due to failure to maintain the achieved osseointegration (late failure).
Mechanical	Failure resulting from fracture of implants, connecting screws, and framework
Latrogenic	Failure as a result of nerve damage and mal-positioning of the implant.
Patient Adaptation	This category includes patient issues with phonetics, aesthetics or other psychological issues.

The section below would be mainly focused biological causes of implant failure, mainly a peri-implant infection (peri-implantitis).

1.4.1 Peri-implant Infection

Peri-implant infection refers to a collection of conditions resulting in an inflammatory reaction in the tissues surrounding the dental implant [10]. It can be further divided into two different categories: peri-implant mucositis and peri-implantitis. Peri-implant mucositis is a reversible inflammatory reaction mainly affecting the surrounding soft tissue, without the loss of supporting bone. Peri-implantitis, in contrast, can be defined as an inflammatory process affecting the tissues around the implant, resulting in the loss of the supporting bone [46, 47]. Moreover, peri-implantitis being more destructive in nature,

affecting the surrounding bone and soft tissue, replacement implants are pretty much difficult to place and are associated with reduced implant survival rates [48].

Zitzmann *et al*,. conducted a review to determine the prevalence of patients affected by peri-implant infection and they reported that while peri-implant mucositis was prevalent in 79% of the subjects and 50% of all implants while; peri-implantis was found to be present in 28-56% of the patients and 12-43% of the affected implants [49]. But as very few studies currently exists with data on peri-implant diseases, with even fewer studies actually looking at its prevalence, it has been suggested that the peri-implant disease might be grossly under-estimated [6]. And with the increasing number of implants being placed each year, it is expected that the total number of patients suffering from this condition would rise in the near future. Amongst the multiple factors causing peri-implant infection, oral biofilm formation and dental plaque play an essential role on the initiation and progression of peri-implant diseases [50].

Oral biofilm formation

Microbes possess an ability to colonize surfaces of the oral cavity including the Ti, gold and acrylic surfaces of dental prostheses. Oral biofilms comprise of complex microbial communities and its formation involves multiple steps: 1) pellicle formation; 2) reversible attachment; 3) irreversible attachment; 4) co-adhesion and multiplication of attached colonies; 5) maturation of biofilm; 6) cell detachment [51].

The acquired pellicle formation can occur within a few minutes from the introduction of clean implant surface into the oral environment. It is composed of host derived molecules mainly coming from saliva (proline-rich proteins, statherin, *a*-amylast, mucins and other glycoproteins [52]. Adsorption and de-sorption of these saliva derived molecules regulate

the thickness of the pellicle layer [52], which then plays an important role in the establishment of the microbial community. Generally, bacteria are known to make contact with the implant surface, rather passively, via flow of saliva and other fluids, though in some species, flagella might assist in the transport of bacteria to the surface [51]. The interaction of bacterial cells towards the acquired conditioning film results in the formation of long-range, reversible, physicochemical adhesive forces generated by negatively charged bacterial cells and the hydrophobic surface components of the pellicle [51]. Along the process, irreversible attachment occurs via strong but short-range interactions between the microbial cell surface components called adhesins and the complementary receptors on the surface of the acquired pellicle [53].

The primary/ early colonizers recognize specific host-derived salivary pellicle molecules and hence this step marks one of the most important steps in biofilm formation. These early colonizers are resistant to high oxygen concentrations and some physical challenges like chewing and fluid flow [54]. Moreover, they offer additional receptors for adhesion of secondary colonizers, who on their own are unable to bind to the implant surface. This process is called coadhesion [51]. In short, early colonizers adhere directly to the salivary pellicle and this is followed by subsequent growth of the biofilm upon recruitment of secondary colonizers, which co-adhere with the early ones.

The maturation of the biofilm is associated with decreased growth rates and formation of a biofilm matrix, that further contributes towards the structural integrity of the biofilm, making it more resistant to environmental stress, local host conditions and anti-microbials [51, 55]. The final step of the biofilm life-cycle involves detachment of cells from the implant surface as a response to unfavorable local host conditions. This is considered to

be an effective survival strategy as by doing this, the biofilm escape the unfavorable environment and re-colonize in a more favorable location [51].

These biofilms are capable of developing on all the surfaces of the oral cavity including titanium substrates. Ti on its own does not possess any anti-microbial activity and is not known to alter the colonization of bacterial cells onto its surface [56]. Infact, bacteria are known to colonize the Ti surface within 30 minutes after surgical placements of the dental implants [57]. This suggests that interaction of host saliva and Ti implants is indeed one of the most important steps, that lays the foundation for the formation of the biofilm onto the implant surface. Therefore, it is very important to study these titanium-implant interactions at the molecular level and use this knowledge to come up with effective decontamination strategies, eventually aiding in preventing peri-implant diseases.

1.5 Decontamination of Titanium Implants

Contamination of dental implants is known to be one of the major factors influencing the success of any implant [58, 59]. Dental implants with peri-implantitis are therefore commonly decontaminated using various chemical and physical methods with an aim to minimize their failure rate [58, 59]. But the lack of knowledge and consensus about an optimum method or disinfectant to decontaminate these affected implants poses a major challenge.

1.5.1 Chemical decontamination of dental implants

Some of the most commonly used chemical reagents used for implant surface decontamination include sodium bicarbonate ($NaHCO_3$), hydrogen peroxide (H_2O_2),

chlorhexidine (CHX), Ethylene -diamine -tetra-acetic acid [EDTA], and tetracycline [58-61]. Selection of one or more of these solutions to decontaminate implants might have different levels of toxicity effects on the cells and hence one must be really careful while choosing these solutions [61]. Moreover, different types of implants vary considerably regarding their ease of decontamination, Indeed, Osseotite implant discs are easier to disinfect than the Nanotite discs [62]. This suggesting that surface modification to achieve faster osseointegration can also affect the ability of chemicals to decontaminate the implants. Moreover different chemical contamination reagents can have varying degrees of effectiveness [11, 62] and hence there is a need to further focus our research on identification and validation of new chemical decontamination methods.

1.5.2 Physical and mechanical decontamination of dental implants

With the advent of new technologies, various novel methods have been tested for their potential utility in decontamination of dental implants. Some of the these include laser based disinfection [62], electric current disinfection [63]. And air abrasion + brushes [64] among other methods.

Though these methods look promising not there is a big knowledge vacuum in the literature about the effectiveness of the method for optimum decontamination of dental implants.

Moreover, many of these methods have been thought to potentially enhance the effectiveness of chemical reagents to decontaminate dental implant surfaces, though not much research has been focused to assess the utility of such combinations. In general clinical practice, most dentists end up using CHX as their first preference for disinfection of implant surfaces, mainly when diagnosed with peri-implantitis [65]. The other

common reagents for decontamination include citric acid and H2O2 [62]. All these chemical reagents can be easily activated using an ultrasonic tip or a laser that can impart energy in these solutions, creating fluid movement [62]. The increased movement of these fluid disinfectants might therefore increase their decontamination efficiency. Similarly, a Ti brush is sometimes used to clean the implant surface [66].

Chapter 2: Hypothesis and Objectives

2.1. Hypothesis

We hypothesize that saliva might interfere with the interaction of Ti-implants with blood, hence significantly limiting osseointegration. Furthermore, by characterizing the surface of saliva-contaminated Ti, we might be able to come up with more targeted and specific decontamination strategies for Ti-surfaces.

2.2. Objectives

The objective of this thesis was to characterize the physical properties of saliva-contaminated titanium surfaces and further assess the different chemicals that could be used for decontamination.

These objectives were addressed in two manuscripts presented in chapter 4 and 5 respectively.

Chapter 3: Materials and Methods

3.1 X-ray Photoelectron Spectroscopy (XPS)

X-ray Photoelectron Spectroscopy (XPS), also generically known as Electron Spectroscopy for Chemical Analysis (ESCA), is considered as the gold standard technique for assessing and characterizing solid surfaces [67]. When the solid surface to be analyzed is irradiated with X-rays, the surface atoms absorb these X-rays leading to the ejection of core electrons having binding energy less than the X-rays. The photoelectron energy liberated during this process is known to be unique for each surface element and sensitive to the chemical state of the element. XPS is capable of sensitively detecting these photoelectron energy signatures from almost all the elements in the periodic table to effectively provide quantitative information on the elemental and chemical composition of the solid surfaces [67-70].

XPS mainly comprises an X-ray source, a photoelectron energy analyzer and an ultra-high vacuum [67]. The most common X-ray sources used for XPS are Al Kα or Mg Kα owing to their suitable line energy and width for such applications [67, 70]. Also, in order to maintain high level of sensitivity and protect samples from surface contamination, XPS analyzer utilizes vacuums of the order of 10^{-8} Pa or lower [67]. The photoelectron analyzers in XPS detect energies from the ejected photoelectrons and relay this information to the detector system, which further yields data in the form of specific peaks.

In order to identify the surface elements and quantify each atom on the surface, a low-resolution general survey scan is performed that spans the entire binding energy (0 to 1000 eV) of a surface. Following this initial scan, a high-resolution scan is then

performed to obtain more precise XPS spectra for each element in that specific region [67, 70]. The high precision and sensitivity of XPS allows it to assess approximately 1^{-10} outermost atomic layers (10nm or less) of a solid surface with the detection limit of 0.1-1.0 %.

In the both the chapters of this thesis, the surface elemental composition of the titanium samples was characterized using a monochromatic X-ray photoelectron spectrometer K alpha (Thermo Fischer Scientific Inc. East Grinstead, UK) equipped with an Al Kα X-Ray radiation source (1486.6eV, 0.834nm), and an ultrahigh vacuum chamber (10^{-9} torr). Survey scans were obtained for various test groups in manuscript 1 (polished control, saliva-contaminated, listerine treated, chlorhexedine treated, citric acid treated, saline treated, PBS treated, phosphoric acid treated) and manuscript 2 (control, saliva contaminated, saliva and then blood contaminated and blood contaminated) over the range of 0 –1350eV with a pass energy of 100eV at a step of 2.0eV, while high resolution scans of C and Ti were collected with a pass energy of 50eV at a step of 0.1eV. The carbon bonded hydrogen or carbon (C- (H, C)) at 285.0eV was set as a reference for all samples. Data analysis and peak fitting were performed using Avantage chemical surface analysis software (5.41v, Thermo Fischer Scientific Inc, East Grinstead, UK).

3.2 Contact Angle Measurement

Solid surface contact angle measurement is one of the most common techniques used to evaluate the wettability and hydrophobicity of solid surfaces and this is done by quantifying the angle formed by a liquid droplet onto a given solid surface [71].

In our studies, we used contact angle measurements to assess the hydrophobicity/ hydrophilicity of Ti specimens before and after contamination by body fluids. Using a contact angle meter (OAC 15, Data Physics, Germany), static contact angle measurements were recorded with the sessile drop method at room temperature. Briefly, a drop of distilled water was deposited on the Ti control and treated specimens and the angle formed between the liquid drop and the specimen's surface was measured. Values were reported as averages of 5 drops of distilled water per sample. The side view images were captured and the static contact angle was automatically calculated using video based software (SCA 20, Dataphysics Instruments, Germany). We used this analysis to distinguish the properties of the first layer of different Ti surfaces with regards to surface hydrophobicity.

3.3 Fluorescence Microscopy

With the advent of new methods to fluorescently label different microbial components with different dyes, fluorescence microscopy has come up as a new technique to evaluate the microbial burden on a given surface. In our experiments, reverse light fluorescence microscope (Carl Zeiss Microscopy GmbH, Gottingen, Germany) equipped with a AxioCam digital camera (MRm Rev. 3, Carl Zeiss Microscopy, Gottingen, Germany) was used to assess the microbial load on the contaminated Ti specimens as well as to evaluate the effect of various cleaning agents on microbial decontamination of these contaminated specimens.

More specifically, in manuscript 1, Ti specimens belonging to different test conditions (polished control, saliva contaminated, Listerine treated, Chlorhexedine treated, citric

acid treated, saline treated, PBS treated and phosphoric acid treated) were fluorescently stained using Live/Dead staining kit (BacLight Bacterial Viability Kit, Molecular Probes, Carlsbad, USA) and assessed under fluorescence microscope to evaluate the effect of saliva contamination as well as chemical treatment with different solutions on the surface microbial load. Similarly, in manuscript 2, fluorescence microscopy was used to evaluate the effects of acetic acid, acetone and tween-20 on microbial decontamination of saliva contaminated Ti implants.

The Live/Dead BacLight kit has found routine application in various fields of research encompassing microbial analysis. The kit consists of 2 fluorescent dyes SYT09 and propidium iodide (PI) capable of staining nucleic acids in two different colors. The red fluorescing PI dye is used to estimate the dead microbial load as it can only enter cells with damaged cytoplasmic membranes while the green SYT09 is mainly used to assess total microbial count owing to its ability of entering all cells. The fluorescent staining intensities of these two dyes are therefore detected and analysed to obtain an estimate of the microbial load on a given surface.

For our experiments, the Live/Dead staining was performed as per the manufacturers protocol. Briefly, the live/dead stain was prepared by diluting 1µL of SYTO 9 (excitation (λ) = 485 nm, emission = 498 nm) and 1µL of propidium iodide (excitation = 535 nm, emission = 617 nm) in 1 mL of distilled water. Specimens were placed in 24-well plate, and 500µL of the reagent mixture was added to each well followed by incubation at room temperature and in the dark for 15 min. Each Ti sample was positioned on a glass slide and then covered with mounting oil and stored in a dark space at 4˚C until further processing. Ti specimens were assessed using a fluorescence microscope (Carl Zeiss

Microscopy GmbH, Gottingen, Germany) equipped with a digital camera (AxioCam MRm Rev. 3, Carl Zeiss Microscopy, Gottingen, Germany) in combination with image processing software (ZEN; Carl Zeiss Microscopy GmbH, Gottingen, Germany). For each Ti sample, five randomly selected sites were captured from the surface using a 10x objective. Median of red fluorescent areas (dead cells), green fluorescent areas (viable cells), and total attached bacteria were calculated (expressed as A.U.) using cell profiler image analysis software (Broad Institute of MIT and Harvard, Massachusetts, USA).

Chapter 4: Manuscript 1

Evaluation of Efficacy of Different Chemical Reagents for Decontamination of Titanium Implant Surfaces

Azam Fayezi Sisi, Mohamed-Nur Abdallah, Ashwaq A. Al-Hashedi, Rubens Albuqurque, Faleh Tamimi

Abstract:

Biological fluids, pH fluctuations, diet and the microbes can contaminate the dental implant surfaces. These contaminants may alter the implant surfaces ultimately leading to pre-implantitis and the loss of osseointegration. Though several chemical agents are routinely used in clinics for implant decontamination, their exact effect on the implant surfaces is not well known and hence identifying their effect on Ti and oral contaminants is critical for developing better treatments for decontamination of Ti dental implants.

We evaluated the effect of 6 different solutions that are commonly used to manage peri-implantitis (Listerine, 0.2% Chlorhexidine, 50% citric acid, 0.9% saline, PBS and 35% phosphoric acid) on saliva-contaminated implant surfaces. We used x-ray photoelectron spectroscopy (XPS) to assess the elemental composition of the surfaces and fluorescence microscopy to assess the bacterial load. XPS analysis revealed that amongst all the solutions assessed, citric acid and saline were the most effective in decontaminating Ti and partially restoring the original implant surface chemistry. Although none of the solutions was able to fully recuperate the original surface chemistry, fluorescence microscopy showed that citric acid was effective in reducing the microbial load. These results indicate that amongst the solutions tested, citric acid and saline could be an option for clinical application.

4.1 Introduction

Biological fluids, microbes, pH fluctuations as well as food debris in the oral environment, can introduce different types of contaminants (hydrocarbons, molecules, elements, biofilm) onto the surface of dental implants [1-5]. These contaminants may alter the superficial properties of the implants and lead to peri-implantitis, a condition characterized by an inflammatory reaction around partially osseo-integrated implants that eventually results in the loss of the supporting bone [4].

Various cleaning solutions have been explored for implant surface decontamination [6]. Some of the most commonly used solutions include Chlorhexidine, hydrogen peroxide (H_2O_2), citric acid, phosphoric acid, delmopinol, Listerine R, iodine, phosphate buffered saline (PBS), beta-isodona, and chloramine-T [6-10]. Of these, Chlorhexidine (CHX), one of the most important antiseptic solutions used in dentistry [11], has been employed for the treatment of peri-implantitis and as an implant irrigation solution [12, 13]. A large number of *in vitro* and *in vivo* studies have indeed highlighted the role of CHX for implant surface decontamination and its contribution to re-osseointegration [14-20]. Likewise, citric acid (CA) is also used for Ti surface decontamination during the surgical treatments of peri-implantitis [21, 22]. Various *in vitro* studies have demonstrated that CA treatment alone or in combination with H_2O_2 can significantly decrease the viable microbial load [15, 23, 24]. However, it has been claimed that, in failed Branemark implants, CA treatment alone did not reduce the concentration of elemental surface contaminants like carbon and nitrogen [25]. However despite the extensive literature on decontamination of Ti and management of peri-implantitis, progress in this filed has been very limited and treatment of pre-implantitis remains very unpredictable. One reason for such limited progress is the fact that very little is known about the interaction of

decontamination solutions with the implant surface as well as their precise mode of action [6, 11-13].

Though the above mentioned solutions are commonly used in clinical dental practices, no ideal treatment for decontamination of Ti exists. Many of these solutions have been used on an empirical basis with many protocols combining different solutions and cleaning methods [6-9, 26]. To develop an optimum protocol for decontamination of oral implants, it is very important to precisely understand the effect of these solutions on implant surface as well as on the biofilm.

We hypothesized that different chemical cleaning solutions might interact differently with contaminated implant surfaces. Hence our aim was to precisely characterize these interactions and assess the exact effect of these cleaning solutions on the composition and bacterial load saliva-contaminated Ti-implant surfaces. This way, we would be able to pave the way for more predictable treatments of peri-implantitis.

Biological fluids such as saliva are one of the most common sources of implant surface contamination [27] and hence characterizing the interaction of saliva with Ti implant surface and identifying optimum chemical treatment/ cleaning methods to decontaminate these Ti-implants is a major focus of research in these areas. Indeed if a cleaning method is unable to clean saliva-contaminated Ti, it will be unrealistic to expect it to decontaminate Ti-implants with active biofilm. Therefore, our study aimed at characterizing the physico-chemical properties of Ti implants surfaces following contamination with saliva and assessing the effectiveness of different routinely used chemical solutions on this decontamination of these surfaces.

4.2 Materials and Methods

In this study, Ti-samples were contaminated with saliva and treated with different solutions to assess their efficacy in decontaminating saliva contaminated Ti-surfaces. Ethical approval was obtained from the McGill University Ethics Board (14-464 GEN).

4.2.1 Sample Preparation

Ti samples (Ultra-Corrosion-Resistant Titanium, grade 2, McMaster-Carr, Cleveland, OH) were obtained as rectangular bars (6.4, 12.7 and 305.0 mm) and cut into smaller sections (12.7, 6.4 and 6.4 mm) using an abrasive cutter (Delta AbrasiMet, Buchler, Whitby, ON). In order to obtain flat surfaces, the Ti samples were first polished using a water-cooled trimmer and 240-to-600 grit silicon carbide papers (paper-c wt, AA Abrasives, Philadelphia, PA). Then, the samples were polished on a polishing wheel (LapoPol-5, Struers, Rodovre, Denmark) using two polishing clothes: rough to intermediate polishing cloth (15-0.02μm; TexMet C) and final polishing cloth (1-0.02μm; ChemoMet) with colloidal silica (≤ 0.06μm; MasterMet; Buchler, Whitby, ON). All samples were cleaned in an ultrasonic bath (FS20D Ultrasonic, Fisher Scientific, Montreal, Canada) with acetone, ethanol and distilled water for 5 minutes in each solution at room temperature. The samples were vacuum dried over-night before further analyses.

4.2.2 Surface Contamination with Saliva

For contamination of the titanium samples with saliva, unstimulated saliva samples were collected from a healthy non-smoking volunteer in sterile tubes. Single titanium samples were immersed in each of the saliva tubes for 15 minutes followed by ultra-sonication in distilled water for 15 minutes. All samples were vacuum dried over-night before any further analyses.

4.2.3 Surface Decontamination with various solutions

In order to assess the effect of commonly used cleaning solutions on Ti implants, saliva-contaminated Ti specimens were cleaned in an ultrasonic bath for 15 minutes with 10ml of one of the following reagents: Listerine, 0.2% Chlorhexidine, 50% citric acid, 0.9% saline solution, phosphate buffered saline (PBS) or 35% phosphoric acid (for each condition n=9).

Samples were then ultra-sonicated in 10ml distilled water for 15-minutes and vacuum dried, before analyzing their surfaces. All samples were analyzed for surface composition and bacterial load before and after saliva-contamination and subsequent decontamination with the different solutions.

4.2.4 Surface composition:

The surface elemental composition of the different titanium samples was characterized using a monochromatic X-ray photoelectron spectrometer(XPS)(K alpha, Thermo Fischer Scientific Inc., East Grinstead, UK) equipped with an Al Kα X-Ray radiation source (1486.6eV, 0.834 nm) and an ultrahigh vacuum chamber (10^{-9} torr). Survey scans were obtained with pass energy of 100eV at a step of 2.0eV and high-resolution scans of C was collected with pass energy of 50eV at a step of 0.1eV. The reference for all the samples was set as carbon bonded hydrogen or carbon (C-(H, C)) at 285.0eV. Data analysis and peak fitting were performed using Avantage chemical surface analysis software (5.41v, Thermo Fischer Scientific Inc., East Grinstead, UK).

4.2.5 Bacterial load

We used fluorescence microscopy in order to assess the microbial load on the saliva-contaminated Ti specimens as well as to evaluate the effect of the various cleaning agents on microbial decontamination. Saliva contaminated-Ti specimens (n=9) were probed

using the Live/Dead staining kit (BacLight Bacterial Viability Kit, Molecular Probes, Carlsbad, USA) and analyzed by fluorescence microscopy. The live/dead stain was prepared by diluting 1µL of SYTO 9 (excitation/ emission (λ) = 485/498 nm) and 1µL of propidium iodide (excitation/ emission= 535/617nm) in 1mL of distilled water. The specimens were placed in a 24-well plate and 500µL of the above reagent mix was added to each well followed by incubation in dark for 15 min. at room temperature. These Ti samples were then positioned on a glass slide, covered with mounting oil and stored in a dark at 4˚C until further processing. An upright fluorescence microscope (Carl Zeiss Microscopy GmbH, Gottingen, Germany) equipped with a digital camera (AxioCamMRm Rev. 3, Carl Zeiss Microscopy, Gottingen, Germany) in combination with image processing software (ZEN; Carl Zeiss Microscopy GmbH, Gottingen, Germany) was used to analyze these samples. For each Ti sample, five randomly selected sites were captured from the surface using a 10x objective. Median of red fluorescent areas (dead cells), green fluorescent areas (viable cells), and total attached bacteria were calculated (expressed as A.U.) using cell profiler image analysis software (Broad Institute of MIT and Harvard, Massachusetts, USA).

4.2.6 Statistical analysis

Statistical analysis for XPS and bacterial load results was performed using Origin 9.0 (Origin lab, Northampton, MA). All the data were analyzed using Tukey Post Hoc test and one-way ANOVA test and the significance level was set at $p<0.05$.

4.3 Results

4.3.1 Surface elemental composition of Ti before and after contamination

XPS analysis demonstrated that surfaces contaminated with saliva showed significantly higher concentrations of C and N and significantly lower concentrations of Ti and O compared to the control uncontaminated surfaces (Fig.1, $p<0.05$). All of the tested cleaning agents were able to substantially reduce the concentration of C on the surface of saliva-contaminated samples. Out of all, citric acid and saline stood out in this ability to reduce the concentration of C (fig. 1-B, $p<0.05$), though no significant difference was observed between C-cleaning efficiency of the two.

Moreover, amongst all the solutions tested, citric acid was the only one able to reduce the surface concentration of N of the saliva-contaminated samples (Fig.1C). Listerine, chlorhexidine and phosphoric acid treatments resulted in even higher surface N-concentrations as compared to saliva-contaminated specimens.

With regards to O-concentration, all solutions were effective in raising the O-concentration as compared to saliva-contaminated specimens; though saline was the only solution able to bring the levels similar to that prior contamination (Fig.1E).

Furthermore, all the cleaning solutions were found to be effective in increasing the concentration of Ti on the saliva-contaminated surfaces, although again, the treatment with saline solution was the most effective in raising the surface concentration of Ti as compared to other modalities (Fig.1D).

All these results suggests that citric acid and saline could be more effective in cleaning saliva-contaminated implant surfaces as compared to other cleaning reagents like Listerine, PBS, phosphoric acid and chlorhexidine.

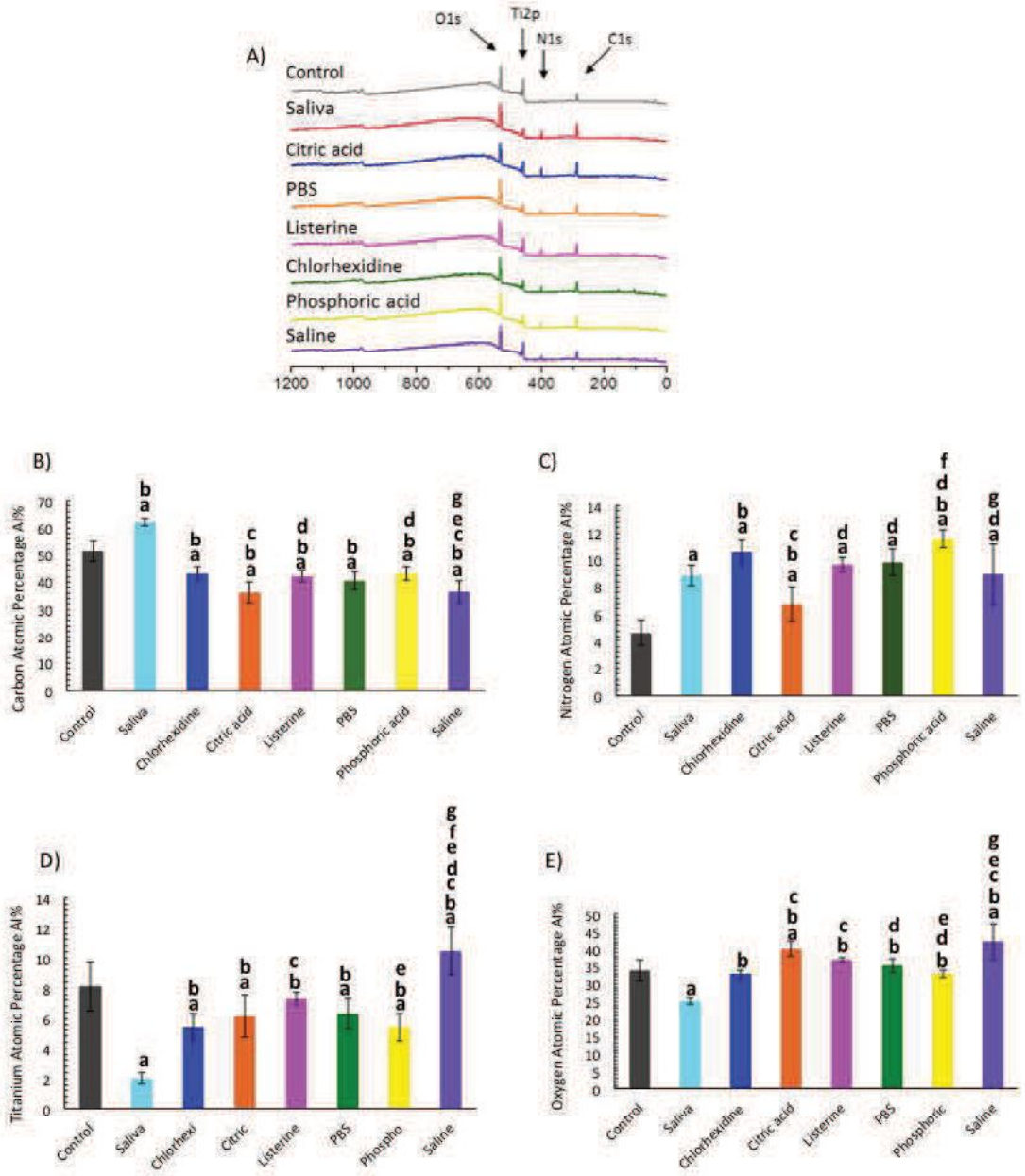

Figure 1. A) XPS survey spectra of the different surfaces: polished control, saliva-contaminated and saliva-contaminated after treatment with cleaning agents: (Listerine, Chlorhexidine, citric acid, saline, PBS and phosphoric acid). Carbon (C1s), nitrogen (N1s), oxygen (O1s), and titanium (Ti2p) are shown. B-E) letters in the bar chart indicate significant differences from: control (a), Saliva (b), chlorhexidine (c), citric acid (d), Listerine (e), PBS (f), phosphoric acid (g) and saline (h) $p<0.05$).

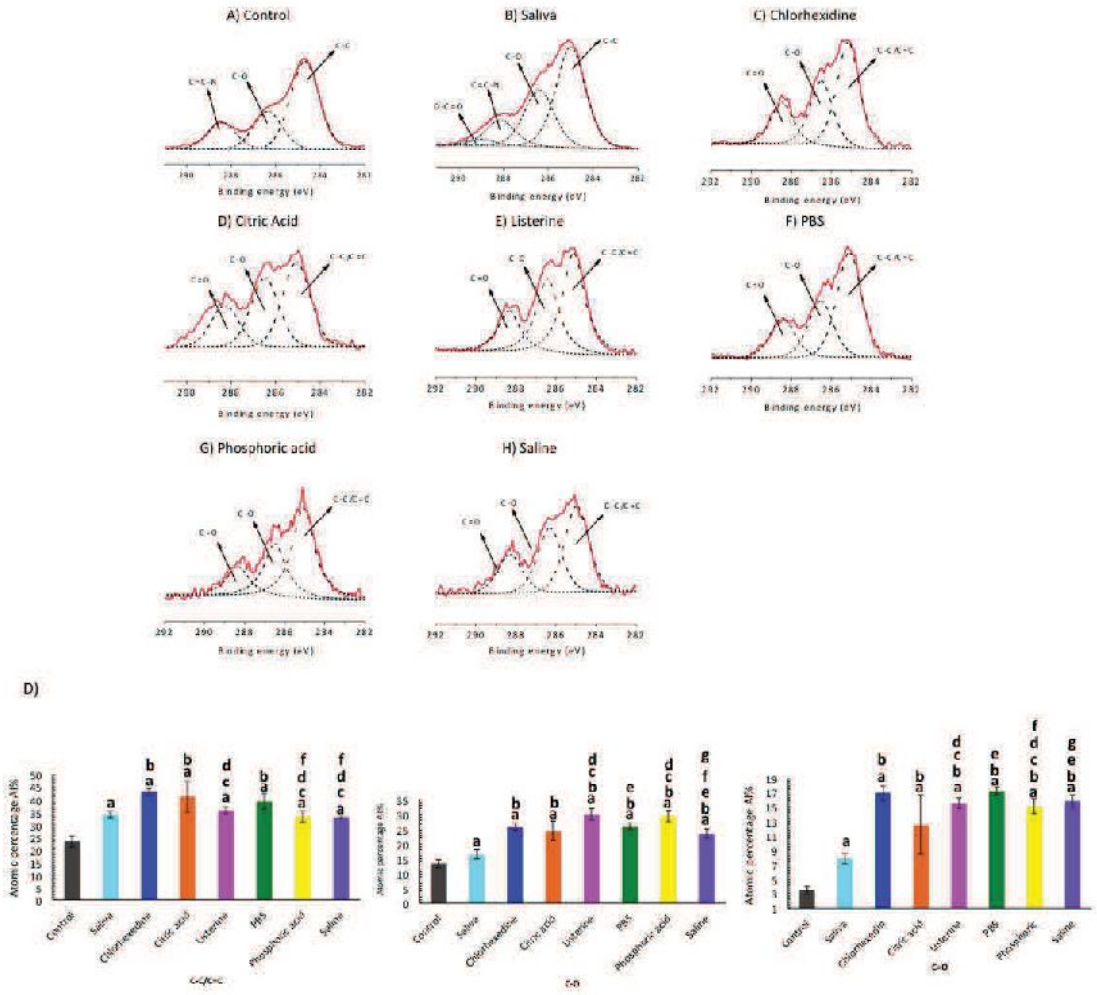

Figure 2. Peak fitting of the XPS high-resolution C1s spectra of the control (A), saliva-contaminated Ti specimens (B), and contaminated samples treated with cleaning solution: Chlorhexidine (C), citric acid (D), Listerine (E), PBS (F), Phosphoric acid (G) and saline (H). I-K) letters in the bar chart indicate significant differences from: control (a), Saliva (b), chlorhexidine (c), citric acid (d), Listerine (e), PBS (f), phosphoric acid (g) and saline (h) $p<0.05$).

4.3.2 XPS Deconvolution

The XPS-deconvolution data shows significant differences in the C-C peaks in the saliva-contaminated samples as compared to the control uncontaminated samples. No treatment was able to reduce the level prior to contamination, though saline solution was the most effective in reducing the C-C peak levels.

Similarly for O=C, saliva contaminated samples had high concentration of C=O as compared to the controls. Again, no solution was able to reduce the levels prior to contamination. In fact, almost all the solutions tested seemed to maximally increase the O=C peaks. The saliva-contaminated samples had significantly higher concentration of C-O as compared to the control samples.

4.3.3 Microbial Analysis

Fluorescence microscopy analysis demonstrated that the overall microbial load (live and total respectively) on the Ti specimens significantly increased upon exposure to human-saliva as compared to the control specimens. Moreover, almost all the different cleaning solutions were able to reduce the load of live bacteria on the saliva-contaminated Ti specimens (Fig.3A). Among all the tested solutions, citric acid was the most effective in reducing the load of live bacteria. Phosphoric acid and PBS was equally good in decontaminating live bacterial load, with no significant difference with citric acid. Listerine and saline were least effective in this context.

Moreover, none of the tested solutions demonstrated potent bactericidal activity. CHX seemed to reduce the dead bacterial count though not significantly, as compared to saliva contaminated-Ti specimens. Looking at the total bacterial load, we again observed no significant effect with all the 6 solutions tested as compared to saliva-contaminated Ti-surfaces. But we observed that citric acid and Listerine treatment significantly reduced the live/dead ratio, while the other solutions did not have any significant effect.

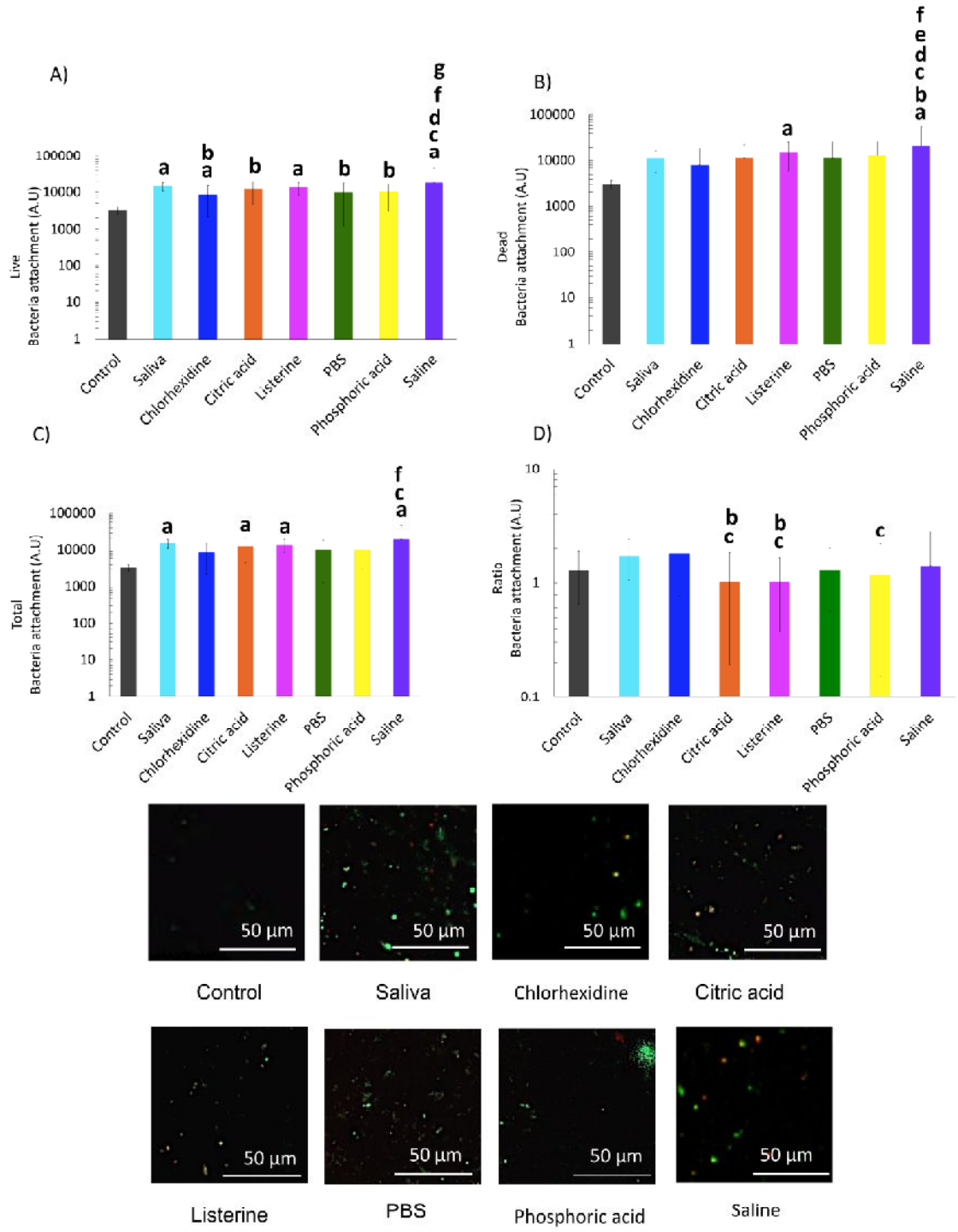

Figure 3. Fluorescence Microscopy analysis to assess the microbial load on the Ti specimens. A-D) Latters in the bar chart indicate significant differences from: control (a), Saliva (b), chlorhexidine (c), citric acid (d), Listerine (e), PBS (f), phosphoric acid (g) and saline (h) $p<0.05$).

4.4 Discussion

In this study, we used XPS analysis and fluorescence microscopy to assess the effects of 6 different routinely used solutions (Listerine, 0.2% Chlorhexidine, 50% citric acid, 0.9% saline, PBS and 35% phosphoric acid) on surface composition and microbial load of saliva-contaminated Ti. Our data demonstrated that amongst the 6 different solutions assessed, treatment with the 50% citric acid and 0.9% saline solutions respectively were most effective in partially restoring the implant surface chemistry prior to saliva contamination. Similarly, treating saliva-contaminated Ti with citric acid solution significantly reduced the surface microbial load of live bacteria.

4.4.1 Saliva contamination of titanium

Saliva contamination of Ti implants, can cause the loss of the beneficial effects of the Ti-oxide layer, resulting in reduced biocompatibility and corrosion resistance [28]. Moreover, saliva contamination constitutes the initial phase of pathological biofilm formation [27] and hence effective decontamination of saliva contaminated Ti implants is vital for implant success. In our study, saliva-contaminated Ti surfaces were enriched with high concentrations of the elements C and N, even after ultra-sonication in distilled water. This suggests that some components of saliva (e.g. proteins, microorganisms) might strongly adhere to the implant surface modifying their surface composition. Moreover, the low surface Ti concentration of saliva-contaminated Ti indicates that the constituents of saliva might be completely covering the Ti-surface, restricting the Ti exposed for interaction. Furthermore, fluorescence microscopy of saliva-contaminated Ti demonstrated a significant increase in the surface microbial load as compared to untreated polished Ti controls. Indeed, adsorption of salivary proteins onto the implant surface facilitates the adsorption of bacterial biofilms leading to lack of osseointegration

[5,27-28]. Our data therefore indicates that saliva contamination might potentially interfere with the overall osseointegration process by reducing the availability of Ti on the implant surface as well as by increasing the microbial load on these implant surfaces.

4.4.2 Decontamination of Ti-surfaces with routinely used chemical agents

Our results show that saliva-contaminated Ti implants cannot be cleaned completely with any of the tested solutions. In this study, we chose 6 different chemical cleaning solutions routinely used in clinics [6-10] to assess their ability to optimally decontaminate the saliva-contaminated Ti specimen and restore their surface chemistry. Our XPS analysis demonstrated that treatment with all these 6 solutions resulted in significant decrease in the level of C on the Ti-surface with citric acid and saline treatments working the best. Citric acid effectively reduced surface N-concentration on saliva-contaminated samples. The treatments increased the total exposed Ti surface, potentially making it available for bioactive interactions while saline increased the concentration of available surface Ti. However, none of the solutions were able to restore the surface concentration of Ti and N to the levels prior to saliva contamination. All solutions were able to reduce the bacterial load on the contaminated Ti-surfaces; however no solution was able to eliminate completely the saliva live bacteria. Therefore, taking into account that these experiments were done on polished surfaces, it would be expected that the tested solutions would probably be unable to completely eliminate bacteria on rough implants contaminated with complex pathological biofilm [6, 26]. With regards to its effect on implant surface composition, one study suggested that citric acid treatment on failed contaminated smooth surface implants did not lead to the reduction of elemental surface contaminants like carbon and nitrogen [10]. This difference in observations might be due to the large difference between the uses of failed contaminated implants *vs* the *in vitro* contaminated

implant specimen's, as used in our study. More or less, our results are in coherence with another study which demonstrated that combination of citric acid with hydrogen peroxide (H_2O_2) and a CO_2 laser, significantly reduced the elemental surface contamination and bacterial load [23].

Of note, Listerine, CHX and phosphoric acid had little or no effect of N and Ti concentration, suggesting that these solutions might be sticking to the Ti-surfaces, some of them even increasing surface contamination. It might therefore not be a good idea to use them in clinics for decontamination purpose. Moreover, these solutions are hydrophobic and they might have limited effect on dissolving hydrophobic substances.

One of the most abundant salivary proteins adsorbed to Ti implants *in vivo* and *in vitro* is the hydrophobic albumin [29, 30]. Moreover, divalent cations like Ca^{+2} and Mg^{+2} can serve as bridging agents in the adsorption of albumin onto the Ti surfaces [31]. Interestingly, both the citric acid and saline solutions are effective Ca^{+2} ion chelators [32], meaning that their presence can trap free calcium ions in the solution. Hence, treatment of saliva contaminated Ti specimens with these solutions might limit the availability of free Ca^{+2} ions ultimately resulting in significant reduction in the adsorption of salivary protein onto the Ti surface. Moreover, these divalent Ca^{+2} ions may also serve as a bridging agent in the adhesion of bacteria to the Ti surfaces [33, 34]. This might further explain the enhanced efficacy of citric acid solution in reducing live microbial load on the saliva treated Ti-specimens. This is again in coherence with various *in vitro* studies demonstrating that citric acid treatment leads to significant decrease in the viable microbial load on the implant surfaces [15, 24].

Our data therefore supports the use of citric acid and saline for Ti surface decontamination as routinely done during surgical treatments of peri-implantitis [21, 22].

Hence, our results points towards the more pronounced use of citric acid and saline solutions to decontaminate the Ti-implant materials in an attempt to restore their normal surface chemistry and enhance implant success. None of the solutions were able to kill bacteria and completely decontaminate the implant surface suggesting that none of these solutions individually are enough to completely restore the Ti-surface. Indeed it would be interesting to evaluate the combination of different decontamination strategies in order to obtain optimum surface decontamination.

4.5 Conclusion

This study demonstrated for the first time that citric acid and saline solutions can optimally decontaminate saliva-contaminated Ti implants suggesting their potential utility for clinical applications.

4.6 Acknowledgements

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Chapter 5: Manuscript 2

Saliva Contamination of Titanium can be Cleaned with Acetic Acid and Tween20

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Abstract

Ti implants can get easily contaminate with saliva during surgery or after placement. The nature of saliva contamination of Ti implants and its impact on osseointegration is largely unknown. In this study, we assessed how saliva interacts with titanium and its subsequent implications on Ti-blood interaction. We used contact angle measurements (CAM), x-ray photoelectron spectroscopy (XPS) and fluorescence microscopy to characterize Ti samples before and after exposure to human saliva. The effect of saliva contamination on blood- implant interaction was further investigated.

Our analysis revealed that saliva formed a hydrophobic organic layer on the Ti surfaces that was rich in bacteria, and interfered with Ti-Blood interaction. After revealing the hydrophobic nature of saliva surface contaminants, we explored the use of solvents (acetic acid and acetone) and detergents (tween20) for Ti surface decontamination. Indeed, our analysis demonstrated that acetic acid and tween-20 achieved substantial elemental as well as microbial decontamination, suggesting that they can be potentially useful for Ti-implant decontamination. This study therefore demonstrates that saliva interacts with Ti-implants and interfere with blood Ti interaction but this saliva contamination can be managed with the use of acetic acid and tween-20 for Ti decontamination.

Keywords: Titanium implants, saliva, blood, contamination, decontamination.

5.1 Introduction

Titanium (Ti) medical devices are widely used to replace missing, injured or altered biological structures [1]. Ti and its alloys are considered to be among the best available materials for the fabrication of dental implants [2, 3]. This is due to their excellent biophysical and mechanical properties such as high strength to weight ratio, low modulus of elasticity, high resistance to corrosion and more importantly, their excellent biocompatibility and osseointegration properties [3, 4]. The outstanding biocompatibility of Ti implants is mainly attributed to its surface which is composed of a Ti-oxide layer that facilitates the deposition of extracellular matrix and bone adhesion while preventing surface corrosion and toxicity [5, 6].

Osseointegration is defined as a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant [7] and comprises of different phases such as osteo-phylic phase (primary inflammatory cell response, blood clot formation etc.); osteo-conductive phase (lamellar bone formation) and osteo-adaptive phase (bone remodeling, reorientation of vasculature) [8]. During the surgical placement, implants interact physically with the surrounding tissues. In this sense, interaction of Ti implants with blood constitutes the initial step towards osseointegration, wherein, blood clot formation initiates the wound healing process at the implant site [7].

Dental implants are prone to contamination by bacteria and organic substances, which can alter the implant surface chemistry and saturate the available chemical bonds offered by the Ti surface [9, 10]. These contaminants therefore limit the actual surface area available for active interaction with the surrounding environment [6, 11, 12], ultimately restricting osseointegration and causing implant failure [2, 11, 13-16]. Saliva is a

potential source of microbes and is known to play an important role in biofilm formation, further leading to peri-implantitis [15]. Moreover, low saliva pH increases Ti- surface corrosion [17]. However, knowledge about how and to what extent saliva alters the Ti-surface chemistry and its functional characteristics is very limited [12]. Various chemical agents have been proposed for decontaminating implant surfaces without affecting their topography. These include among others chlorhexidine, citric acid, phosphoric acid etc. Though these treatments have shown to be partially beneficial, they are unable to achieve re-osseointegration [18].

Hence, we aimed to characterize the physicochemical properties and bacterial load of Ti implant surfaces following contamination with saliva, and investigate how this contamination affects blood-implant interaction. Optical microscopy and Scanning Electron Microscopy (SEM) have been used extensively to characterize the contaminated Ti surfaces. Though these techniques are useful to observe bacteria on the surface, their resolution does not allow assessment of very thin layers of contaminants [3, 19, 20]. In this regards, x-ray photoelectron spectroscopy (XPS) analysis is a gold standard technique for detailed characterization of the outermost surface layer ranging in the nanometer or angstrom scale. Hence, we used XPS analysis along with contact angle measurements for detailed surface characterization of saliva-contaminated Ti surfaces. The information gathered from these analyses was used to develop new potential strategies for chemically decontaminating implant surfaces.

5.2 Materials and Methods

In this study, Ti-samples were contaminated with saliva and treated with different solutions to assess their efficacy in decontaminating saliva contaminated Ti-surfaces. Ethical approval was obtained from the McGill University Ethics Board (14-464 GEN).

5.2.1 Sample Preparation

Ti samples (Ultra-Corrosion-Resistant Titanium, grade 2, McMaster-Carr, Cleveland, OH) were obtained as rectangular bars (6.4, 12.7 and 305.0 mm) and cut into smaller slices (12.7, 6.4 and 6.4 mm) using an abrasive cutter (Delta AbrasiMet, Buchler, Whitby, ON). In order to obtain consistent flat surface, the cut Ti-surfaces were mirror polished using 240-to-600 grit silicon carbide papers (paper-c wt, AA Abrasives, Philadelphai, PA, LapoPol-5, Struers, Rodovre, Denmark) and two polishing clothes: rough to intermediate polishing cloth (15-0.02μm; TexMet C) and final polishing cloth (1-0.02μm; ChemoMet) with colloidal silica suspension (≤0.06μm; MasterMet; Buchler, Whitby, ON). All samples were cleaned in an ultrasonic bath (FS20D Ultrasonic, Fisher Scientific, and Montreal, Canada) with acetone, ethanol and distilled water for 5 minutes in each solution at room temperature. The samples were then vacuum-dried over night before further analyses.

5.2.2 Sample Contamination

Ti samples were contaminated with: human saliva or human blood alone as well as with human blood after exposure to saliva. For saliva contamination, unstimulated saliva samples were collected from a healthy non-smoking volunteer in sterile tubes (n=10). Single titanium samples (n=10) were immersed in each of the saliva tubes for 15 minutes followed by ultra-sonication in distilled water for 15 minutes [21]. For blood

contamination, whole blood was collected from the index finger of a healthy, non-smoking volunteer using a sterile lancet (OAC 15, Data Physics, Germany) [22]. And 50 µl of the collected blood was applied onto the surface of each Ti sample (n=10) for 15 min. before ultra-sonicating in distilled water for 15 min.

To assess the effect of saliva contamination on Ti-blood interaction, Ti samples (n=10) were first contaminated with saliva (as mentioned above), before contaminating them with blood (as mentioned above). All samples were vacuum dried over-night before any further analyses.

5.2.3 Surface de-contamination

To further characterize the surface contaminants and explore new ways to decontaminate Ti, Ti-samples contaminated as mentioned above (saliva, saliva and blood and blood alone; n=10 respectively) were cleaned in an ultrasonic bath for 15 minutes with one of the following reagents: acetic acid (50%), acetone (100%) and tween20 (0.5%) respectively. Samples were then ultra-sonicated in distilled water for 15-minutes and vacuum dried before analyzing their surfaces.

5.2.4 Surface Analysis

X-ray Photoelectron spectroscopy

The surface elemental composition of the different titanium samples was characterized using a monochromatic X-ray photoelectron spectrometer K alpha (Thermo Fischer Scientific Inc., East Grinstead, UK) equipped with an Al Kα X-Ray radiation source (1486.6 eV, 0.834 nm) and an ultrahigh vacuum chamber (10^{-9} torr). Survey scans were obtained for all samples (control, saliva, saliva+ blood and blood) with pass energy of 100 eV at a step of 2.0 eV and high-resolution scans of C were collected with pass energy

of 50 eV at a step of 0.1 eV. The reference for all the samples was set as carbon bonded hydrogen or carbon (C- (H, C)) at 285.0 eV. Data analysis and peak fitting were performed using Avantage chemical surface analysis software (5.41v, Thermo Fischer Scientific Inc., East Grinstead, UK).

Contact angle measurement

The hydrophobicity/ hydrophilicity of the Ti samples before and after contamination was assessed by contact angle measurements (CAM). Using a contact angle meter (OAC 15, Data Physics, Germany), static contact angle measurements were recorded with the sessile drop method at room temperature. The values were reported as averages of at least five drops (1µl) of distilled water per sample. The measurements were done using video-based software (SCA 20, Dataphysics Instruments, Germany).

Bacterial load

We used fluorescence microscopy to assess the microbial load on the saliva-contaminated Ti specimens as well as to evaluate the effects of the various cleaning agents on microbial decontamination. Saliva contaminated Ti specimens (n=9) were stained using the Live/Dead staining kit (BacLight Bacterial Viability Kit, Molecular Probes, Carlsbad, USA) and assessed by fluorescence microscope. The live/dead stain was prepared by diluting 1µL of SYTO 9 (excitation (λ) = 485 nm, emission = 498 nm) and 1µL of propidium iodide (excitation = 535nm, emission = 617nm) in 1 mL of distilled water. The specimens in each group were then placed in 24-well plate, and 500µL of the reagent mixture was added to each well followed by incubation in the dark at room temperature for 15 min. Each Ti sample was then positioned onto a glass slide and covered with mounting oil and stored in a dark space at 4˚C until further processing. Ti specimens

were assessed using an upright fluorescence microscope (Carl Zeiss Microscopy GmbH, Gottingen, Germany) equipped with a digital camera (AxioCam MRm Rev. 3, Carl Zeiss Microscopy, Gottingen, Germany) in combination with an image processing software (ZEN; Carl Zeiss Microscopy GmbH, Gottingen, Germany). For each Ti sample, five randomly selected sites were captured from the surface using a 10x objective. The Median of red fluorescent areas (dead cells), green fluorescent areas (viable cells), and total attached bacteria were calculated (expressed as A.U.) using cell profiler image analysis software (Broad Institute of MIT and Harvard, Massachusetts, USA).

5.2.5 Statistical analysis

Statistical analysis for XPS and bacterial load results was performed using Origin 9.0 (Origin lab, Northampton, MA). All the data were analyzed using Tukey Post Hoc test and one-way ANOVA test and the significance level was set at $p<0.05$.

5.3 Results

5.3.1 Surface elemental composition of Ti before and after contamination

XPS analysis demonstrated that Ti surfaces belonging to all the three test groups (saliva contaminated; saliva + blood and blood alone) had significantly higher concentrations of C, N and significantly lower concentrations of Ti and O as compared to the control untreated surfaces ($p<0.05$). The specimens contaminated with blood after saliva contamination tended to have the lowest concentration of Ti and O as compared to control and other test groups respectively, suggesting that prior saliva contamination indeed interferes with the blood-Ti interaction. Specimens treated with human blood with or without saliva demonstrated higher concentration of N and lower levels of O as compared to saliva contamination ($p < 0.05$).

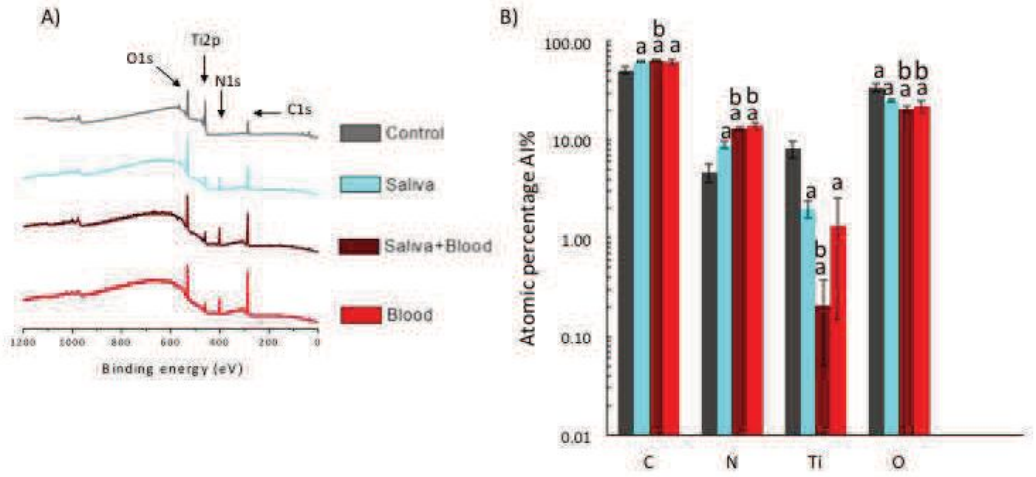

Figure 1. A) XPS survey spectra of the different surfaces: control, saliva alone, saliva + blood and blood alone. Peaks of the elements: carbon (C1s), nitrogen (N1s), oxygen (O1s) and titanium (Ti2p) are shown. B) Bar charts illustrating the surface elemental composition of the different surfaces. a, b, c indicate significant differences from control, saliva and saliva+ blood groups, respectively ($p<0.05$).

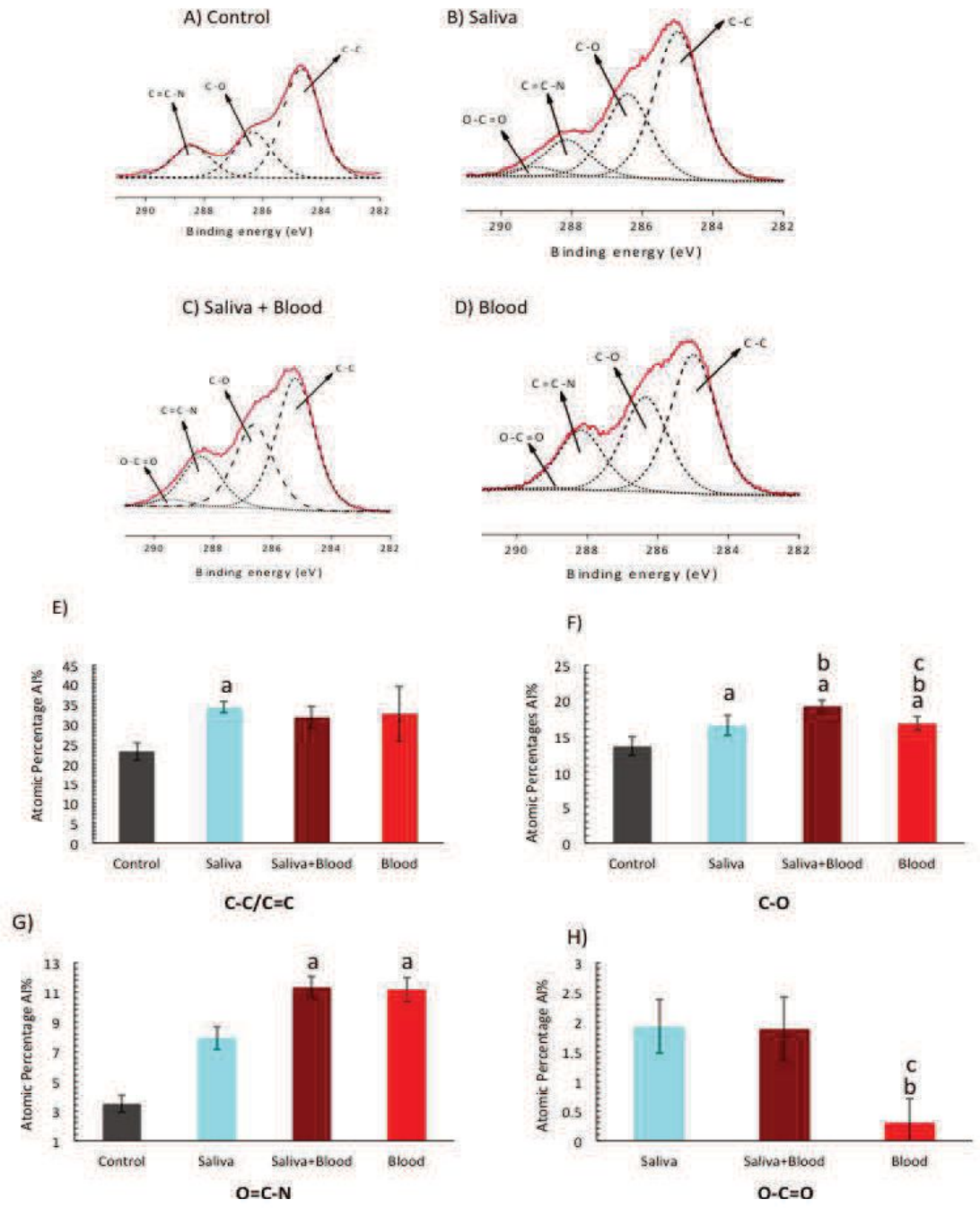

Figure 2. Peak fitting of the XPS high-resolution C1s spectra of: A) control, B)-saliva, C) saliva+ blood and D) blood samples. E-H) Bar charts illustrating the relative amounts of the different components identified in the C1s spectra. a, b, c indicate significant differences from control, saliva, saliva+ blood groups, respectively ($p<0.05$).

The XPS-deconvolution data shows significant increase in the C-C peaks in the saliva-contaminated samples as compared to the control uncontaminated samples. The C-O peaks were significantly higher in the saliva + blood treated group as compared to blood alone, suggesting that prior contamination with saliva interferes with the interaction of blood with the Ti implants. Moreover, as compared to controls, O=C-N peaks were enriched in specimens treated with blood alone or with saliva followed by blood. Most importantly, O-C=O peaks were significantly higher in the saliva+ blood group as compared to specimens treated with blood alone, suggesting that prior saliva contamination indeed alters with the interaction of blood with Ti-surface.

5.3.2 Contact angle measurements

The contact angle of the control polished Ti surface before contamination (45.7 ± 14.8°) was significantly lower than that of the samples contaminated with saliva (66.1 ± 3.1°), saliva+ blood (80.2 ± 4.5°) and human blood alone (78.4±3.3°). This result indicates that contamination with these substances resulted in the formation of a hydrophobic layer on the Ti surface. Furthermore, significant difference was observed between the contact angle measurements of Ti-surfaces treated with blood vs those treated with saliva and blood (Fig.3) further suggesting that saliva contamination prior to blood exposure significantly interference with the interaction between Ti-surfaces and blood constituents.

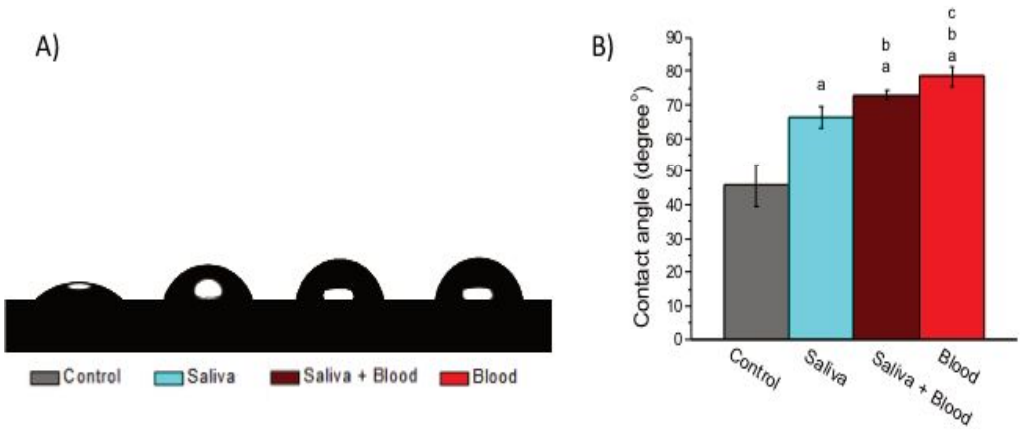

Figure 3. A) Photographs of water droplets placed on different Ti groups. B) Bar charts illustrating the contact angle measurements on the different surfaces. a, b, c indicate significant differences from control, saliva, saliva and blood groups, respectively ($p<0.05$).

5.3.3 Effects of solvents and detergent on the contaminated Ti Surfaces

Given the hydrophobic nature of the contaminated surfaces, we assessed the decontamination efficiency of two organic solvents (acetic acid, 100% acetone) and one detergent (0.5% tween-20) using XPS analysis. The analysis demonstrated that both the solvents (acetic acid and 100% acetone) and the detergent (0.5% tween-20) were able to decrease the surface concentration of C and increase the surface concentration of Ti and O in saliva contaminated Ti-surfaces, as well as in surface exposure to saliva + blood and blood. Moreover, amongst the three reagents tested, acetic acid treatment was the most effective in surface decontamination as it significantly reduced the surface concentration of C increased surface levels of Ti and O beyond their levels prior to saliva contamination (Figure 4 and 5). Acetic acid was also the only solution able to reduce the contamination of N in the contaminated surfaces

5.3.3.1 Deconvolution analysis following various treatments

Saliva-contaminated Ti specimens

The treatment of acetone and tween-20 upon saliva contamination conversely resulted in a significant decrease in the C-C peaks when compared to those of the control specimens. But the acetic acid treatment significantly restored the level of C-C peaks similar to the polished controls, differing from saliva contaminated as well as acetone and tween-20 treated specimens. The C-O peaks were significantly higher for all the treatment groups as compared to saliva contaminated as well as control specimens, though we did not observe any significant difference amongst the three treatment group specimens. The O=C-N/C=C-N peaks for acetone and tween-20 treated specimens were significantly higher than the controls and saliva contaminated Ti-surfaces, while those for acetic acid were lower from control and tween-20 groups (Fig.5D).

Saliva-contaminated Ti specimens treated with blood (Saliva + blood)

For this group also, acetic acid treatment was found to be significantly different from the control, saliva+ blood, acetone treated and tween-20 treated specimens respectively, whereas the deconvolution profile for acetone and tween-20 treated specimens were significantly different from controls and saliva+ blood treated specimens. Moreover, saliva+ blood treated specimens significantly differed from the controls with regards to C-O peaks, whereas the acetone and tween-20 had higher values as compared to controls and saliva+ blood group. The acetic acid treated specimens showed altogether different C-O deconvolution profile as compared to all the other groups. Similarly, the O=C-N/C=C-N peaks significantly varied between acetic acid treated group and control, acetone treated and tween-20 treated specimens (Fig. 5E).

Ti specimens treated with blood alone

For this group, we observe that the acetone and tween-20 treated specimens differed significantly with the controls with regards to C-C peak profile, while the acetic acid treatment seems to restore the C-C peaks, with no significant difference between the acetic acid treated and control groups. For C-O deconvolution profile, all the treatment groups significantly varied from the control and blood treated specimens respectively. Moreover, acetic acid treatment specimens demonstrated significantly different C-O deconvolution peaks than all the other groups respectively. Similarly, tween-20 treated specimens seemed to have significantly different O=C-N/C=C-N peaks as compared to control, blood treated and acetone treated specimens (Fig.5F).

5.3.3.2 Contact angle analysis following various treatments

The treatment of the contaminated surfaces with the solvents and the detergent significantly reduced the contact angle, almost restoring it to the original level of the untreated/ uncontaminated controls (Figure 6). Acetic acid treatment was particularly good for decontaminating saliva contaminated Ti-surfaces, because it achieved the exactly same levels as of uncontaminated controls.

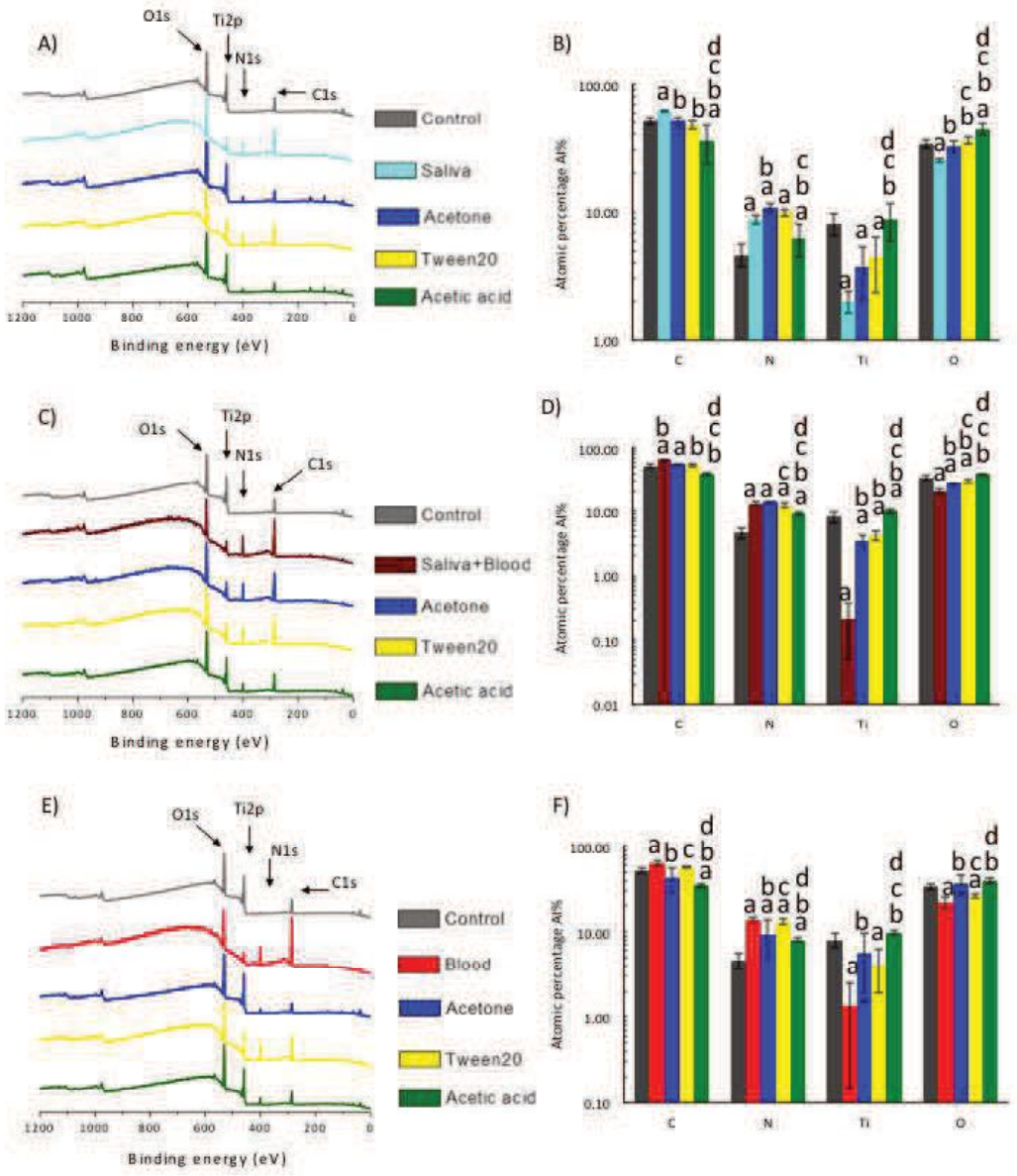

Figure 4. A to C) XPS survey spectra of the different surfaces: control, saliva, saliva+ blood, blood, acetone, tween 20 and acetic acid. Peaks of the elements: carbon (C1s), nitrogen (N1s), oxygen (O1s), and titanium (Ti2p) are shown. D to F) Bar charts illustrating the surface elemental composition of the different surfaces. a, b, c, d indicate significant differences from control, contaminant, acetone, tween 20, acetic acid groups, respectively ($p<0.05$).

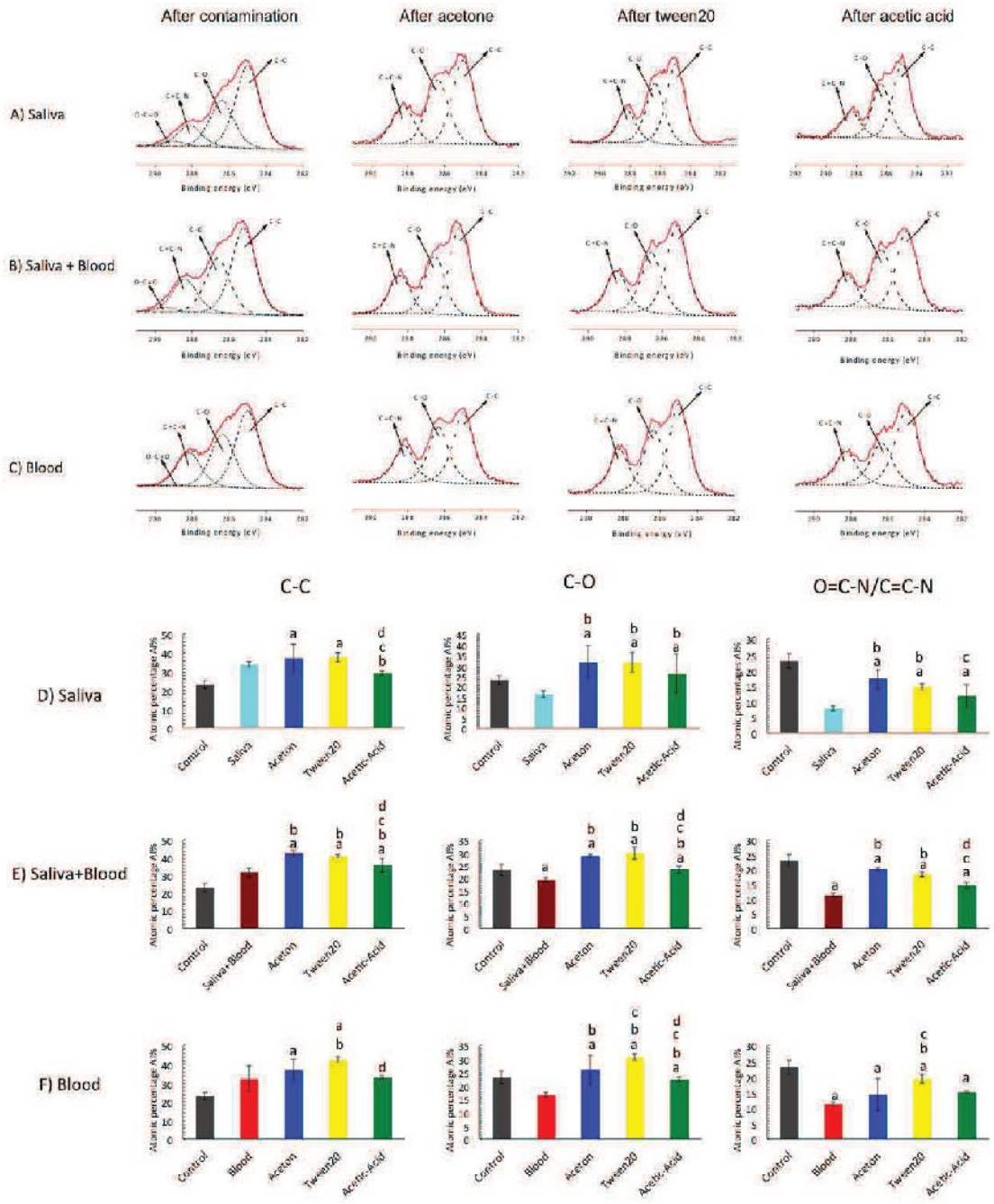

Figure 5. Peak fitting of the XPS high resolution C1s spectra of the different groups: A) saliva, B) blood+ saliva, C) blood; after contamination and after cleaning with each one of the chemical reagents (acetone, tween20, acetic acid); D-F) Bar charts illustrating the relative amounts of the different components identified in the C1s spectra after cleaning the different contaminants (saliva, saliva and blood, blood) with one of the chemical reagents (acetone, tween20, acetic acid) . a, b, c, d indicate significant differences from control, contaminant, acetone, tween-20 and acetic acid treated specimens respectively ($p<0.05$).

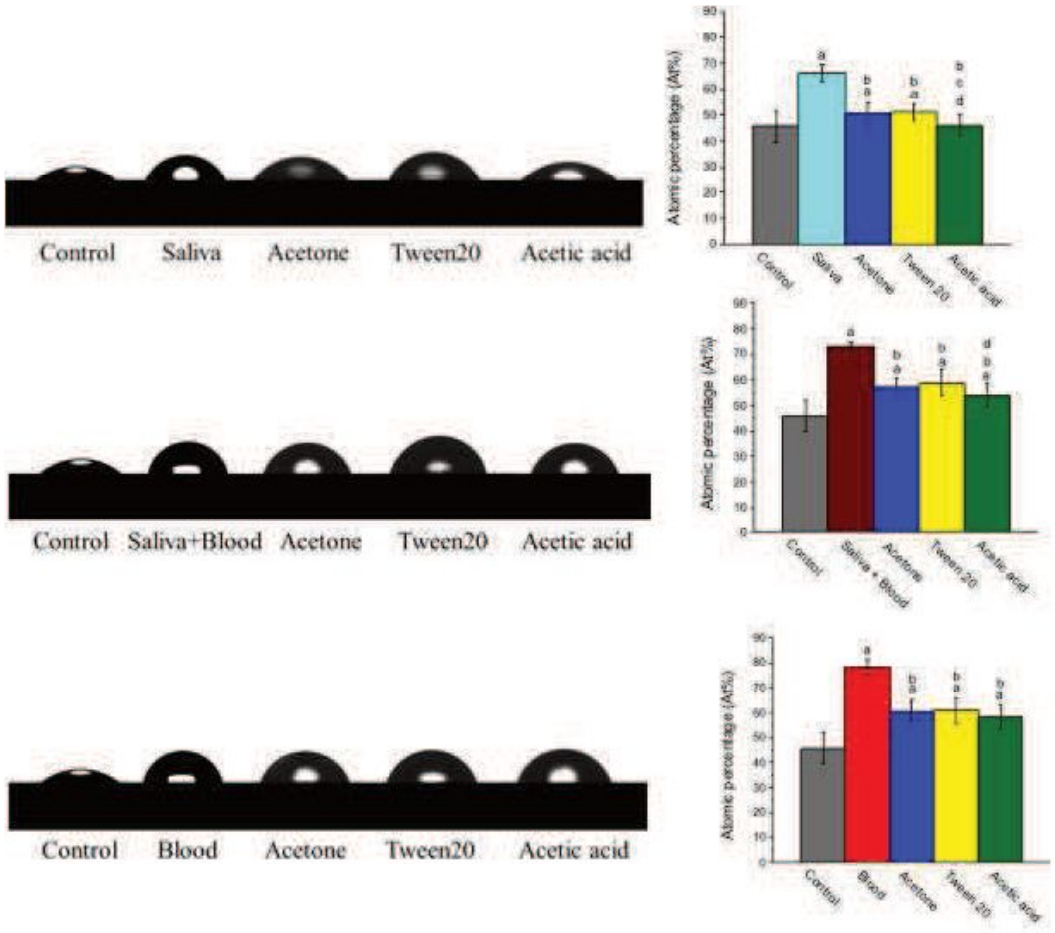

Figure 6. Photographs of water droplets places on different Ti groups (Left) and Bar charts illustrating the contact angle measurements on the different surfaces (right). a, b, c, d indicates significant differences from control, contaminant (saliva, blood and saliva + blood), acetone and tween 20 groups, respectively ($p<0.05$).

5.3.4 Fluorescence microscopy

We used fluorescence microscopy to assess the bacterial load on the Ti surfaces following saliva contamination as well as post-surface decontamination using the different cleaning agents. Fluorescence microscopy analysis demonstrated that the microbial load on the Ti specimens' increased significantly upon exposure to human

saliva as compared to the polished control specimens Figure 7. Interestingly, when these saliva contaminated Ti specimens were treated with the different cleaning agents (acetic acid, acetone and tween-20) we observed an overall reduction in the surface microbial load as compared to the saliva contaminated samples in term of live bacteria, dead bacteria and total amount of bacteria on the surface Figure 7A. However, acetic acid was the only solution able to restore the surface live microbial load similar to level prior to contamination. Similarly, the bead bacterial load decreased significantly with acetone and acetic acid treatment but did not decrease following tween20 treatment. Moreover, all three solutions significantly decreased the total bacterial load, wherein acetic acid treatment was found to be the best. However, when we assessed the ratio of live to dead bacteria, we observed that the ratio increased slightly with saliva contamination. But treatment with acetone and tween20 surprisingly further increase the live/dead ratio. Treatment with acetic acid seemed to decrease this ratio though not significantly.

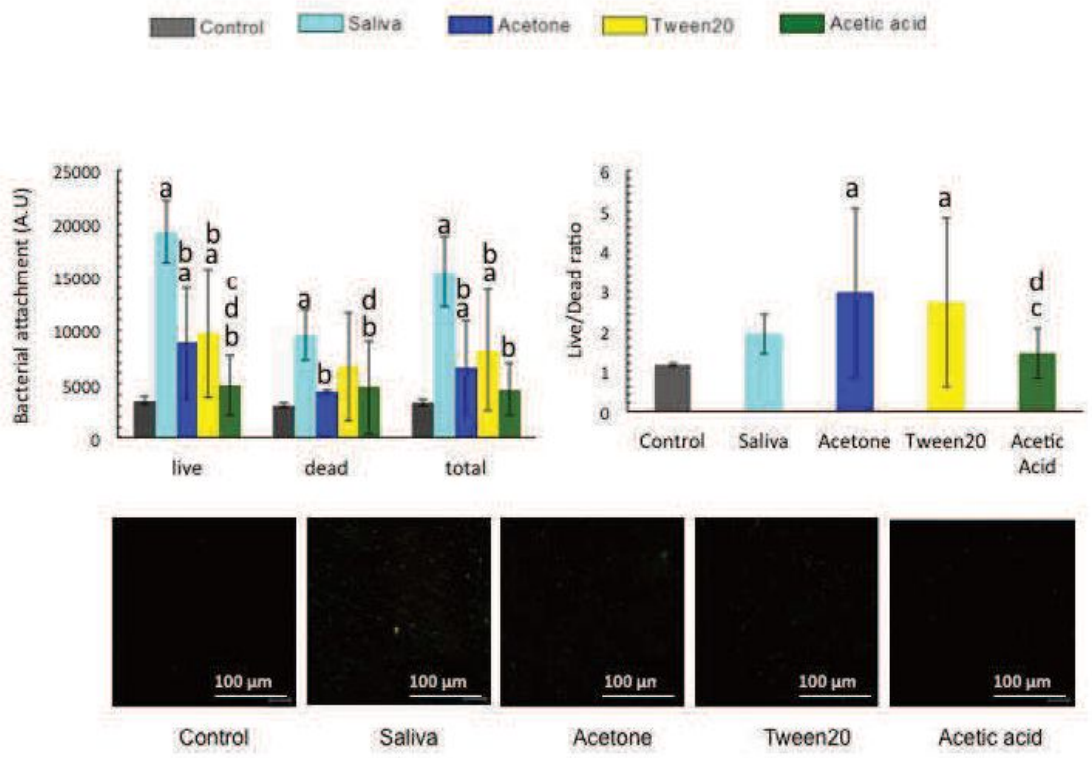

Figure 7. Fluorescence Microscopy analysis to assess the microbial load on the Ti specimens. Latters in the bar chart indicate significant differences from: control (a), saliva (b), acetone (c), tween20 (d) and acetic acid ($p<0.05$).

5.4 Discussion

Our analysis demonstrates that saliva contamination results in the formation of hydrophobic organic layer on to the Ti-surface, which is rich in bacteria. Moreover, this interferes with the interaction of Ti-surfaces with blood, restricting the overall osseointegration process. Understanding the hydrophobic nature of the saliva surface contaminants, we explored the utility of acetic acid and acetone (solvents) as well as tween-20 detergent for Ti-surface decontamination. Our observations suggest that acetic acid and tween-20 could substantially achieve elemental as well as microbial

decontamination, further suggesting that they can be potentially useful for Ti-implant decontamination. Therefore, this study reveals that saliva interaction with Ti-implants interferes with their interaction with blood and acetic acid and tween-20 can be potentially used to decontaminate these Ti implants.

Contact angle and XPS analyses of the Ti surfaces demonstrated that saliva and blood could change the properties and composition of Ti surfaces. The section below would discuss these interactions and their implications in further details.

5.4.1 Saliva contamination

The surface Ti-oxide layer determines the physico-chemical properties and biocompatibility of Ti implants and is very important for osseointegration of implants [6, 22-27]. However, the Ti-oxide layer loses its protective effects the upon exposure to saliva contamination, rendering it less biocompatible [26] and corrosion sensitive [24]. Moreover, our results show that, saliva-contaminated specimens had a high-concentration of the elements C and N suggesting that the components of saliva (e.g. proteins, molecules, microorganisms) might strongly adhere to the implant surface modifying its surface composition. In our study, surface contamination with human saliva resulted in a significantly lower contact angle as compared to the control untreated Ti surfaces. This suggests that saliva renders the Ti surface hydrophobic as a result of possible interaction between the Ti surface and some of the abundant hydrophobic salivary proteins. This is further supported by the XPS de-convolution data, wherein the saliva-contaminated specimens were enriched in hydrophobic organic domains (e.g. C-C, C-H). Furthermore the low concentration of Ti surface on the saliva contaminated Ti-specimens indicated that the salivary constituents probably covered the Ti surface completely. Moreover,

fluorescence microscopy analysis reveals that saliva contamination of Ti specimens increased the surface microbial load. Hence, our data indicates that saliva contamination might potentially interfere with the overall osseointegration process by reducing the availability of the reactive Ti on the implant surface, rendering them more hydrophobic in nature [25] as well as by increasing the microbial load on these implant surfaces.

5.4.2 Blood – Ti interaction

The interaction of blood with Ti implants is the initial step towards osseointegration, wherein blood clot formation aids the wound healing process at the implant site [7, 8]. Absorption of plasma proteins involved in thrombos formation, such as fibrins, onto the implant surfaces [7] eventually leads to wound healing via clot formation. Along with fibrin, other important plasma proteins like fibronectin and vitronectin are also known to adsorb onto implant surfaces promoting cell adhesion and osseointegration [7]. Our observations support these previous findings as Ti specimens treated with human blood had more hydrophobic surface properties due to the adhesion of hydrophobic plasma proteins (fibrin/ fibronectin) as well as lipids in the blood [23, 27]. Interestingly, the specimens treated with human blood were found to have highest concentration of surface N, further implicating a strong adherence of blood proteins to the Ti implant surfaces.

5.4.3 Interaction of blood with saliva contaminated Ti-specimens

We further studied the effects of saliva contamination on blood-implant interaction. The initial adherence of saliva on to the Ti surface seemed to influence the interaction of blood with the implant surface. We observed that, blood rendered saliva-contaminated Ti-surfaces even more hydrophobic and with organic chemical entities in blood samples

are different from saliva + blood samples on the surface which could reduce osseointegration at the implant-bone interface. Moreover, the different O-C=O peaks amongst saliva contaminated-blood treated specimens were significantly different than just blood treated. Our data therefore indicates that it is important to avoid saliva contamination of Ti implant on the first place, so that it does not interfere between the blood clot formations on the Ti surfaces.

5.4.4 Cleaning contaminated implants

Our results above implicates common body fluids like saliva can contaminate Ti-implant surfaces affecting their physico-chemical properties forming a strong and stable adhesive interaction between the Ti-surface and the contaminants, which is retained even after ultra-sonication with distilled water bath. The inability of the adsorbed contaminant to dissolve in water might also have to do with the hydrophobic nature of these molecules confirming the results of the contact angle measurements and XPS. Therefore, the cleaning of Ti-implants could be more challenging than expected and though the implant surfaces might appear clean, stronger cleaning reagents capable of disrupting these hydrophobic interactions must be evaluated for optimum cleaning. Surprisingly, to this date all chemicals used to clean Ti in the clinic do not have the properties needed to dissolve hydrophobic substances. As evident from the above discussion, contamination with body fluids changes the surface composition of Ti rendering it more hydrophobic [7, 23, 27]. Considering the hydrophobic nature of these contaminated surfaces, inorganic solvents (acetic acid and acetone) and a detergent (tween-20) were evaluated for their ability to disrupt the hydrophobic interactions between the implant surface and the contaminants. Though acetone cannot be used directly in the mouth, it was mainly used

to establish the proof of concept that organic solvents can be effective in cleaning Ti surfaces *ex vivo*. The fact that acetone achieved significant decontamination and confirmed our hypothesis stating that the contaminants are hydrophobic. Furthermore, acetic acid is a mild acidic organic solvent, which is FDA approved for human use [28], and tween20 is a non-toxic biocompatible detergent [29-31]. Both of these reagents can therefore be safely used *in vivo* and could possibly be a potential clinical treatment option for decontaminating Ti implants. Indeed, all the three cleaners were able to significantly decontaminate the saliva-contaminated Ti surfaces. But amongst the 3 chemical cleaners, acetic acid was found to be one of the best options.

All of the above mentioned cleaning agents were effective in reducing the microbial load from the saliva contaminated Ti specimens. However, acetic acid was the best microbial decontaminant of all as it was able to reduce the number of live bacteria to the levels prior to contamination. Furthermore, fluorescence microscopy analyses suggests that acetic acid treatment did not kill the live bacteria on the implant surface but indeed disrupt the biofilm, washing it away from the implant surface.

Our results therefore suggest that implant surfaces are highly susceptible to surface contamination with saliva. Hence, one should be very careful and avoid contamination with saliva during dental implant procedures. Furthermore, our work indicates that just cleaning implant surfaces with water does not decontaminate the implant surfaces, whereas solvents such as acetic acid or detergents such as tween-20 can be very effective for decontaminating these Ti-implants. Hence, these solvents and detergents should be further explored as a viable option for clinical use.

One of the major limitations of our study is that we did not use stable established

pathological biofilm and hence future work needs to focus on assessing the effects of acetic acid and tween-20 on pathological biofilms. Also, as opposed to real life screw shaped, surface treated implants; we use uniform polished Ti-specimens for this study. Though we expect these identified solutions to equally penetrate and interact with the real-life complex and rough implant surfaces, more systematic studies needs to be conducted in this context. We found that the polished machine surfaces could not be optimally cleaned with the solutions used. We expect that it would be even more difficult to clean surface treated rough Ti-specimens with these solutions and future studies should address this in further details. Moreover, the saliva proteome and microbiome composition varies significantly amongst different donors and so will be the contamination arising from these saliva samples. Therefore, the future studies should use saliva samples from multiple donors to better assess this issue.

5.5 Conclusion:

Our study demonstrates that contamination with saliva results in the formation of a hydrophobic coating on the Ti surface, which might interfere with its interaction with blood. Moreover, owing to the hydrophobic nature of this layer, optimum decontamination was obtained by using solvents such as acetic acid and detergents such as tween-20. Hence, we precisely demonstrate the hydrophobic nature of the interaction between Ti-implants and saliva and suggest the use of acetic acid and tween-20 for optimum decontamination of Ti-implants.

5.6 Acknowledgements

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Research Ethics Board

JUN 02 2015

DATE OF APPROVAL

June 2, 2015

Dr. Faleh Tamimi
Faculty of Dentistry, McGill University
Room M-51 Strathcona Anatomy & Dentistry
3640 University Street
Montreal, Quebec H3A 2B2

RE: 14-464 GEN entitled "Removal of Oral Biofilm from Dental Surfaces."

Dear Dr. Tamimi:

The following documents in relation to the research proposal entitled above received Full Board review at the convened meeting of the MUHC-Montreal General Hospital Research Ethics Committee on April 14, 2015, and were entered accordingly into the minutes of the Research Ethics Board (REB) meeting.

We are pleased to inform you that the revised documents were found ethically acceptable and we hereby provide you with full approval via review by the Chairman on June 2, 2015, for the following documents:

- Research Protocol (dated May 15, 2015)
- Informed Consent Forms (French and English dated May 15, 2015)

At the MUHC, sponsored research activities that require US federal assurance are conducted under Federal Wide Assurance (FWA) 00000840.

APPROVAL JUNE 2, 2015
EXPIRATION JUNE 1, 2016

It is important to note you may initiate the study only after all required reviews have been completed and all decisions are favorable. At that time you will receive MUHC Authorization to conduct the study in correspondence issued by the Research Institute of the MUHC.

All research involving human subjects require review at a recurring interval and the current study approval. It is the responsibility of the principal investigator to submit an Application for Continuing Review to the REB prior to the expiration of approval to comply with the regulation for continuing review of "at least once per year".

It is important to note that validation for the translated version of the consent document has been certified by an MUHC translator. Any further modification to the REB approved and certified consent document must be identified by a revised date in the document footer, and re-submitted for review prior to its use

The Research Ethics Boards (REBs) of the McGill University Health Centre are registered REBs working under the published guidelines of the Tri-Council Policy Statement, in compliance with the "Plan d'action ministériel en éthique de la recherche et en intégrité scientifique" (MSSS, Qc) and the Food and Drugs Act (17 June, 2001); and acting in conformity with standards set forth in the (US) Code of Federal Regulations governing human subjects research, functions in a manner consistent with internationally accepted principles of good clinical practice.

We wish to advise you that this document completely satisfies the requirement for Research Ethics Board Attestation as stipulated by Health Canada.

The project was assigned MUHC Study Number 14-464 GEN that is required as MUHC reference when communicating about the research.

Should any revision to the study, or other unanticipated development occur prior to the next required review, you must advise the REB without delay. Regulation does not permit initiation of a proposed study modification prior to REB approval for the amendment.

Good luck with your study.

Sincerely,

Dr. Terry Chow
Chairman
GEN Research Ethics Board
MUHC-Montreal General Hospital

McGill University Health Centre
Genetics/Population Research
Investigator Initiated Studies
Research Ethics Board

JUN 0 2 2015

DATE OF APPROVAL