Decontamination of Titanium Dental Implants Using Physical Methods

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DEDICATED TO

My family

for their endless love and support

"If opportunity doesn't knock, build a door"

Milton Berle

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Abstract

Bacterial contamination of titanium (Ti) implants is a major cause for peri-implant infections and eventual implant failure, a problem that could affect ~ 5 million patients every year worldwide. Many implant decontamination techniques have been assessed to manage these infections. However, they all present inconsistent clinical outcomes especially when it comes to achieving complete re-osseointegration. The lack of knowledge on the effect of the available techniques on implant contaminants could be the reason behind these unpredictable results. We hypothesized that even though these techniques could be useful in elimination of bacteria, they might be unsuccessful in removing organic contaminants and restoring the original surface composition.

To test this hypothesis, we measured the level of Ti surface contaminants before and after contamination using X-ray photoelectron spectroscopy (XPS); the most sensitive technique available for characterization of surface chemistry. Then we used XPS to evaluate and compare the decontamination efficiency of commonly employed methods (metal and plastic curettes, Ti brush and laser). The effects of these methods on the bacterial load and Ti surface morphology were also evaluated. Based on this information, two new techniques specially designed for Ti implant decontamination were then developed and optimized; which are electrochemical treatments and an implant-paste.

In the first study, we were able to demonstrate the superiority of Ti brushes for mechanical decontamination and laser treatment for bacterial eradication from Ti surfaces, indicating that different decontamination techniques interact in a different manner with the Ti surface contaminants. In addition, this study demonstrated that complete elimination of bacteria does not necessarily indicate complete decontamination of the Ti surfaces, and all the tested decontamination techniques failed to remove the organic contaminants or restore the original

properties of Ti surface. Subsequently, it could be recommended that an efficient clinical protocol for the management of peri-implant infections should involve an initial cleaning of contaminated implant surfaces with Ti-brushes to eliminate bacteria and organic contaminants followed by a laser treatment to eradicate the remaining bacteria.

The second study presented a new decontamination approach (the optimized electrochemical treatment) that was able to disinfect contaminated Ti surfaces using alternating currents (-2.3mA, +22.5μA) and voltages as low as the titanium standard electrode potential (1.8V). We demonstrated that this method is bactericidal and able to completely decontaminate saliva-contaminated titanium within 5 minutes while preserving surface integrity. Furthermore, with the aid of mechanical brushing, this optimized electrochemical treatment was able to achieve complete decontamination of biofilm-contaminated Ti surfaces.

In the third study, we demonstrated that a novel inorganic implant-paste developed by us had superior decontamination efficiency compared to prophylaxis brushes and a commercial toothpaste. The implant-paste was able to remove biofilm from contaminated Ti without affecting its surfaces integrity. This is the first prophylaxis paste specially designed to decontaminate implant surfaces, although future studies will be needed to assess its efficiency for surgical decontamination of implant surfaces or implant maintenance therapy.

Résumé

La contamination bactérienne de titane (Ti) implants est une cause majeure d'infections péri-implantaires et l'insuffisance éventuelle implant, un problème qui pourrait affecter presque 5 millions de patients chaque année dans le monde entier. Beaucoup de techniques de décontamination d'implants ont été évalués pour gérer ces infections. Par contre, ils présentent tous les résultats cliniques incompatibles surtout quand il vient à la réalisation complète re-ostéointégration. Le manque de connaissances sur l'effet des techniques disponibles sur les contaminants implant pourrait être la raison de ces résultats imprévisibles. Nous émettons l'hypothèse que, bien que ces techniques puissent être utiles dans l'élimination des bactéries, ils pourraient être réussir à éliminer les contaminants organiques et la restauration de la composition de la surface d'origine.

Pour tester cette hypothèse, nous avons mesuré le niveau de Ti contaminants de surface avant et après la contamination en utilisant la spectroscopie photoélectronique par rayons X (SPX); la technique la plus sensible disponible pour la caractérisation de la chimie de surface. Ensuite, nous avons utilisé SPX pour évaluer et comparer l'efficacité de la décontamination des méthodes actuellement utilisés (métal et plastique curettes, Ti brosse et laser). Les effets de ces méthodes sur la charge bactérienne et Ti morphologie de surface ont également été évalués. Basé sur cette information, deux nouvelles techniques spécialement conçues pour Ti décontamination de l'implant ont ensuite été développés et optimisés; qui sont des traitements électrochimiques et un implant-pâte.

Dans la première étude, nous avons été capable de démontrer la supériorité des brosses Ti mécanique pour la décontamination et le traitement au laser pour l'élimination des bactéries à

partir de surfaces en titane, ce qui indique que différentes techniques de décontamination interagissent de manière différente avec les contaminants de surface Ti. En plus, cette étude a démontré que l'élimination complète des bactéries ne signifie pas nécessairement la décontamination complète des surfaces en titane, et toutes les techniques de décontamination testés n'a pas réussi à éliminer les contaminants organiques ou restaurer les propriétés originales de surface Ti. Subséquemment, il pourrait être recommandé qu'un protocole clinique efficace pour le traitement des infections péri-implantaires doit inclure un premier nettoyage de surfaces d'implants contaminés avec Ti-balais pour éliminer les bactéries et les contaminants organiques, suivi par un traitement au laser pour éliminer les bactéries restantes.

La deuxième étude a présenté une nouvelle approche de décontamination (le traitement électrochimique optimisée) qui était capable de désinfecter les surfaces Ti contaminés à l'aide de courants alternatifs (-2.3mA, + 22.5μA) et tensions aussi basses que le potentiel d'électrode standard de titane (1,8 V). Nous avons démontré que cette méthode est bactéricide et capable de décontaminer complètement titane de salive contaminée dans les 5 minutes, tout en préservant l'intégrité de la surface. En plus, à l'aide d'un brossage mécanique, ce traitement électrochimique optimisé a pu réaliser une décontamination complète de surfaces contaminées par Ti biofilm.

Dans la troisième étude, nous avons démontré qu’un nouveau inorganique implant-pâte développé par nous avait l'efficacité de décontamination supérieure par rapport aux brosses de prophylaxie et un dentifrice commercial. L'implant-pâte était en mesure d'éliminer le biofilm de Ti contaminés sans affecter l'intégrité des surfaces. Ceci est la première prophylaxie pâte conçus spécialement pour décontaminer les surfaces implantaires, bien que des études futures seront nécessaires pour évaluer son efficacité pour la décontamination chirurgicale des surfaces implantaires ou le traitement d'entretien de l'implant.

Originality & Author Contributions

This thesis is composed of three manuscripts prepared for publication by the candidate as the primary author. The work presented in this thesis represents original contributions to knowledge. A statement of the contribution of the candidate and the co-authors is provided below for each of these manuscripts:

Chapter 4: Decontamination of titanium implants using physical methods.

Ashwaq Ali Al-Hashedi, Marco Laurenti, Veronique Benhamou, Faleh Tamimi

The candidate conducted the experimental work, analyzed the data and drafted the manuscript. ML provided the scanning electron microscopy (SEM) images presented in the manuscript. VB performed the experimental procedures of laser therapy with the assistance of the candidate. FT supervised and designed the project, and corrected the manuscript. All authors reviewed the manuscript.

Originality: All content of this work is original; this is the first report that evaluates the effect of available decontamination techniques on implant contaminants through the measurement of titanium surfaces contaminants before and after decontamination using X-ray photoelectron spectroscopy (XPS). It is also the first study that used XPS to compare the decontamination outcomes of several physical decontamination methods (metal and plastic curettes, Ti brush and laser) on titanium surfaces. We were able to demonstrate that complete elimination of bacteria does not necessarily indicate cleanliness of the titanium surfaces, and all the tested decontamination techniques fail to restore the Ti original surface properties.

This manuscript has been submitted to the journal of ***Clinical Oral Implant Research***.

Chapter 5: Electrochemical treatment of contaminated titanium surfaces. An approach for implant surface decontamination.

Ashwaq Ali Al-Hashedi, Marco Laurenti, Mohamed-Nur Abdallah, Rubens F. Albuquerque Junior, Faleh Tamimi

The candidate performed the experimental work, analyzed the data and wrote the manuscript. ML assisted in the electrochemical experiments and preparing the electrolytes, MA assisted in collecting and analyzing the XPS data. RA and FT planned and supervised the project, and corrected the manuscript.

Originality: This is the first study showing that by studying the electrochemical properties of biofilm growing on titanium surfaces, it is possible to develop optimized electrochemical decontamination treatments. We discovered that very low frequency alternating currents are highly effective in decontamination of Ti. This project sets a strong base towards the development of new technologies dedicated to electrochemical decontamination of Ti implants. It would allow clinicians to control infections around implants and protect them from failure.

This manuscript has been submitted to the journal of ***ACS Biomaterials Science &Engineering.***

Chapter 6: From toothpaste to "implant-paste": A new product for cleaning dental implants.

Ashwaq Ali Al-Hashedi, Marco Laurenti, Faleh Tamimi

The candidate prepared and performed all the experiments, analyzed the data and wrote the manuscript. ML assisted the candidate in preparing the implant-paste and taking the SEM images. FT planned and supervised the project, and reviewed the manuscript.

Originality: This work is original and innovative; it reports the process of development and optimization of the implant-paste; the first inorganic paste specially designed for the decontamination of titanium implant surfaces.

Report of Invention has been filed for the "implant-paste".

CHAPTER 1: Introduction

1.1 Background and Literature Review

1.1.1 Dental implants

Dental implant, also referred to as endosseous dental implant, is an alloplastic material placed into the bone of the jaw or skull for the replacement of missing teeth or to act as an orthodontic anchor [1, 2]. It is composed of the implant body or fixture, which is the portion designed to be surgically placed in the bone and may extend slightly below or at the crest of residual ridge, and abutment, which is the portion supporting and retaining the dental prosthesis [3].

Dental implants are commonly manufactured from commercially pure titanium (c.p. Ti) and Ti alloys due to their high corrosion resistance, thermal stability, and appropriate mechanical properties (i.e. high strength, high fracture toughness and relatively low modulus of elasticity) [4]. Biocompatibility of Ti owed to the stable passive oxide layer (TiO_2; 3-10 nm thick) on its surface that forms immediately upon exposure to the atmosphere [5].

The American Academy of Implant Dentistry (AAID) reported that, as of 2015, "more than 30 million Americans are missing all their teeth in one or both jaws; 15 million people have crowns and bridges replacements for missing teeth and 3 million have implants. The number is growing by 500,000 a year. The estimated US and European market for dental implants is expected to reach $4.2 billion by 2022" [6]. Globally, more than 12 million implants are placed every year and $3.4 billion is estimated for current implant market [7], with an anticipated annual growth rate of 7.2% [8].

These numbers demonstrate that dental implants are a well-accepted treatment for replacing missing teeth. This is attributed to their high satisfactory outcomes regarding restoration of the patient's function and aesthetics, as well as long-term survival [9]. The success rate of dental

implants has been reported in the scientific literature as 98 % [6]. However, their success is extremely dependent on the establishment and maintenance of a successful osseointegration with the surrounding bone [10-12].

1.1.2 Osseointegration

Several definitions have been used to describe the phenomenon of osseointegration. In the initial concept described by Branermark and co-workers, osseointegration was defined as: "*a direct functional and structural connection between living bone and the surface of a load bearing implant, which is apparent at the light microscope level*" [12]. A clinical definition of osseointegration was suggested as: "*a process whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved and maintained in bone during functional loading*" [13]. Osseointegration was also defined as "functional ankylosis of the implant in bone without any intervening soft tissue" [14].

The osseointegration process starts immediately after implantation upon wetting the implant surface with blood followed by the deposition of proteins, coagulation, inflammation and tissue formation [15]. Within seconds of blood and interstitial fluids contact with the implant surface, proteins adsorb to the implant surface and platelets are activated, forming a blood clot (hemostasis phase). The clot contains many signalling molecules that trigger the migration of monocytes, neutrophils and mesenchymal cells towards the implant surface [12]. When neutrophils and macrophages are activated, they migrate to the implant site from nearby capillary beds and release inflammatory mediators that are necessary to kill bacteria but they are also toxic for the host cells and can enhance tissue damage (inflammatory phase).

After elimination of the bacteria, concentration of the tissue growth factor β (TGF - β) superfamily increases, including bone morphogenetic proteins (BMPs), growth and

differentiation factors (GDFs) [16]. These signaling molecules allow for the recruitment, migration, and differentiation of mesenchymal cells, which take part in the formation of woven bone (proliferation phase).

Bone attaches to implant surfaces in two modes; the first is defined as distance osteogenesis, when the newly formed osseous tissues grow from the original bone surfaces towards the implant surface. The second is called contact osteogenesis and it occurs when the implant surface is colonized by osteogenic cells that produce extracellular bone matrix leading to formation of bone directly on the implant surface [17]. Woven bone is later on removed by osteoclasts and replaced by lamellar bone, and remodelled to form mature load-oriented bone (remodelling phase) [18].

Osseointegration around dental implants was initially elucidated as a pure wound healing process, considering Ti a bioinert material that did not provoke any positive reactions in the surrounding biological environment or affect the process of bone healing [19]. However, nowadays the osseointegration is described as a dynamic process that results from a complex set of reactions that involve two main mechanisms; wound healing and host immune response to the biomaterial [10].

The host reaction to implant is determined by tissue characteristics and implant characteristics. Implants characteristics such as surface topography or coating influence the pattern of protein adsorption onto the surface [20]. Moreover, the way these materials affect the host-adsorbed proteins determines the subsequent molecular and cellular responses, and the type of tissue formed around implants [21]. This indicates that the dental implants are immunomodulatory rather than inert materials [22, 23].

Subsequently, the success of osseointegration depends on implant material biocompatibility, chemical composition, design (macrostructure), surface characteristics (microstructure), status of

implant bed, adjunctive surgical techniques and loading conditions [12, 16, 24-26]. The factors inhibiting osseointegration include excessive implant mobility, radiation therapy, pharmacological agents such as cyclosporine A [27], and patient related factors such as smoking, osteoporosis and renal insufficiency [16, 28].

1.1.3 Implant success and survival

Several authors have proposed success criteria for dental implants [29-31], however, the most recognized criteria for assessing osseointegrated implants are the criteria outlined by Albrektsson et al.[29] as follows: absence of implant mobility; absence of irreversible signs and symptoms such as pain or paresthesia; absence of peri-implant radiolucency; absence of progressive vertical bone loss exceeding 0.2 mm/year following the implant first year of service; success rate of implants, defined as mentioned, is 85% after 5 years and 80% after 10 years. Yet, the most clinically reported criterion is the implant survival, which describes whether the implants are still in the mouth or have been removed, even if they are not in function [32].

Recently, implant quality of health scale has been developed to assess the success or survival of dental implants based on the clinical and radiographic evaluation [33, 34]. The scale was established and approved by the International Congress of Oral Implantologists, into 3 main categories that describe the conditions of implant success, survival, and failure. The implant success category defines implants with optimal conditions of health. Implants survival was further classified into two categories; satisfactory survival that illustrates implants with less than ideal conditions but do not require clinical management, and compromised survival that includes implants with less than ideal conditions and require clinical management to increase the chance for success. The category of failed implant includes implants that necessitate removal or have been already removed [35].

Generally, studies have shown that dental implants achieve more than 90% success and 95.7% survival rates after 10-15 years of implantation [36-38]. This indicates the high predictability of this treatment modality in rehabilitation of partial and complete edentulism.

1.1.4 Dental implants failure

In every year 12 million implants are being placed worldwide [39]. With this huge number of dental implantations, the estimated 2- 10 % failure rate over10 years of function [40, 41], translates into a large number of failure cases (~ 1 million) every year.

Implant failure defined as "*the inadequacy of the host tissue to establish or maintain osseointegration*" [42]. Dental implants fail for several reasons; aseptic loosening or early implant failure due to incomplete osseointegration, before or after the functional loading of the implant. Such failures could be caused by early loading, surgical contamination, poor compatibility of the implanted material, or inefficient healing. In these scenarios, bone does not integrate with the implant surface, leading to implant mobility, foreign body reaction, infection and tissue necrosis [43, 44].

After successful osseointegration, dental implants failure occurs either due to excessive occlusal load or chronic peri-implant infections [44, 45]. Normal masticatory forces on dental implant prosthesis should not cause implant failure, provided that the implants are placed in a favourable position to mastication and surrounded with adequate supporting bone and healthy gingiva [46]. However, inadequate distribution of mastication forces on the bone surrounding implants causes bone loss and implant loosening. This creates spaces for bacterial invasion that promote the progression of bone loss and end up in implant failure [35, 47].

Nevertheless, loss of osseointegration due to bacterial infections (Peri-implantitis), is the primary cause of dental implant failure [47, 48]. As soon as the implant fixture is exposed to the oral

environment, a saliva pellicle adsorbs to its surface followed by bacteria colonization and biofilm formation [49]. As a response to the accumulation of biofilm on the implant surfaces, the body immune system induces cell lysis and damage to the surrounding tissue, resulting in progressive destruction of theses tissues and implant failure [50]. Microbiological studies showed that infected implant pockets harbor bacteria similar to that found in chronic periodontitis, such as *P. gingivalis, P. intermedia, P. nigrescens, T. forsythia, C. rectus and A. actinomycetemcomitans* [51]. Peri-implantitis is mainly caused by infection with *P. gingivalis* [52, 53], although some cases may be associated with specific species of bacteria such as *staphylococcus or preptostreptococcus* [54]. Implant surfaces are more prone to infections compared to natural tooth surfaces due to their high surface energy and microroughness, which facilitate bacteria attachment and colonization [55]. Indeed, rough implant surfaces can carry 25 times more bacteria than root surfaces of equal surface area [56].

1.1.5 Peri-implant infections

Inflammatory diseases of tissues surrounding dental implants include peri-implantitis and peri-implant mucositis. Peri-implantitis is defined as an inflammatory process affecting hard and soft tissues around an osseointegrated functional implant, resulting in pocket formation and loss of supporting bone beyond biological remodelling. Peri-implant mucositis is defined as reversible inflammatory lesions in the soft tissues surrounding a functional implant without causing bone loss [48, 57].

Probing depths, radiographic bone changes and signs of mucosal inflammation were used for the diagnosis and comparison of cases with peri-implant infections, although there has been little agreement among studies on these diagnostic criteria [58]. Recently, Froum and co-workers have proposed a classification for peri-implantitis based on severity and extent of bone loss as follows:

Early peri-implantitis is when probing depths are ≥ 4mm, with bone loss not exceeding 25% of the implant. Moderate peri-implantitis is when the implant has probing depths ≥ 6mm and bone loss of 25-50 %. Advanced peri-implantitis is where probing depths exceed 8mm and bone loss >50 %, which usually requires implant removal [59].

Studies vary widely in their reports on the prevalence of peri-implant diseases due to the use of different definitions for the affected cases [60]. Mombelli et al. reported that prevalence of peri-implantitis could be in the order of 10% of the implants and 20% of the patients during 5 to 10 years of follow up [61]. Koldsland et al. [62] reported the prevalence of peri-implantitis to range between 11.3% and 47.1% in their subjects. Moreover, the prevalence of peri-implant mucositis and peri-implantitis was 31% and 37% of the study subjects, respectively [63]. Furthermore, other studies estimated that peri-implant mucositis affects 80% of the subjects with dental implants and 50% of the placed implants, while peri-implantitis affects 28–56% of the subjects and 12–43% of the implants [64].

Several factors may influence the pathogenesis of peri-implantitis, accelerating the tissue destruction such as poor oral hygiene, previous history of periodontitis, occlusal overloading, and cigarette smoking [65]. Interestingly, diabetes and alcohol consumption have demonstrated less association with the etiology of this disease [66] while the possible role of other factors, such as genetic traits, the implant surface or the lack of keratinized mucosa, is not confirmed [65, 67].

1.1.6 Management of the peri-implant infections

Peri-implant mucositis can be successfully treated by personal or professional mechanical debridement procedures with or without adjunctive antimicrobial agents. It was reported that inflammation is significantly reduced after treatment, which is expressed as reduction of

bleeding on probing, which indicates the reversible nature of this disease [60, 64, 68]. On the other hand, no method is able to achieve predictable and complete resolution of peri-implantitis lesions [57, 64, 65, 69]. The successful treatment of peri-implantitis should achieve the following goals: removal of the peri-implant biofilm and inflamed tissues, complete decontamination of the implant surface to render it biocompatible, regeneration and re-osseointegration of the lost peri-implant bone, and maintain the implant function with healthy peri-implant tissues [51].

To date, many nonsurgical and surgical approaches have been attempted to treat peri-implantitis and promote bone growth on the affected implant surfaces [57, 69-71]. Nonsurgical therapy involves mechanical instrumentation of the implant surfaces alone or combined with antimicrobial agents. Radiographic and histologic data demonstrated that the nonsurgical approach is not effective in resolving the peri-implantitis lesion as only limited improvement in the clinical parameters has been achieved after treatment. Subsequently, in the treatment of peri-implantitis, surgical therapy is usually recommended [64, 70, 71].

Surgical therapy involves an access flap, a resective or regenerative approaches. Access flap surgery is usually performed to maintain the soft tissues around implants and getting access for the proper decontamination of implant surface [64]. Resective procedures are recommended when there is minimal bone loss around implants, to enhance the self-performed oral hygiene measures and reduce the sulcus depths. They involve traditional osteoplasty, ostectomy and apical positioning of gingival flaps after implant surface decontamination [64, 72]. Regenerative approaches are considered with moderate bone loss to support the tissue dimensions during healing and to enhance the possibility of achieving re-osseointegration through the use of bone grafts and membranes [65]. However, when the implant loss is severe, removal of the infected implant and subsequent regeneration of the deficient ridge may be indicated [57]. The surgical

techniques provide easy access for implant surface decontamination, visibility and ability to perform regenerative procedures. Therefore they demonstrate significant improvement in the clinical and microbiological parameters, promote bone fill and could achieve re-osseointegration, though there is no solid evidence of obtaining true re-osseointegration with any technique [71, 73] .

Implant surface decontamination is performed to remove the biofilm from implant surface and facilitate healing with surrounding tissue. Hence, it is critical whether surgical or non-surgical treatment is employed. It is also critical that the decontamination technique does not damage the implant surface or increases its roughness, favouring the biofilm accumulation [74].

Decontamination techniques can be classified as mechanical, chemical and optical techniques (laser assisted) [57]. Mechanical decontamination includes the use of ultrasonic scalers with plastic or carbon fiber tips, stainless steel, titanium curettes, brushes and scalers, air abrasive powders and pumice polishing cups [57]. Chemical decontamination using local or systemic antimicrobials such as chlorhexidine, tetracycline, citric acid, hydrogen peroxide and phosphoric acid have been used alone or in combination with mechanical debridement, in order to decrease the bacterial load and accelerate healing [57, 74, 75].

Laser decontamination involves irradiation of the contaminated implant surfaces with multiple lasers such as CO_2, diode, erbium doped yttrium aluminium garnet (Er: YAG), and neodymium-doped yttrium–aluminium–garnet (Nd: YAG) [76-78]. Er: YAG laser has shown the highest potential to remove subgingival plaque and calculus efficiently without significantly damaging the implant surface or surrounding bone [76, 79]. The CO_2 laser has also been reported to be safe and does not negatively affect osteoblastic attachment to implant surfaces [80]. However, the irradiation of implant surface with Nd: YAG laser is contraindicated because of its moderate

absorption by titanium, causing thermal reactions such as melting and cracks formation on the surface [81]. Diode laser does not damage the implant surface but it has the risk of heat generation on peri-implant tissues [82]. Even though lasers studies show favourable outcomes, mostly for Er: YAG and CO_2 lasers, lasers have not demonstrated additional advantages over conventional mechanical measures in terms of implant surface decontamination [83-85].

In general, there is no consensus over the optimal decontamination method or combination protocol that would achieve complete resolution of the peri-implantitis lesions [57, 64, 65]. Moreover, decontamination studies are heterogeneous and they evaluate different treatment combinations and techniques, however, no single protocol was reported to be optimal or superior [71, 73].

1.2 Research Rationale and Thesis Outlines

Bacterial contamination of implants surfaces leads to peri-implantitis that negatively affects the osseointegration and cause implant failure. Considering the huge number of dental implants placed annually and the high prevalence of this disease, implant loss has a devastating psychological impact on patients' lives as well as a significant financial loss to families and healthcare agencies. Moreover, implant surface decontamination remains challenging with lack of evidence on the most effective decontamination protocol that could resolve these infections. The development of new, effective methods is therefore necessary. Accordingly, this thesis was designed to address the aforementioned issue through evaluating four commonly used decontamination methods in order to understand the mechanism of their treatment and explore their effect on biofilm-contaminated implant surfaces (described in chapter 4). Based on this knowledge, we developed and optimized new efficient decontamination methods that are able to

control surface contaminants, bacterial infection and restore the original implant surface properties (described in chapter 5 & 6).

CHAPTER 2: Characterization Methods

2.1 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy is the most widely used surface analysis technique because it can detect all elements on the surfaces except hydrogen and helium [86, 87]. This technique, also described as electron spectroscopy for chemical analysis (ESCA), analyzes the surfaces chemistry, chemical state and electronic state of the elements using ultrahigh vacuum. It can also measure the elemental composition as a function of depth into the sample, and relative concentration of one or more elements as a function of lateral position on the surface, through depth profiling and 3D mapping respectively [86, 88]

The basic principle of XPS work is the photoelectric effect outlined by Einstein in 1905, in which the sample surface is irradiated with low-energy X-rays. These X-rays excite the samples atoms leading to ejection of electrons (photoelectrons), mostly from the uppermost layer (～10-100 Å) of the analyzed surface.

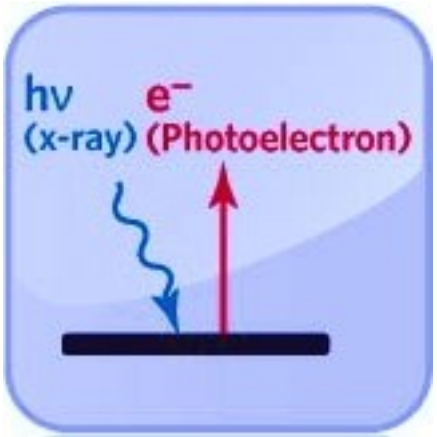

Fig. 2.1. Schematic diagram of the photoelectric effect that occurs in XPS, illustrating the ejection of electron after irradiating the surface with low energy x-ray.

XPS analyzer determines the kinetic energies of the emitted photoelectrons, which are directly

related to the binding energies of the elements from which they are ejected, thus the elements present in the sample can be identified. The energy intensity of these photoelectrons provides information about the relative concentration of the elements in the samples [89, 90].

XPS is a non-destructive surface analytical technique with a detection limit of ~ 0.1- 1.0 atomic % and a relative error of 20 % [91-93]. However, it is a surface sensitive technique, contaminated surfaces and non-conducting or poorly conducting materials may produce additional XPS signals, causing incorrect analysis of the surface composition. To overcome this drawback, ultrahigh vacuum pressure is always used for XPS analysis that help to eliminate the excessive surface contamination, and recently most spectrometers are equipped with charge compensation tools [86, 94, 95].

2.2 Live / Dead Bacterial Assays and Fluorescence Microscopy (FM)

Live/ dead bacterial assays are rapid fluorescence-based assays that evaluate and quantify the viability and number of attached bacteria using fluorescence microscopy [96]. These techniques have become highly accepted tools over the traditional microbial quantification methods, i.e. direct colony counting [97]. They are simple, precise, reproducible, and highly sensitive in quantification of adhering microorganisms [98-100]. Therefore, they have been used to evaluate bacterial viability in biofilms, differentiate pathogenic from non-pathogenic bacteria, and count viable water-borne bacteria [96, 101, 102]. In our studies, we used the newly developed two-color live/dead staining technique that provides a visual differentiation between living and dead bacteria [103].

LIVE/DEAD BacLight Bacterial Viability Kit (L7012, Molecular Probes, Carlsbad, USA) employs two different nucleic acid-binding stains; the green-fluorescent SYTO 9 and the red-

fluorescent propidium iodide stains. These stains differ in their ability to penetrate healthy bacterial cells. When used alone, SYTO 9 stains both live and dead bacteria while propidium iodide penetrates and stains only bacteria with damaged membranes. When both dyes are present, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red.

Live and dead bacterial cells are viewed with a fluorescence microscope, which refers to an optical microscope that uses fluorescence to produce an image. Fluorescence is a molecular phenomenon in which certain material absorbs light of specific wavelength and almost instantly emits light of longer wavelength detectable as visible light. The sample can either be fluorescing in its natural form like chlorophyll and some minerals, or treated with fluorescing chemicals (fluorophores)[104, 105]. Hence, the basic function of the fluorescence microscope is to illuminate the stained samples with a light of a specific wavelength or (wavelengths) that is absorbed by their fluorophores, and then to separate the much weaker emitted fluorescence from the excitation light through the use of an emission filter. In a properly configured microscope, only the emission light should reach the eye or detector so that the resulting fluorescent structures are visualized with high contrast against a very dark (or black) background [106].

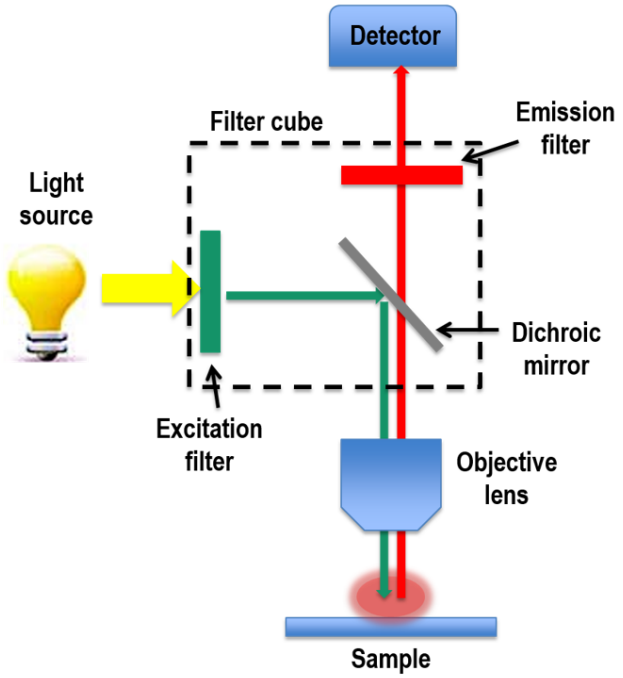

Fig. 2.2. Schematic diagram of a Fluorescence Microscope

2.3 Scanning Electron Microscope (SEM)

A scanning electron microscope scans a focused electron beam over a given surface to create an image. It is useful for inspecting topographies of samples at very high magnifications (microscale and nanoscale levels). During SEM examination, a beam of electrons is focused on a spot volume of the sample, resulting in the transfer of energy to that spot. The collision between the beam electrons (primary electrons) and the sample causes dislodgement of electrons from its surface. The dislodged electrons (secondary electron) are attracted and collected by a positively biased grid or detector, and then translated into a signal. The sweep of the electron beam across the area being examined produces many such signals. These signals are then amplified, analyzed, and translated into images showing the topography and morphology of the inspected area [107].

In addition to secondary electrons, the primary electron beam causes the emission of backscattered (or reflected) electrons, characteristic X-rays and visible light. Backscattered

electrons are most valuable for illustrating contrasts in composition in multiphase samples. They have higher energy than secondary electrons and a definite direction so they cannot be collected by a secondary electron detector. All emissions above 50 eV are considered to be backscattered electrons. Characteristic X-rays are used for the elemental analysis and characterization of a material [108, 109].

Some samples require special preparation before observation, because SEM operates under high vacuum and uses high-energy electron beam to create images. One of these preparations is to remove water from the samples because the water would vaporize in the vacuum causing distortion of the samples. Also all non-metal samples need to be made conductive by covering the sample with a thin layer of a conductive material, through a process known as sputtering [110].

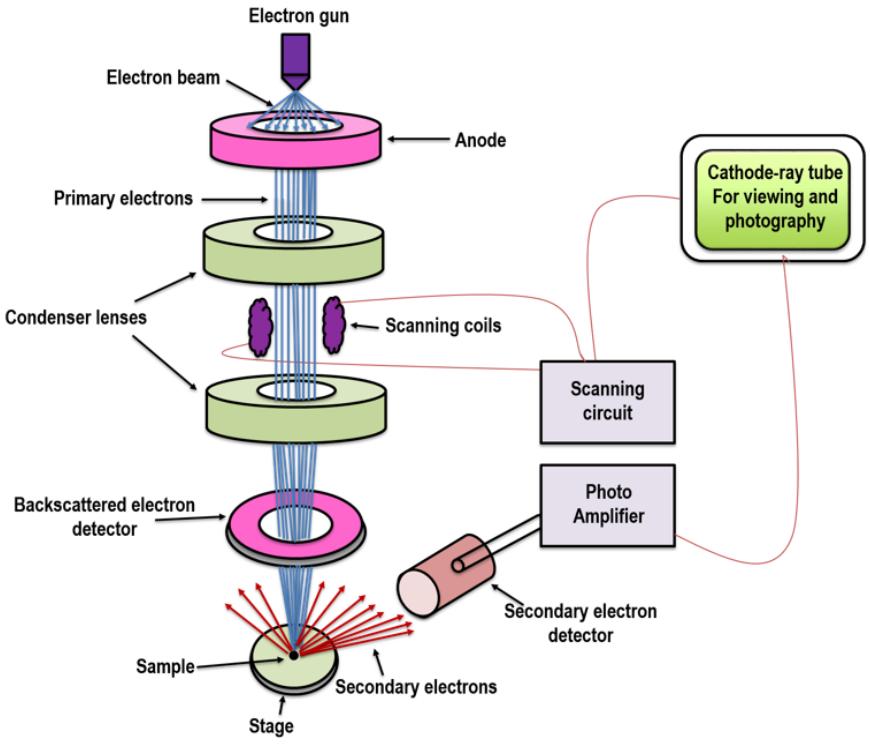

Fig. 2.3. Schematic diagram of a Scanning Electron Microscope

2.4 The Potentiostat and the Electrochemical Cell

A potentiostat is an electronic instrument used to control a three-electrode electrochemical cell. This device can run a very wide range of electrochemical experiments. The three-electrode electrochemical cell includes; a working electrode where the potential is controlled and where the current is measured, a reference electrode that is used to measure the working electrode potential, and a counter electrode that completes the cell circuit. All electrodes are immersed in an electrically conductive solution (electrolyte).

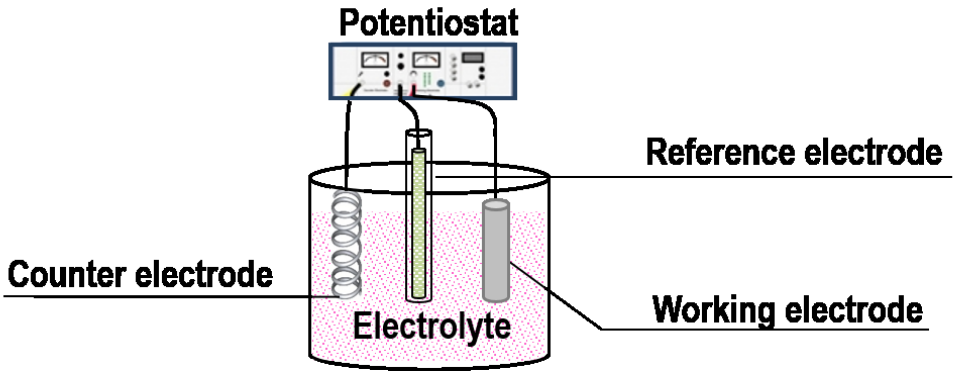

Fig. 2.4. Schematic diagram representing the three-electrode electrochemical cell setup and potentiostat.

Basically the potentiostat controls the potential difference between the working and the reference electrode. It implements this control by applying the current into the cell through the counter electrode. In almost all applications, the controlled variable in a potentiostat is the cell potential (potential difference between the working and the reference electrode) and the measured variable is the cell current (the current flow between the working and counter electrodes)[111].

In our experiments (Chapter 5), we used two electrochemical techniques; measurement of open circuit potential to evaluate the corrosion properties of Ti samples before and after contamination and chronoamperometry technique to apply electrochemical treatments to the contaminated Ti samples.

The open circuit potential (OCP) is the potential of the working electrode relative to the reference electrode when no potential or current is applied to the electrochemical cell. It is also called the corrosion potential and used as a criterion for the corrosion behaviour of the inspected metal. In corrosion experiments, a sample of the metal is set up as the working electrode then the potential is measured with respect to a stable reference electrode without affecting the electrochemistry reactions on the metal surface. Any change in the measured potential, therefore, can be attributed to changes at the metal/ solution interface [112]. The chronoamperometry (CA) is an electroanalytical technique in which the potential of the working electrode is changed stepwise and the resultant current is monitored as a function of time [113].

CHAPTER 3: Thesis Hypotheses and Objectives

3.1 Study Working Hypotheses

- Current methods used for dental implant decontamination are not able to completely remove Ti surface contaminants.
- The electrochemical treatment of the Ti implants with alternating potential is able to completely remove bacterial and organic contamination from their surfaces.
- A prophylaxis paste free of fluoride and organic molecules would be able to decontaminate Ti implants efficiently and create environment suitable for re-osseointegration.

3.2 Objectives

- To evaluate the decontamination effectiveness of four commonly used decontamination methods (Metal curettes, Plastic curettes, Ti-brushes, Er: YAG laser) on biofilm-contaminated Ti implant surfaces.
- To assess the electrochemical properties of Ti implant surfaces contaminated with biofilms in order to develop an optimized electrochemical method for complete implant surfaces decontamination.
- To develop and optimize new prophylaxis pastes specially designed for biofilm removal from Ti dental implants.

CHAPTER 4: Decontamination of Titanium Implants Using Physical Methods

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4.1 Abstract

Introduction: Current decontamination methods of titanium (Ti) implants present limited success in achieving predictable re-osseointegration. We hypothesized that even though these techniques could be useful in elimination of bacteria, they might be unsuccessful in removing organic contaminants and restoring the original surface composition.

Objectives: The aim of this study was to assess the effect of four decontamination methods on the surface chemistry and bacterial load of biofilm-contaminated implant surfaces in order to improve implant surface decontamination.

Materials and Methods: The ability of clinically available methods such as metal and plastic curettes, Ti brushes and Er: YAG laser to decontaminate Ti implant surfaces was assessed. Surface morphology, chemical composition and properties of machined Ti discs (Ø 5.0 and 1.0 mm thick) were analyzed before and after oral biofilm contamination using scanning electron microscope and x-ray photoelectron spectroscopy. The presence and viability of bacteria were evaluated with live-dead assays

Results: Biofilm contamination created an organic layer rich in hydrocarbons and bacteria that covered entirely the Ti surfaces. This organic layer has tightly adhered to Ti surfaces and could not be completely removed with any of the methods assessed. Ti brushes achieved greater elimination of organic contaminants and bacteria than curettes and Er: YAG laser, however, none of them was able to restore the original surface chemistry. Alternatively, Er: YAG laser-treated surfaces showed the lowest live-to-dead bacterial ratio.

Conclusions: Ti brushes were more effective than curettes (metal or plastic) and Er: YAG laser in decontaminating Ti implant surfaces, although none of these techniques was able to

completely eliminate surface contamination. Er: YAG laser was more effective than curettes and Ti brushes in killing the biofilm bacteria.

Clinical significance: The combination of Ti brushes and Er: YAG laser could be a good decontamination protocol that eliminates surface contaminants and kills bacteria on implant surfaces.

Keywords: Dental implants, Decontamination, Mechanical techniques, Er: YAG laser, Surface chemistry, Peri-implantitis.

4.2 Introduction

Peri-implantitis is an inflammation of tissues surrounding dental implants associated with bleeding, suppuration and bone loss, which eventually results in implant failure [48, 114]. It affects up to 47% of implant patients [115], and up to 43% of the implants placed [64]. Peri-implantitis is associated with accumulation of microbial biofilm on the exposed implants surfaces, mainly gram negative anaerobic microbiota [54, 116].

Many techniques have been assessed and used to manage peri-implantitis. They include non-surgical and surgical decontamination of implants with mechanical instruments, antimicrobial therapies or lasers [57, 70]. Bone regenerative and supportive therapies have also been used in combination with surgical decontamination to enhance the bone re-osseointegration [69, 71].

Mechanical decontamination involves the scaling and polishing of the contaminated implants surfaces using curettes, polishing brushes, ultrasonic devices or air-abrasive powder systems [117, 118]. These methods demonstrate success in debriding the contaminated surfaces but they also cause damage of the implants microstructures. In order to avoid surface damage, modified tips and gentle abrasive powders were introduced to clean implants. Nevertheless, the success of the mechanical methods has been always limited by their inability to access deep and narrow bony defects [119]. Lasers have also been used to control peri-implantitis especially carbon dioxide (CO_2) and erbium-doped yttrium–aluminium–garnet (Er: YAG) lasers, with better decontamination results obtained by the latter [120, 121]. The cost of laser therapies, however, should be weighed because their use has not shown additional benefits over the cheaper traditional mechanical treatments [70].

Currently, a gold standard for the management of peri-implantitis does not exist [122]. It seems that so far the proposed treatments cannot achieve complete debridement of the bony defect or

decontamination of the implant surfaces [122]. Indeed re-osseointegration fails to occur on implant surfaces exposed to bacterial contamination following traditional treatment of peri-implantitis lesions. However, re-osseointegration can be consistently achieved in sites previously affected by peri-implantitis as long as a pristine implant surface is used [123]. This indicates that in peri-implantitis, the quality of the Ti surface determines whether re-osseointegration will occur or not. Moreover, this findings could suggest that restoring the implant surfaces to their original condition could be of great importance for achieving true re-osseointegration.

Also, the analytical techniques used in most decontamination studies are not sensitive enough to evaluate the removal of the implant surface contaminants. The majority of these studies assessed bacterial removal at a macroscopic level using light and fluorescence microscopy, or at a microscopic level using Scanning Electron Microscopy (SEM) [124-127]. Others used Colony Forming Unit and bacterial smear tests to examine the presence of viable bacteria and their regrowth after treatment [76, 128]. However, even though these qualitative examinations provide valuable information on the presence and viability of bacteria, they offer no clue on the ability of the decontamination techniques to remove bacterial toxins or residual biofilm. The assessment of bacterial organic products on titanium (Ti) implant surfaces might require a higher magnification at the nanoscale level.

More importantly, despite the importance of surface chemistry in implants osseointegration [129], no emphasis has been given to the chemical changes that might occur to implants surfaces after decontamination. Therefore, it is critical to investigate in depth the effect of the physical decontamination methods on surface contaminants, and their ability to create an environment suitable for re-osseointegration.

X-ray photoelectron spectroscopy (XPS) was used in this study to identify and quantify the elements present on Ti surfaces (outermost 5–10 nm) and their chemical state. XPS has been widely used for the chemical analyses of Ti surfaces [130, 131]; however, it has barely been used to assess decontamination of Ti implants. XPS is a very sensitive technique that can measure surface elements including the adsorbed hydrocarbons. Hence, it could detect any change in the surface elemental composition due to contamination, and give an extremely accurate evaluation on the degree of surface cleanliness after decontamination. We hypothesized that analyzing the surface chemistry of Ti surfaces before and after decontamination with physical methods could accurately assess their ability to remove surface contaminants. Therefore, we aimed at evaluating the effect of four commonly used decontamination methods on the surface chemistry and bacterial load of biofilm-contaminated Ti implants.

4.3 Material and Methods

The study protocol was approved by the Ethics Board of McGill University (application 14-464 GEN). Four healthy non-smoker subjects volunteered for this study and signed an informed written consent before participation. A total of 48-machined Ti discs (Institut Straumann AG, Basel, Switzerland; 5.0 mm in diameter and 1.0 mm in thickness) were used in the experiments directly after removal from the original packaging without further processing. The discs were characterized before and after biofilm contamination, and after decontamination (Fig. 4.3.1).

4.3.1 Samples contamination

Dental biofilm was developed on the machined discs using intraoral maxillary splints following a previously described protocol [117, 126, 132, 133]. Maxillary impressions were taken to fabricate the acrylic splints to which the Ti discs were fixed; each splint accommodated 12 discs. Participants were asked to wear the splints/ discs for 24 hours and only remove them for eating

or drinking while keeping them in phosphate buffered saline. The splints / discs were then collected and stored for further analysis.

4.3.2 Decontamination procedures

The contaminated discs were randomly and equally allocated to 4 treatment groups (Fig. 1). The treatment groups were cleaned and instrumented as follows:

4.3.2.1 Metal Curettes*:* Stainless steel metal curettes (Gracey 5/6, Hu-Friedy, Chicago, IL) were used to scale the samples from bottom to top under water irrigation [134].

4.3.2.2 Plastic Curettes: Manual plastic curettes made of high-grade resin (Implacare ™ II, Hu-Friedy) were also used in a scaling mode with water irrigation [134].

4.3.2.3 Titanium brush*:* Rotary brushes made of titanium (Ti Brush, 2.5 mm in diameter and 8 mm in length; Institute Straumann AG, Basel, Switzerland) were used at a rotation speed of 920 rpm under irrigation with water [124].

4.3.2.4 Laser*:* erbium: yttrium-aluminium-garnet laser device (AdvErL Evo Er: YAG; J. Morita, Irvine, CA) emitting pulsed infrared radiation at a wavelength of 2,940 nm was used following the manufacturer's recommendation. Laser parameters were set at 100 mJ/pulse and a pulse rate of 20 Pulses per second. Samples surfaces were irradiated using C600F fiberoptic tip (J. Morita) at an incidence angle of 45° and a distance of 0.5-1 mm from the sample surface. To cover the whole sample surface, the tip was moved from the bottom to the top of the disc in parallel motion at a constant speed. Water was irrigated at a rate of 5 mL/ minute.

The cleaning with curettes and brushes was performed homogeneously over the entire surfaces with a constant force of 0.23 ± 0.05 N that was calibrated and controlled using a mechanical testing system (MACH-1; Biomomentum Inc., Laval, QC, Canada) and repeated before each cleaning procedure. The time needed to complete the removal of biofilm upon visual

examination was measured. One skilled operator (A.A) carried out all the cleaning procedures in the same session to assure the reproducibility of the treatments.

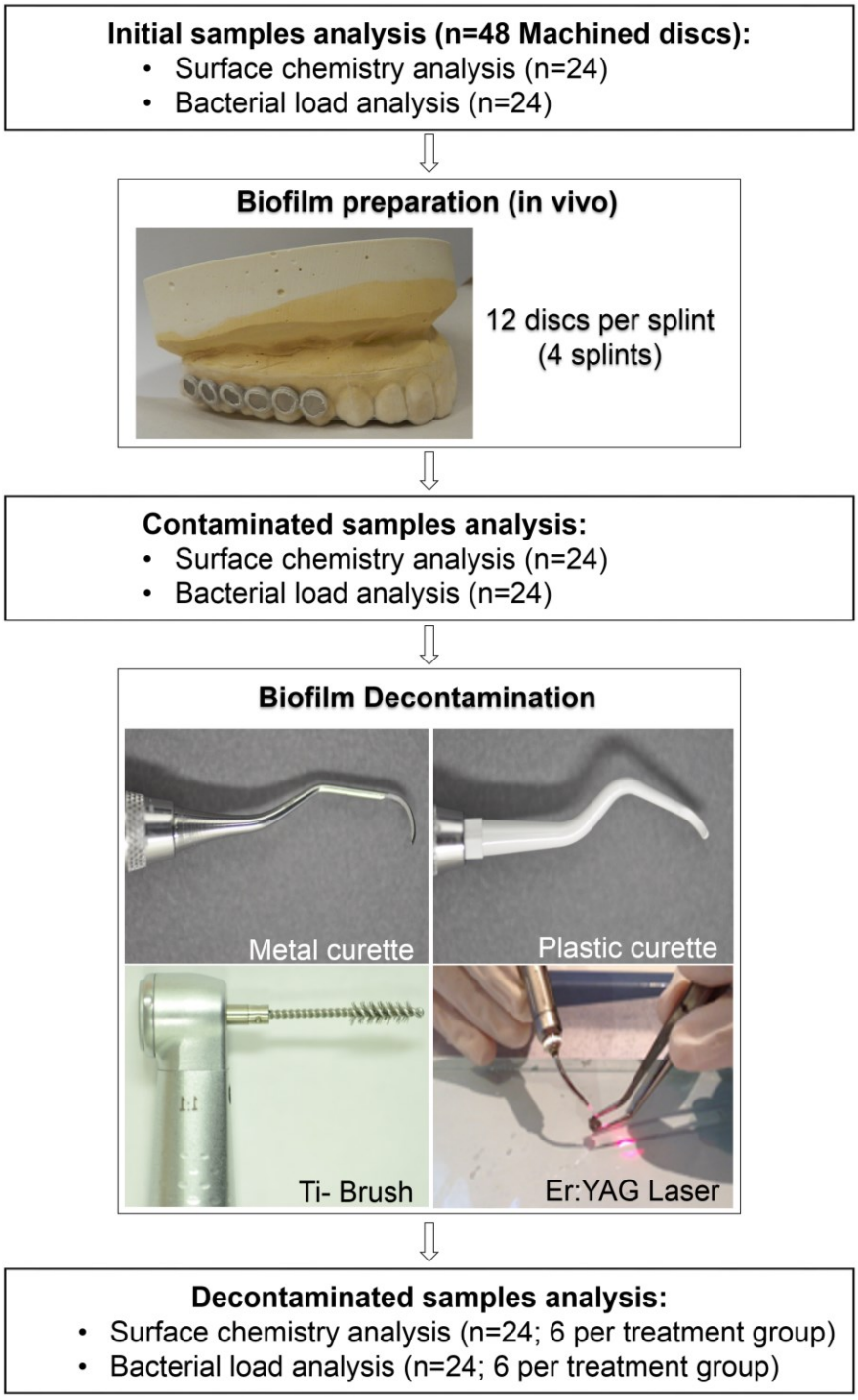

Fig. 4.3.1. Flowchart of materials and methods

4.3.3 Surface Analysis

Since XPS analysis would be affected by live- dead bacterial assays on the surface, contaminated Ti discs of each splint were randomly and equally allocated to 2 main groups, therefore a total of 24 discs was allocated to each group. One group was used to evaluate the change in surface chemistry using XPS and the other group was used to assess the change in bacterial load using live dead assays and SEM (Fig. 1). The discs were evaluated as received from the company, after contamination and after decontamination using the following techniques:

4.3.3.1 X-ray Photoelectron Spectroscopy (XPS)

X-ray Photoelectron Spectrometer (Thermo Fischer Scientific Inc., East Grinstead) was used to analyze the surface chemistry of all samples (n= 6 for each group). The instrument is equipped with a monochromatic Al *Kα* X-Ray source (1486.6 eV, (λ) 0.834 nm) and an ultrahigh vacuum chamber (10^{-9} torr). The size of the analysed spot was 400 μm. Survey scans were recorded over the range of 0 −1200 eV with a pass energy of 200 eV and a resolution of 1.0 eV. Elements quantification, binding energies and peak areas were acquired using Avantage analysis software (5.932v; Thermo Fisher Scientific, Waltham, MA).

4.3.3.2 Live/ dead bacterial assays and Fluorescence Microscopy (FM)

LIVE/DEAD BacLight bacterial viability kit (L7012, Molecular Probes Inc., Carlsbad) and fluorescence microscopy were used for the quantitative assays of bacteria on both contaminated and decontaminated discs (n= 6 for each group). The live/dead stain was prepared by diluting 1 μL of staining component A (SYTO 9; excitation (λ) = 485 nm, emission = 498 nm) and 1 μL of staining component B (Propidium Iodide; excitation = 535 nm, emission = 617 nm) in 1 mL of distilled water. Discs were placed in 48-well plates and covered with 500 μL of the reagent mixture before incubating them at room temperature and in the dark for 15 min. Each disc was

then carefully positioned on a glass slide, covered with component C (mounting oil) and stored in the dark at 4˚C until further processing.

Samples were evaluated under a fluorescence microscope (Carl Zeiss Microscopy GmbH, Gottingen) operated with a ZEN image processing software (Carl Zeiss Microscopy GmbH, Gottingen), using an AxioCam digital camera (MRm Rev. 3, Carl Zeiss Microscopy, Gottingen). For each disc, fluorescent images of five randomly selected sites were captured with a 20 x objective. Live (fluorescence green) and dead (fluorescence red) bacterial cells in the same microscopic field were viewed separately with different fluorescence filters and then digitally combined into one picture. Medians of green, red and total fluorescence per the microscopic field area (448 × 335 μm= 0.15 mm^2) were then calculated using Cell Profiler image analysis software (Broad Institute of MIT and Harvard, MA).

4.3.3.3 Scanning Electron Microscope (SEM)

Surface morphology of clean Ti samples was scanned using a field emission SEM (FE-SEM S-4700, Hitachi, Japan) without further preparation. The biofilm-contaminated samples were fixed in 2.5% glutaraldehyde (PAA Laboratories GmbH, Pasching, Austria) for 2 hours then dehydrated using ascending series of ethanol concentrations (30 –100 v/v %) for 15 min each. After that, samples were dried using critical point drying (Ladd Research Critical Point Dryer) and mounted on SEM-sample stubs where they were sputter-coated with gold and examined. SE mode with and acceleration voltage of 20 kV were selected for analysis, and x 10,000 magnification was chosen for the direct comparison of all samples.

4.3.4 Statistical analysis

Sample size was computed by Power and Sample Size Calculations software (Version 3.0, Vanderbilt University, Germany) to achieve a study power of 80 % at a significant level of 0.05.

A sample size of 6 Ti discs was required to assess changes induced by a decontamination procedure to compare differences in the surface chemistry or bacterial load between contaminated and cleaned surfaces using paired sample t test. Since 4 decontamination methods were studied, a total of 48 titanium discs were required for this study.

For the statistical analysis, the SPSS software (version 22; SPSS Inc., IBM Corporation, NY, USA) and Origin (version 9.0; Origin lab, Northampton, MA, USA) were used, describing the Ti discs as statistical units. Means and standard deviations were calculated for all groups. The normality of distribution was tested using Shapiro-Wilk and all data displayed a normal distribution. Within each decontamination group, differences in the surface chemical composition (n = 6) and bacterial load (n = 6) were assessed before and after contamination, and decontamination using repeated measures ANOVA. In order to compare all treatment methods, the percentage changes in the chemical composition and bacterial load were calculated and compared among the different decontamination groups using one way ANOVA and post hoc testing. Results were considered statistically significant at a $P < 0.05$.

4.4 Results

The time needed for cleaning the samples visually with the metal and plastic curettes was 90.0 ± 4.0 sec and 90.2 ± 3.1 sec respectively while it was 60.5 ± 3.5 sec for Ti brushes and 49.7 ±1.6 sec for Er: YAG laser.

4.4.1 Surface Chemistry

Surface chemistry of Ti samples before and after decontamination was analyzed using XPS (Fig. 4.5.1). The surfaces of as-received samples were examined directly after removing the sterile package, and it consisted primarily of 4 elements; oxygen (41.6 ± 1.2 %), carbon (40.7 ± 2.6 %), Ti (16.2 ± 0.8 %) and nitrogen (1.5 ± 0.3 %). Biofilm contamination significantly increased the

carbon and nitrogen levels to 71.6 ± 2.7 % ($P < 0.001$) and 8.8 ± 1.6 % ($P < 0.001$) respectively, at the expense of a decrease in the oxygen (18.5 ± 1.3 %; $P < 0.001$) and Ti levels (1.1 ± 0.5 %; $P < 0.001$), indicating that the organic compounds completely covered the underlying Ti surfaces.

On the other hand, all decontamination methods were able to significantly increase the levels of oxygen to 23.5 ± 4.5 % with metal curette ($P < 0.001$), 27.5 ± 4.4 % with plastic curette ($P < 0.001$), 39.4 ± 4.4 % with Ti brush ($P < 0.001$) and 20 ± 2.6 % with laser ($P < 0.01$). And decrease that of carbon in comparison to their levels on biofilm-contaminated surface (metal curette: 65 ± 6.7 %, $P < 0.001$; plastic curette: 58 ± 7.6 %, $P < 0.001$; Ti brush: 43 ± 6.4 %, $P < 0.001$; laser: 66.6 ± 4.2 %, $P < 0.01$). All techniques significantly increased the levels of Ti to 2.3 ± 0.2 % with metal curette ($P < 0.01$), 4.4 ± 1.2 % with plastic curette ($P < 0.01$); 9.7 ± 3.3 % with Ti brush ($P < 0.001$) except the laser treatment. Laser did not induce any change in the surface Ti compared to its level on the contaminated surfaces (0.5 ± 0.3 and 0.2 ± 0.1 %; $P > 0.05$). Ti brush was the only method that significantly decreased the levels of nitrogen (5.9 ± 1.5 %; $P < 0.05$). Obviously, no method was able to restore surface elements to their levels prior to contamination.

(Fig. 4.5.2) shows the comparisons of the Ti surfaces chemistry between the different decontamination techniques (curettes, Ti brush and laser treatment). Ti levels were significantly higher in Ti brush-treated samples ($P < 0.01$) than in laser-treated group. This could indicate that the Ti brushes removed the surface contaminants and expose the underlying Ti surface while lasers did not. With regards to the levels of other surface elements (oxygen, carbon and nitrogen), Ti brushes induced the highest significant change in favour of decontamination ($P < 0.01$) while laser treatments induced the lowest

4.4.2 Bacterial Assays

The surfaces treated with metal curettes and Ti brushes showed a comparable number of attached bacteria ($74.9\times10^3 \pm 21.6\times10^3$ /mm^2 and $70.9\times10^3 \pm 31.7\times10^3$ /mm^2 respectively) to that found on uncontaminated surfaces ($50.9\times10^3 \pm 19.5\times10^3$ /mm^2; $P > 0.05$), indicating their ability to remove bacteria (Fig. 4.5.3). Alternatively, the surfaces treated with lasers did not show any change in the number of total bacteria ($268.7\times10^3 \pm 45.3\times10^3$ /mm^2; $P > 0.05$) with respect to that on the contaminated samples ($296.8 \times10^3 \pm 16.1 \times10^4$ /mm^2) but they demonstrated a significant increase in the number of dead bacteria ($403.2\times10^3 \pm 26.8\times10^3$ /mm^2; $P < 0.01$), thus a significant decrease of live /dead ratio (2.2 ± 1.2; $P < 0.01$) (Fig. 4.5.3 and 4.5.4). Fluorescence images (Fig. 4.5.5 a) showed that the surfaces treated with Ti brushes were almost as clean as uncontaminated surfaces while those treated with laser were entirely covered with dead bacteria (fluorescence red).

4.4.3 Surface Morphology

SEM images of the contaminated surfaces showed a homogenous biofilm layer that completely masked the machining marks of Ti discs (Fig. 4.5.5 b). After decontamination, surfaces treated with Ti brushes demonstrated obvious morphological changes while surfaces treated with laser showed residues of bacteria and degraded biofilm. These observations support the XPS data and bacterial assays, confirming that laser was not able to remove bacteria and other surface contaminants. SEM images did not show any change in the surfaces treated with metal or plastic curettes, whereas photographs of these surfaces showed pronounced scratch lines. The photographs of Ti brushes- and laser-treated surfaces showed clean surfaces free of scratches (Fig. 4.5.5 c).

4.5 Figures

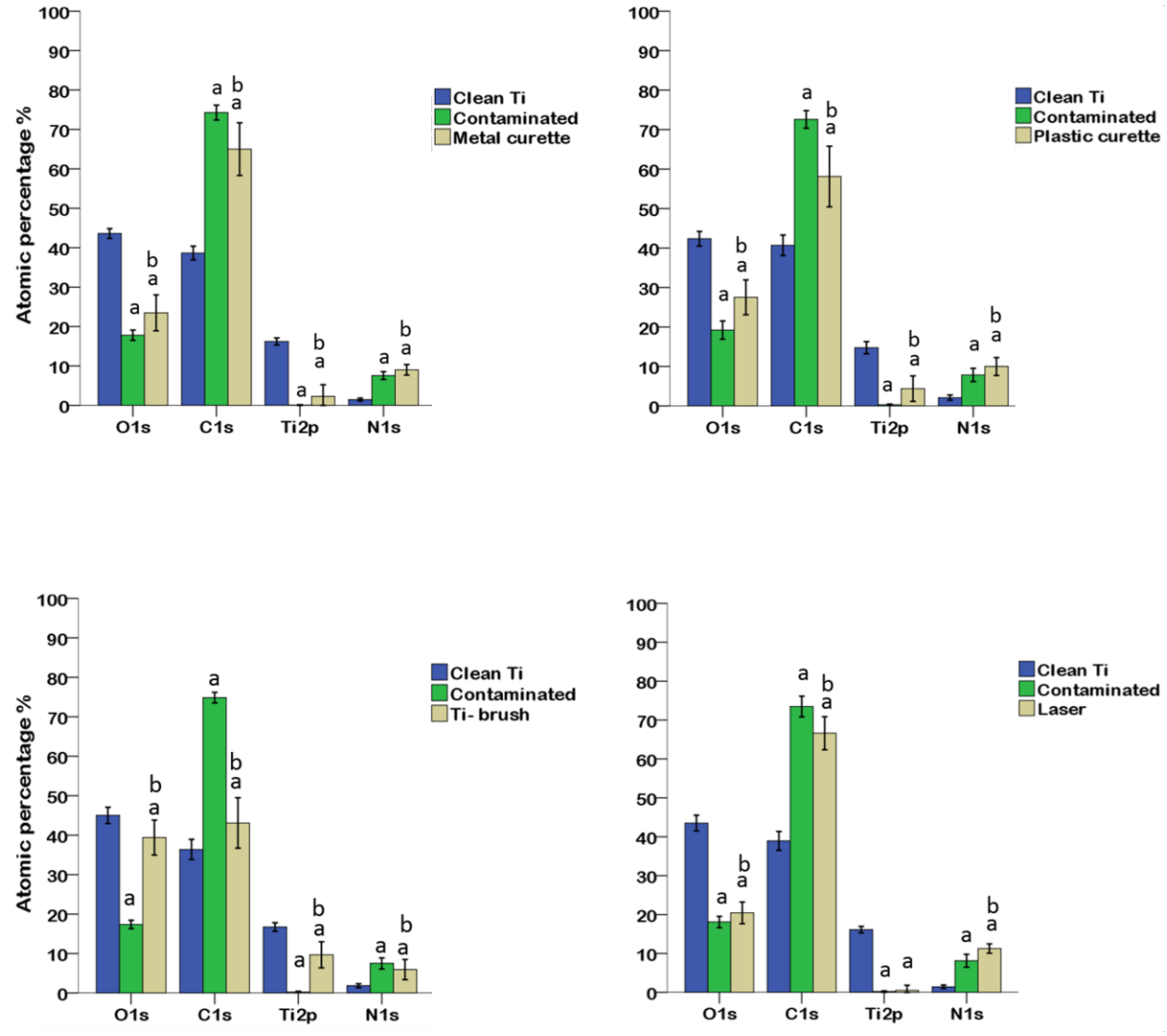

Fig. 4.5.1. Bar charts illustrating the elemental composition of titanium surfaces before and after decontamination as detected with X- ray Photoelectron Spectroscopy (XPS)-low resolution scans. a: Significantly different from clean Ti; b: significantly different from biofilm-contaminated group ($p < 0.05$).

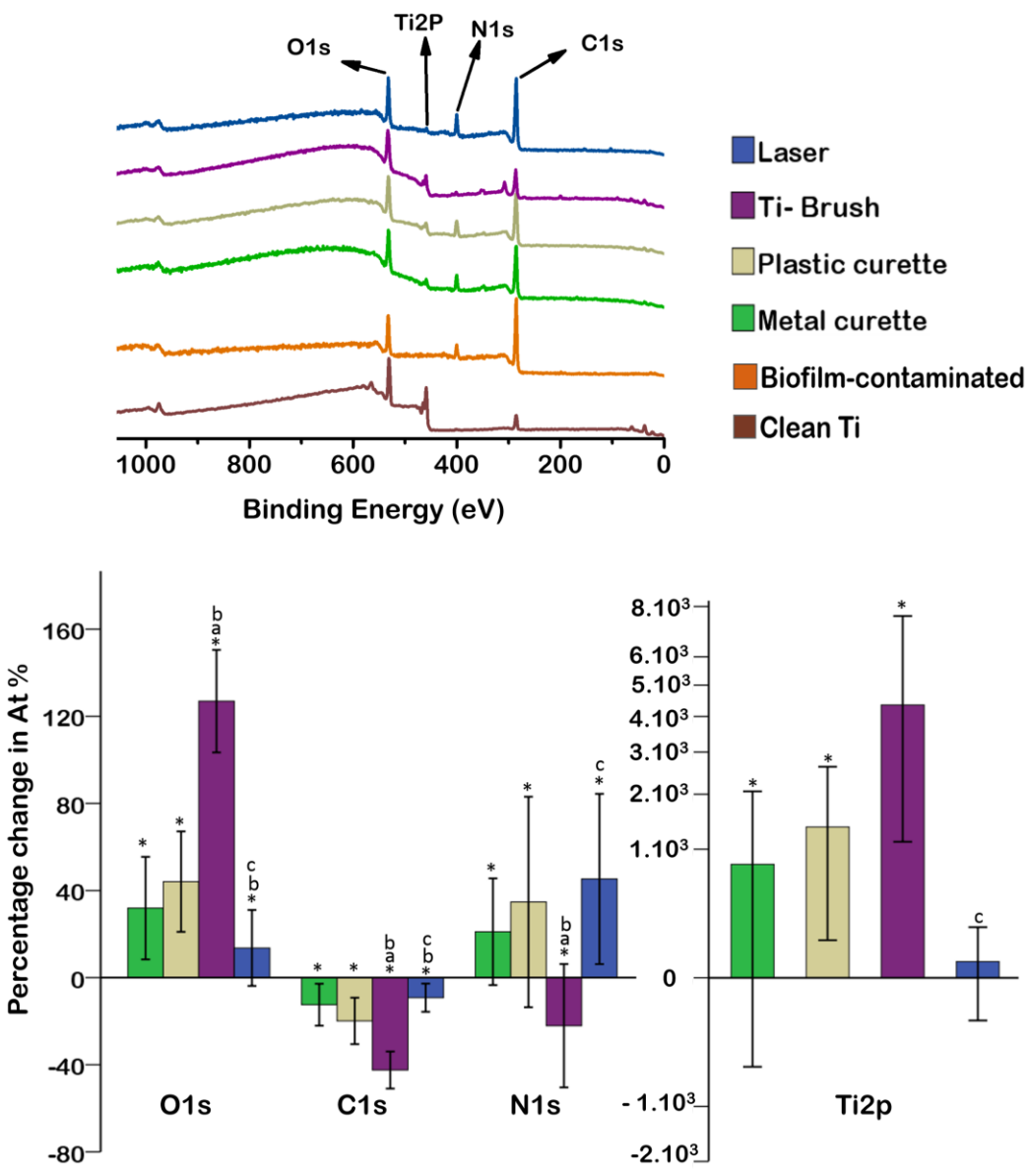

Fig. 4.5.2. XPS surveys (top) and bar charts (bottom) illustrating the comparison of the percentage change in the elemental composition of titanium surfaces between different decontamination methods. * Significantly different from biofilm-contaminated group; a: significantly different from metal curette group; b: significantly different from plastic curette group; c: significantly different from Ti brush group ($p < 0.05$).

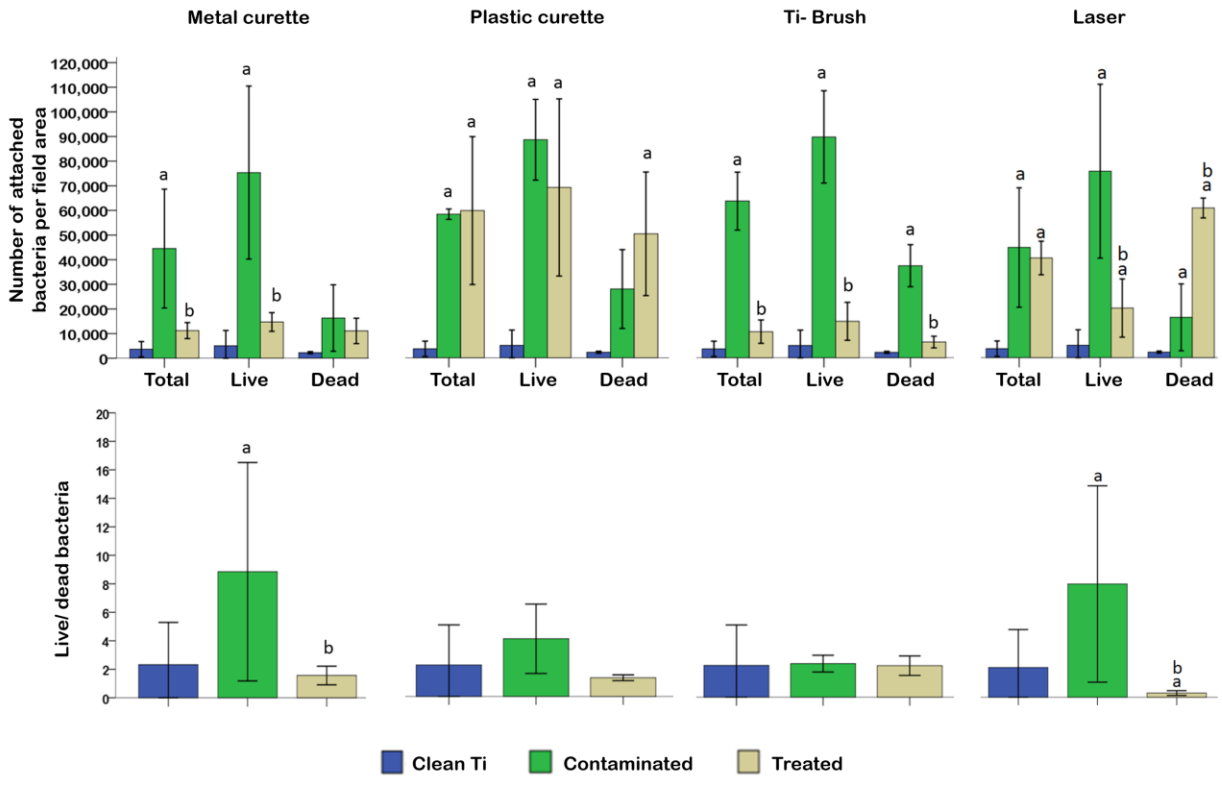

Fig. 4.5.3. Bar charts illustrating the number of attached bacteria (per field area of 0.15 mm^2) and their viability on titanium surfaces before and after each decontamination method. a: significantly different from clean Ti; b: significantly different from biofilm-contaminated group ($p < 0.05$).

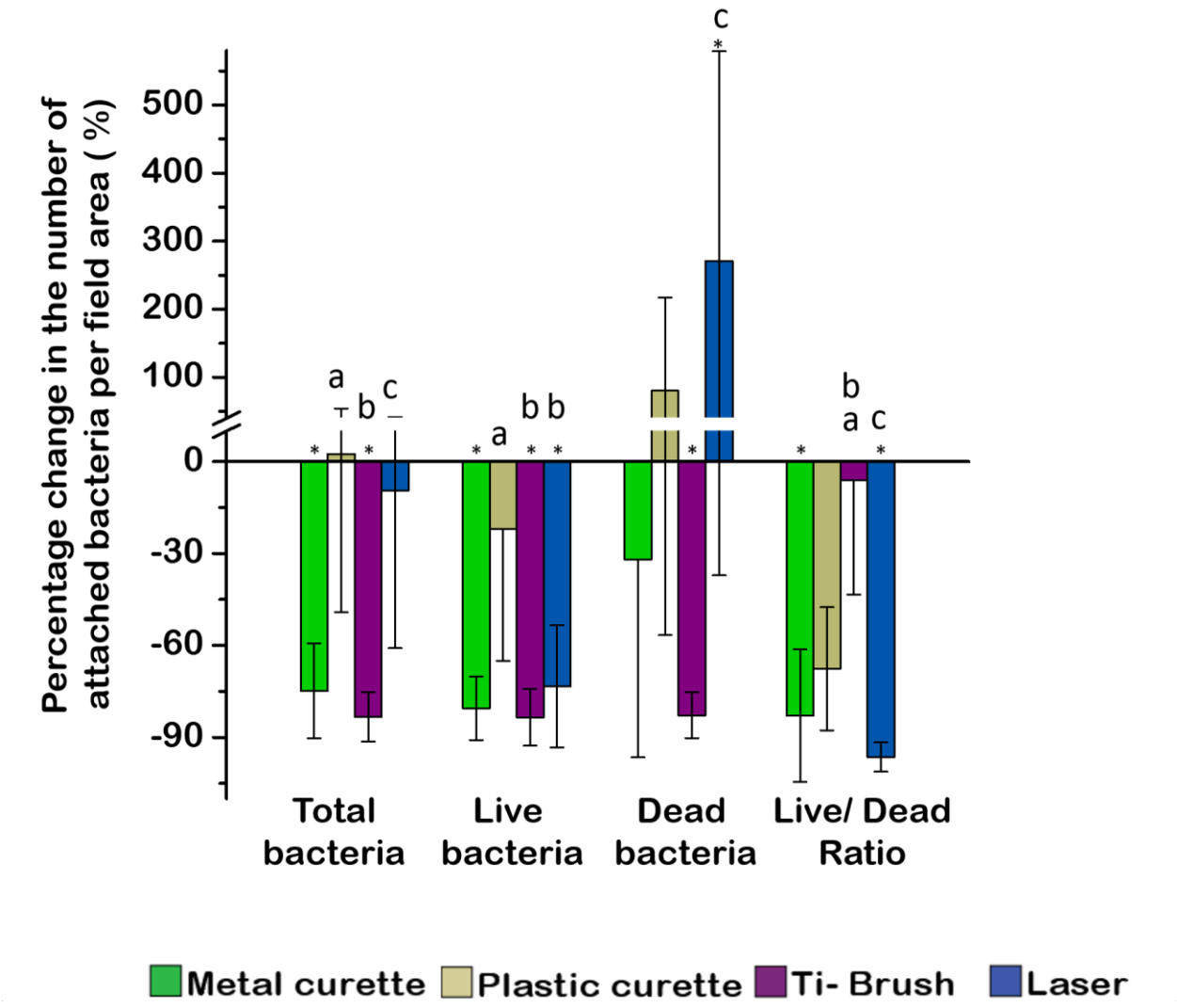

Fig. 4.5.4. Bar chart comparing the percentage change in the number of live and dead bacteria (per field area of 0.15 mm^2) between different decontamination methods. * Significantly different from biofilm-contaminated group; a: significantly different from metal curette group; b: significantly different from plastic curette group; c: significantly different from Ti brush group ($p < 0.05$).

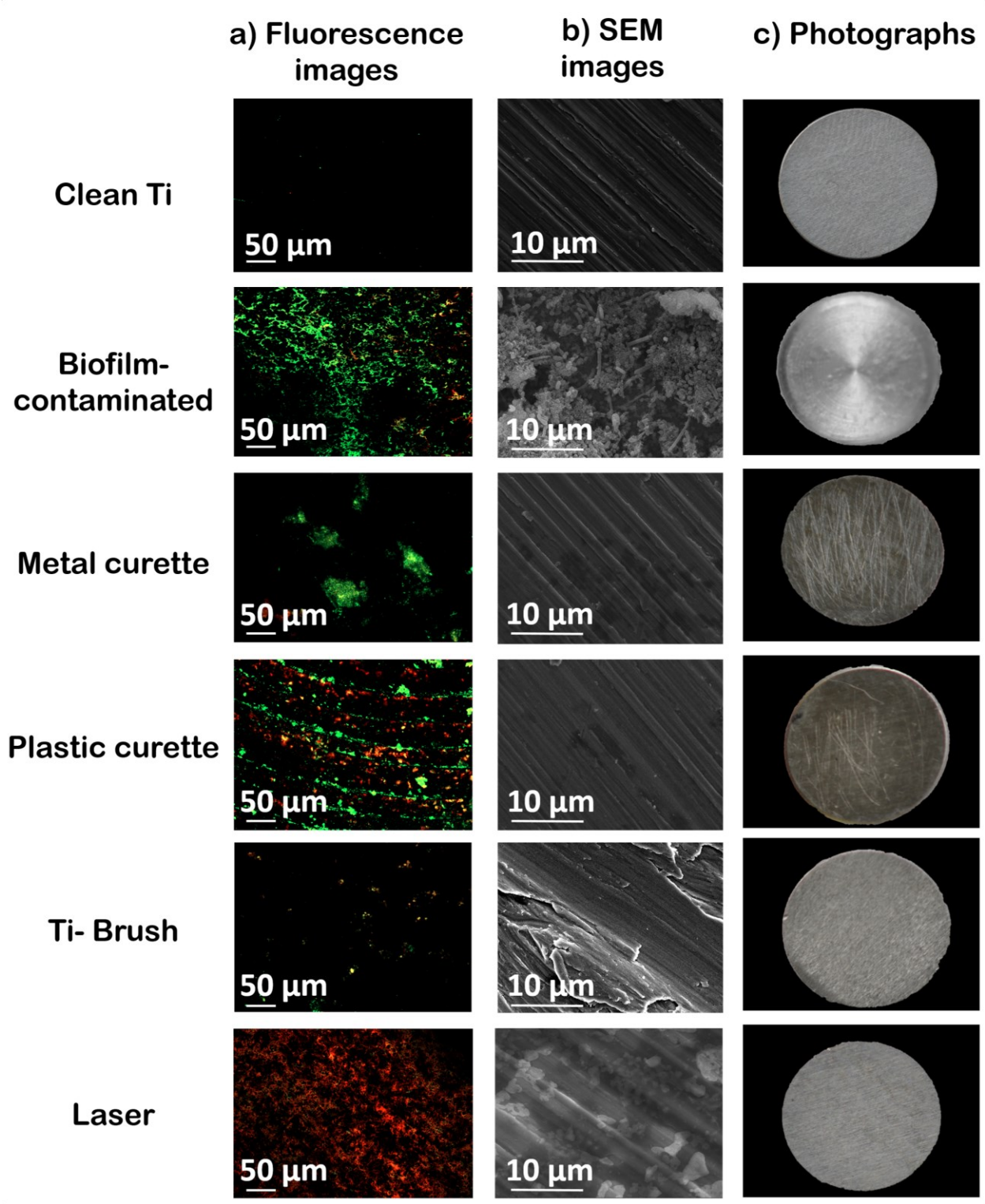

Fig. 4.5.5. (a) Fluorescence (live/ dead staining) images of bacteria on titanium surfaces, (b) Scanning Electron Microscope images illustrating the morphology of the titanium surfaces and (c) photographs of titanium surfaces.

4.6 Discussion

This study provides comparison assessment on the effect of four commonly used decontamination techniques on the surface chemistry, morphology and bacterial load of biofilm-contaminated Ti. Our results demonstrated the superiority of Ti brushes in mechanical decontamination and Er:YAG laser in killing bacteria.

In this study, we used in vivo biofilm model because it offers the opportunity to evaluate implant surfaces in realistic clinical conditions; formation of composite plaque, co-adherence of microorganisms and salivary pellicle under the removal forces of salivary flow and chewing activities [135]. Several in vitro biofilm models have been tested and validated to study the implant surface bacterial interactions [97, 136, 137]. This includes for instance the commonly used microtiter plate-based systems [138]. However, the complex structure of biofilm, the dynamics of its pathogenicity and ecological determinants are not precisely simulated with these models [139, 140].

Most studies assessed decontamination outcomes based on the evaluation of Ti surface morphology or bacterial removal while there has been little emphasis on evaluating the removal of other surface contaminants at the molecular and atomic levels. SEM is useful to visualize the surfaces morphology and the presence of biofilm but this technique does not quantitatively or accurately measure the complete biofilm removal or the changes in the implant surfaces after treatment [141]. Moreover, contamination of the implant surfaces reduces the surface free energy and hinders implant biocompatibility [142], which could negatively affect re-osseointegration. Consequently, this study emphasized on quantification of the Ti surfaces chemistry using XPS, and to evaluate the change that might occur due to decontamination. This would provide clear

understanding on how different decontamination techniques interact with the biofilm accumulated on Ti surfaces, and their ability to restore the original surface properties.

The surface of the clean samples presented high levels of carbon, although the samples were directly examined after unpacking. This result is in agreement with previous studies reporting that surface carbon levels are higher on machined surfaces than on rough surfaces [143]. It could be attributed to contamination during the process of machining and polishing, in which the surfaces were in contact with the machining tools and organic lubricating solutions. The carbon levels could further increase due to the unavoidable adsorption of the airborne hydrocarbons on the Ti surfaces [143, 144].

Exposure of Ti surfaces to the oral plaque for 24h was sufficient to allow for the formation of a homogenous biofilm that completely covered the surfaces of all samples as shown on SEM and fluorescence images (Fig. 4.5.5). These observations corroborated the findings of previous studies that used the same method and time for Ti contamination [118, 145]. XPS data further supported these observations and showed a significant increase in the concentration of organic compounds (expressed as higher carbon and nitrogen), resulting in lower Ti and oxygen elements concentrations. Similar results have also been reported for failed implants contaminated with microbial biofilm [146, 147].

Four decontamination methods that are clinically implemented in the treatment of peri-implantitis were evaluated. The methods include conventional mechanical instruments (metal and plastic curettes), a new mechanical instrument (Ti brush) and Er:YAG laser. Despite the complete removal of surface bacteria by both metal curettes and Ti brushes (Fig. 4.5.3), the XPS data showed that none of these methods was able to completely decontaminate the samples and restore their surface chemistry to its condition prior to biofilm contamination. These results

emphasized on the importance of using XPS analysis to evaluate the decontamination outcomes owed to its ability to accurately detect the surfaces contaminants. Moreover, our results seem to indicate that the reason why the above mentioned decontamination techniques fail to achieve complete re-osseointegration [79, 148, 149] could be their inability to completely remove the organic residuals from the surfaces.

Both types of curettes (metal and plastic) were very limited in their ability to remove organic contaminants, and they also induced visible surface scratches. Metal curettes were able to remove more bacteria than plastic curettes but induced more surface scratches. These results are in agreement with previous reports that indicated the effectiveness of metal curettes in removing soft biofilm from SLA surfaces while the plastic curettes were not effective in removing bacteria from polished or rough implant surfaces [142]. In addition, the surface scratches, damage or increased surfaces roughness have been commonly reported on machined surfaces after instrumentation with metal curettes or scalers [74].

Ti brushes were introduced to decontaminate peri-implant lesions. It was claimed that they are able to easily access and disinfect narrow peri-implant defects due to their capability to adapt more closely to the implant microstructure [150, 151]. However, the cleaning efficiency of Ti brushes could not be demonstrated and literature is still scarce on the decontamination of implant surfaces using Ti brushes. To our knowledge, only one study has tested the decontamination outcomes of Ti brushes in comparison to metal curettes, demonstrating higher capacity of Ti brushes to remove plaque than the metal curettes with being gentler on the Ti surfaces [124]. In this study, the residual plaque area on the Ti surfaces was calculated using histomorphometric analysis, however, the study results could not demonstrate the removal of other surface organic contaminants.

Our study results were consistent with this study, showing that Ti brushes were superior over all other methods in the mechanical removal of bacteria and other surfaces contaminants within a shorter time than cleaning with curettes. However, Ti brushes caused significant change in the Ti surfaces morphology. The surface roughness was not evaluated in the current study but previous study, using both Profilometer and confocal microscopy, found that Ti brushes induce surfaces morphological changes but do not change their roughness [152].

Our study results confirmed the bactericidal activity of Er: YAG laser on bacteria. However, our XPS data did not show the ablation properties claimed for Er: YAG laser as the levels of surfaces organic contaminants were comparable before and after treatment, indicating that the inactivated bacteria and degraded biofilm were not removed from the surface. This could be attributed to the selective absorbance of this laser energy by water in the biofilm, which can cause bacterial inactivation [85, 120] but it is insufficient to cause ablation of the organic materials from the Ti surface.

The remaining degraded bacteria and biofilm on the surfaces can seed further bacterial colonization, encouraging reinfection at faster rate [153] and hinder the interaction of the bone cells with the surfaces, preventing the re-osseointegration. This could explain the reported unstable clinical improvement obtained with laser therapy [64, 154]. The present results are in contrast with previous studies that showed the significant reduction of plaque biofilm after cleaning with Er: YAG laser [118, 155]. However, the surfaces chemistry was not evaluated in these studies, thus the surface cleanliness was not certain.

Subsequently, we can speculate that the combination of Ti brushes and laser therapy could be an efficient protocol for the management of peri-implantitis in dental clinics. The protocol involves an initial cleaning of contaminated implant surfaces with Ti brushes to remove bacteria and

organic contaminants followed by Er: YAG laser treatment to disinfect the surfaces and eradicate the remaining bacteria.

4.7 Study Limitations and future Studies

One limitation of this study could be the use of Ti discs, although they have similar microstructure to implant surface, the cleaning of implant fixtures with screw design and threads is more challenging. However, the methodology used in this study has been proven to be useful for comparisons between different cleaning methods [124, 126, 156].

Another limitation is using in vivo supra-gingival biofilm model instead of complex pathological biofilm that could be more resistant to decontamination. Nevertheless, our results demonstrated the inability of all the tested decontamination methods to remove the soft and less pathogenic biofilm, and thus it could be predicted that they will also be ineffective in removing more complex biofilms.

Furthermore, in this study live/dead bacterial assays were used to investigate the antibacterial efficacy of the tested decontamination methods on the biofilm. However, we recommend future studies to further investigate specific bacterial species using quantitative methods such as fluorescence in situ hybridization (FISH) or quantitative real-time polymerase chain reaction (qPCR) [157-159].

4.8 Conclusions

Within the limitation of this in vitro study, we can conclude that Ti brushes were more effective than curettes and Er: YAG laser in the removal of surface contaminants, whereas Er: YAG laser was more effective than curettes and Ti brushes in killing the biofilm bacteria. None of the methods tested in this study was able to completely eliminate Ti surface contaminants.

4.9 Acknowledgements

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CHAPTER 5: Electrochemical Treatment of Contaminated Titanium Surfaces. An Approach for Implant Surface Decontamination

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5.1 Abstract

Introduction: Bacterial contamination on titanium implants can cause inflammation and eventually implant failure. Currently used methods for decontamination of implants have demonstrated limited success. Metal surfaces can be disinfected electrochemically, however, the effect of electrochemical treatments on biofilm-contaminated titanium is largely unknown.

Objectives: This study was designed to assess the electrochemical properties of bacteria-contaminated titanium in order to develop new treatments to disinfect and clean contaminated implants surfaces.

Materials and Methods: Surface morphology, chemical composition, bacterial load, and electrochemical properties of polished titanium discs were analyzed before and after biofilm contamination and subsequent decontamination with electrochemical methods. The effect of potential charge, voltage, exposure time and electrolyte solution were also evaluated.

Results and Conclusions: Biofilm contamination increased the levels of carbon, nitrogen and live bacteria on titanium surfaces while reducing their open circuit potential and corrosion resistance. Optimized electrochemical treatments with alternating current (-2.3mA, +22.5μA) and voltages as low as the titanium standard electrode potential (1.8V), were bactericidal and able to completely decontaminate saliva-contaminated titanium surfaces within 5 minutes while preserving surface integrity. Furthermore, with the aid of mechanical brushing, the optimized electrochemical treatment was able to achieve complete decontamination of biofilm-contaminated Ti surfaces.

Keywords: Ti implants, Microbial biofilm, Peri-implantitis, Electrochemical treatment, Decontamination.

5.2 Introduction

Osseointegrated implants have been used for rehabilitation of missing biological structures caused by pathological conditions, traumatic injuries, infections, and congenital diseases. Implants are used in orthopedics to fix fractures, joints and limbs, and in dentistry to replace teeth. They are also the best option for defects resulting from cancer surgeries and trauma [160]. Therefore, the number of implant procedures has steadily increased worldwide. In the U.S. alone more than one million dental implantations and about 200,000 hip replacement are performed annually [161, 162] while in Canada, 15,953 hip and 22,545 knee replacements were done in 2009-2010 alone [163].

Biomedical implants are mainly made of Ti or Ti alloys [164] due to their ability to establish and maintain direct contact with bone through the process of osseointegration [12], as well as their excellent biocompatibility and mechanical properties [16]. However, despite the high success rates of implants, complications and failures may occur, which are mostly caused by infections [165-169]. This inflammatory response around the implant, also called peri-implantitis, can be acute or chronic and may lead to progressive bone loss and eventual implants failure [170, 171]. Peri-implantitis is reported to occur at different levels of severity in up to 47.1% of the Ti dental implants [62, 172], about 30 to 40 % of the replaced hip and knee joints[173], and up to 32.2% of femoral fractures with external fixation [174].

In order to manage peri-implantitis, there are two main issues to be addressed. First, the progression of bone loss needs to be stopped; this involves controlling surface contaminants, bacterial infection and surrounding tissue inflammation without damaging or altering the surface integrity of the implants. Second, the original surface properties of the implants need to be restored in order to achieve true re-osseointegration on the decontaminated surfaces [175].

Mechanical, chemical and optical techniques have been attempted to treat peri-implantitis and re-establish bone growth on the affected implant surfaces [57, 69-71]. Mechanical protocols include surgical debridement of contaminated surfaces using curettes, rotary Ti brushes, ultrasonic and air-powered systems [124, 152]. These techniques can only achieve partial removal of bacteria and surface contaminants so they are mainly used in the early stages of infections if the implants are stable without any sign of suppuration [117, 127, 176, 177]. In addition, they present problems in cleaning surfaces that are difficult to access such as deep and narrow bone defects [119].

Antimicrobial agents such as antiseptic (chlorhexidine), local and systemic antibiotics are used as an adjunctive to mechanical decontamination, especially for the treatment of orthopedic implants infections [178-181]. However, neither mechanical nor chemical or their combination are successful to achieve complete resolution of peri-implant infections [156, 181]. Photonic techniques such as lasers and photodynamic therapy are also used to control peri-implantitis and clean infected implant surfaces [118, 182-184]. Yet, in terms of capability to decontaminate Ti surfaces photonic techniques are not superior to conventional mechanical therapies for implant decontamination [70].

The inconsistent outcomes of the available decontamination protocols could be related to the fact that they are mainly modifications of techniques used to manage periodontitis in teeth or infections in bone [70], which are very different from the metallic surfaces of Ti implants. Moreover, most of the current methods are able to eliminate bacteria, but it is not clear if they are able to remove other organic contaminants that tightly attach to the Ti metal and restore the original surface properties [185]. This could be the reason why they too often fail to achieve formation of new bone onto previously biofilm-contaminated implant surfaces, a phenomenon

also known as re-osseointegration [57, 69]. Although some studies reported that re-osseointegration can be achieved with the use of adjunctive surgical regenerative procedures and grafting materials [148], histology often reveals the interposition of connective tissue capsules between regenerated bone and treated contaminated implant surfaces, indicating lack of true re-osseointegration [148, 149].

Electrolytic cleaning of metal surfaces is a well-established technique in many industrial applications [186, 187] and it has recently been used to remove biofilm or prevent its formation [188-190]. The electrochemical polarization of metallic surfaces destabilizes and breaks adhering biomolecules and organisms leading to their detachment from the metallic surfaces [191]. It also causes local change in pH and generates active oxidants and reducing agents, such as oxygen, hydroxide ions and protons that are able to inactivate or reduce the number of viable microorganisms [188, 192, 193]. Another advantage of the electrochemical treatment is that it can access different surfaces with difficult topographies.

Consequently, electrochemical treatments have been used to control bacterial adhesion on electrically conductive surfaces [194, 195] using the bactericidal effect of anodic currents [196, 197] and detachment effect of cathodic currents [198]. Alternating currents, which involve the combination of cathodic and anodic modes, have recently been suggested to induce both bacterial detachment and biofilm degradation [199, 200]. However, this type of currents has barely been explored for decontamination of infected Ti surfaces despite the electro-conductive properties of Ti.

Recently, the disinfection efficiency of low direct current on biofilm-contaminated implant surfaces have been evaluated [201, 202]. The application of direct anodic current of at least 7.5mA for 15 minutes was able to eliminate an Escherichia *coli* biofilm on implant surfaces.

However, this method can produce a dangerously high voltage (ranged between 4 to 20V) [201]. In another study, the removal of multispecies biofilm on implant discs was achieved with the application of 10 mA anodic direct current for 10 minutes [202]. Though, this current seemed to alter the Ti surfaces (blue discoloration) causing delay in maturation of osteoblasts growing on them [130], and also generated a high voltage (ranged between 11-19V) [202].

In the aforementioned studies, implants electrodes were placed in gelatin loaded with NaCl to control the voltage/current. Although, the current used was within the safe range and similar currents have been used for electro-sterilization of root canals of teeth [202], this setup presents some important clinical problems. The presence of saliva and fluid pockets around the contaminated implants dramatically decreases the electrical resistance and makes it difficult to safely control the voltage/current applied to a patient [203]. Furthermore, these exploratory studies did not demonstrate the mechanism by which the electrochemical treatments could remove the biofilm or the most effective parameters for biofilm removal and prevention.

In the present study, we tested the hypothesis that electrochemical treatments with alternating potential are able to both remove organic contamination and bacteria from Ti implant surfaces. To achieve this, we first investigated the open circuit potential of biofilm-contaminated Ti to evaluate the effect of surface contaminants on the corrosion resistance of Ti. This information was then used to optimize the electrochemical decontamination treatments and understand their mechanism of action. We also evaluated the physicochemical properties of Ti surfaces before and after electrochemical treatments to test the capability of this treatment to restore the surfaces original properties.

5.3 Materials and Methods

The study design was reviewed and approved by the Research Ethics Board Committee of McGill University (application 14-464 GEN) and informed written consents were received from all subjects participating in this study.

5.3.1 Samples preparation

Grade 4 titanium (Ti) discs (10-mm diameter and 1 mm thickness; BIOMET 3i LLC, USA) were polished using Silicon Carbide grinding papers (#240, #320, #600, #800; Buehler, Lake Bluff, IL, USA) and polishing cloths (Text Met and Chemo Met I Polishing Cloth; Buehler, US) with colloidal silica polishing suspension (Master Med; Buehler, US). All the samples were then cleaned in sequential ultrasonic baths of acetone, ethanol and deionised water, for 15 minutes each, before drying over-night in a vacuum oven (Isotemp, Fisher Scientific, US).

5.3.2 Samples contamination

Ti samples were contaminated with either saliva or biofilm.

5.3.2.1 Saliva contamination

The Ti samples were contaminated with human saliva collected from a healthy non-smoker individual, at least 2 hours after meal, drinking or brushing. Each disc was immersed in 2 mL of freshly pooled saliva for 20 minutes at 37°C. The samples were then ultrasonicated in deionised water for 15 minutes and dried overnight prior to further analysis [204].

5.3.2.2 Biofilm contamination

Ti samples were contaminated with oral biofilm formed in six human volunteers of both sexes (non-smokers and in good systemic health) as previously described [117, 126, 132, 133]. The discs were then fixed to individual removable acrylic upper jaw splints at the buccal side of premolars and molars. The volunteers were instructed to wear the splints for 24 hours and to

remove them only during drinking or eating, meanwhile storing them in phosphate buffered saline. After that, the splints were removed and the discs washed with sterile saline solution prior to further analysis.

5.3.3 Electrochemical cleaning

A three-electrode electrochemical cell was setup as follows: a saturated Hg/HgCl Calomel reference, a platinum wire counter, and a Ti disc working electrode. All electrodes were immersed in an electrolytic solution and the electrochemical measurements were performed using a potentiostat (PARSTAT 2263; Amtek, Inc. Oak Ridge, Tennessee, US) linked to a computer with Power Suite software (Advanced Measurement Technology, Inc. Oak Ridge, Tennessee, US) for data acquisition (Fig. 5.11.1 Supp.). The chronoamperometry technique was employed for Ti surface cleaning; different potentials were applied to the working electrode to initiate the electrochemical reaction while monitoring the resultant current flow as a function of time at room temperature. Following the electrochemical cleaning, the Ti discs were cleaned ultrasonically in deionised water for 15 minutes, then dried and stored.

5.3.4 Cleaning parameters' optimization

The effect of different parameters on the contaminated Ti discs was assessed and optimized. This included potential charge and magnitude, exposure time as well as the type of electrolyte solution used. A potential of 1.8V was used because it is just above the Ti standard electrode potential. This potential was chosen to ensure that the Ti metal is charged and the following electrochemical (oxidation/reduction) reactions can be induced [111]. These reactions can generate the oxidative species that remove and inactivate bacteria.

Reduction $2H_2O + 2e^- \rightarrow H_2 + 2OH^-$

Oxidation $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$

In order to assess the effect of the direction of the current, the samples were exposed to different charges; a cathodic potential and an anodic potential of 1.8V were initially applied in two separate experiments (single-potential step chronoamperometry) for 5 minutes each. After that, cathodic (2.5 minutes) followed by anodic (2.5 minutes) potentials were applied in the same experiment (double-potential step chronoamperometry) for a total exposure time of 5 minutes. In another experiment the potential charge was reversed to start with anodic potential (2.5 minutes) followed by cathodic potential (2.5 minutes) (Fig. 5.11.2 Supp.).

After optimizing the direction of the current, the exposure time was optimized by adding more chronoamperometry cycles and evaluating their effect on surface decontamination. The effect of the magnitude of the potential on the contaminated surfaces was also evaluated using the optimized exposure time obtained from previous step and 3 different voltages, above and below the standard electrode potential of Ti (1.0, 1.8 and 2.8 V). Finally, three different electrolytes were tested for the cleaning process; sodium chloride (NaCl), potassium hydroxide (KOH) and sodium sulfate (Na_2SO_4) (0.1Molar/20ml).

5.3.5 Ti brush cleaning

The electrochemical treatment of the Ti discs was also combined with mechanical brushing. This was done with brushes made of titanium (TiBrush; Institute Straumann AG) exercising a force of 0.23 N on the samples surfaces and rotating speed of 920 revolution per minute. The samples were instrumented for 60 sec. The cleaning procedure for all discs were carried out by the same investigator.

5.3.6 Analysis Methods

The following methods were used to analyse the chemical, electrochemical and morphological properties of Ti discs before and after contamination, and decontamination.

5.3.6.1 X-ray Photoelectron Spectroscopy (XPS)

The chemical composition of the Ti surfaces was analyzed using a monochromatic X-ray photoelectron spectrometer (Thermo Fischer Scientific Inc., East Grinstead, UK) at three time points: after samples preparation and cleaning (baseline), after contamination with saliva or biofilm, and finally after electrochemical decontamination. The setup was equipped with a monochromatic Al *Kα* X-Ray radiation source (1486.6 eV, (λ) 0.834 nm) and an ultrahigh vacuum chamber (10^{-9} torr). For all discs, survey scans were acquired over the range of 0 −1350 eV with a pass energy of 200 eV and a resolution of 1.0 eV. High resolution spectra of carbon (C1s), titanium (Ti2p) were also obtained with a pass energy of 50 eV and a resolution of 0.1 eV. Binding energies, peak areas and atom concentration ratios were obtained using the curve fitting function of Avantage analysis software (5.932v, Thermo Fisher Scientific, Waltham, MA USA). All experiments were performed in triplets.

In high-resolution spectra, photoelectron binding energies were referenced by setting the peak of carbon bonded to hydrogen or carbon [C-(H, C)] in the resolved C1s spectra to 285.0 eV. High resolution spectra of C1s and Ti2p were deconvoluted into different peaks presenting the possible functional groups on the surface. The C1s high resolution spectra were deconvoluted to the following peaks: carbon single bonded to carbon or hydrogen (C-C,H) at 285.0 eV, carbon single bonded to oxygen (C-O) at 286.4 ± 0.2 eV and carbon double bonded to oxygen (C=O) at 288.4 ± 0.2 eV. The Ti2p high resolution spectra were deconvoluted to: Ti dioxide (TiO_2) at 458.7 ± 0.3 eV, intermediate oxides of Ti_2O_3 at 457.1 ± 0.3 eV and TiO at 455.3 ± 0.1 eV, and metallic Ti at 454.0 ± 0.3 eV [131, 205].

5.3.6.2 Electrochemical measurements

Open circuit potential (OCP) was monitored during a period of 18000 seconds to characterize the electrochemical properties of Ti discs before and after contamination.

5.3.6.3 Live/ dead bacterial assays and Fluorescence Microscopy (FM)

Presence, viability and attachment of salivary/ biofilm microbiota on contaminated and decontaminated Ti discs (n= 8 for each group) were determined using a live/ dead assay (BacLight Bacterial Viability Kit L7012, Molecular Probes, Carlsbad, USA) and fluorescence microscopy. The live/dead stain was prepared by diluting 1 µL of SYTO 9 (excitation (λ) = 485 nm, emission = 498 nm) and 1 µL of propidium iodide (excitation = 535 nm, emission = 617 nm) in 1 mL of distilled water. Discs were placed in 24-well plate, and 1000 µL of the reagent mixture was added to each well followed by incubation in the dark at room temperature for 15 minutes.

Each disc was then carefully positioned on a glass slide covered with mounting oil and stored in a dark space at 4˚C until further processing. Discs were evaluated using an upright fluorescence microscope (Carl Zeiss Microscopy GmbH) equipped with a digital camera (AxioCam MRm Rev. 3, Carl Zeiss Microscopy GmbH) in combination with image processing software (ZEN; Carl Zeiss Microscopy GmbH). For each disc, five randomly selected sites were captured using a 20 x objective. Median of red fluorescent areas (dead cells), green fluorescent areas (viable cells), and total fluorescence per standard microscopic field area (448×335= 0.15 mm^2) were calculated using cell profiler image analysis software (Broad Institute of MIT and Harvard, Massachusetts, US).

5.3.6.4 Scanning Electron Microscope (SEM)

Ti discs were scanned before and after contamination, and after the decontamination to visualize bacteria, surface contaminants, and any morphological changes. Clean Ti discs were scanned with SEM (FE-SEM S-4700, Hitachi, Japan) without further preparation, while contaminated and decontaminated discs were fixed in glutaraldehyde (2.5% in phosphate buffered saline (PBS); PAA Laboratories GmbH, Pasching, Austria) for 2 h and washed 5 times for 10 minutes in PBS, before dehydrating them in ascending concentrations of ethanol (30 –100 v/v %, 15 minutes each) and then drying with critical point CO_2 (Ladd Research Critical Point Dryer). All discs were mounted on SEM-sample stubs and sputtered with gold. The SE mode with an acceleration voltage of 20 kV was selected, and the vacuum pressure was maintained below 1×10^{-5} torr. For direct comparison of surface morphology, SEM images were taken at the same magnification of x20,000 for saliva-contaminated samples and x10,000 for biofilm-contaminated samples.

5.3.7 Statistical analysis

Data were analyzed using the SPSS program (IBM SPSS Statistics 20, IBM Corporation, Somers, NY, USA). One-sample Kolmogorov–Smirnov and Shapiro-Wilk tests, as well as measures of skewness and kurtosis were used to determine distribution normality of all data. P-value of < 0.05 was set to represent a statistically significant difference. Repeated measures ANOVA, one-way ANOVA, Kruskal-Wallis, Mann–Whitney and Paired-sample T tests were performed to compare different groups and experiment time points.

5.4 Results

5.4.1 Surface chemistry and electrochemical properties of clean and contaminated Ti:

XPS analysis showed that clean (control) Ti surfaces were composed of Ti, O, C and traces of N (Fig. 5.5.1 a). C atom concentration ranged from 31.3±2.3 to 47.9±3.0, O atom concentration ranged from 49.8±1.9 to 56.8±1.6, while Ti atom concentration ranged from 14.6±0.6 to 18.6±0.8. These results are in agreement with previously reported findings for the surface chemical composition of BIOMET *3i* dental implants [131, 206].

Saliva contamination of Ti surfaces increased the peak intensities of C1s and N1s up to 73.6±2.4 and 10.7±1.4, while the intensities of Ti2p and O1s decreased to 1±0.3 and 18± 0.9 respectively. The deconvoluted Ti2p and C1s spectra demonstrated a significantly decreased surface concentration of TiO_2 and increase of C-O and C=O concentrations, indicating the presence of an organic layer covering the Ti surfaces (Fig. 5.5.1 a).

OCP measurements showed a significantly higher potential (-0.045V) in clean (control) than in contaminated discs (-0.099V), indicating that saliva contamination changed the electrochemical behaviour of Ti surfaces and reduced their corrosion resistance (Fig. 5.5.1 b and c).

5.4.2 Optimization of the electrochemical decontamination of Ti:

5.4.2.1 Effect of current direction (potential charge)

Exposing saliva-contaminated Ti surfaces to +1.8V potentials for 5 minutes decreased significantly the surfaces concentrations of metallic Ti, TiO and Ti_2O_3 in favor of increasing TiO_2, as shown by the deconvoluted Ti2p spectra. Whereas, the levels of organic compounds did not show a significant change under this potential (Fig. 5.5.2 b and d). On the other hand, exposing the metals to -1.8V for 5 minutes increased the surface levels of O, as seen in XPS surveys spectra, (Fig. 5.5.2 a and c) and reduced the organic compounds from the C=O to the C-

O state, as shown in the deconvoluted spectra. It also decreased the surface concentration of the metallic Ti and Ti_2O_3 in favor of increasing TiO_2 concentrations, although this effect on Ti specially that on TiO_2 was milder than that obtained with other potentials (Fig. 5.5.2 b and d).

Exposing contaminated Ti to +1.8V for 2.5 minutes followed by -1.8V for 2.5 minutes increased significantly the surface concentration of O and Ti and decreased the concentration of C without changing the concentration of N (Fig. 5.5.2 a and c). It also increases significantly the surface concentration of TiO_2 and decreases that of Ti_2O_3, Ti and TiO as shown by the deconvoluted Ti2p spectra (Fig. 5.5.2 b and d). Despite the fact that this electrochemical treatment was able to clean the saliva-contaminated Ti, it was still not able to restore the surfaces elements to their levels prior to contamination.

Exposing the contaminated Ti to the above mentioned treatment in inverted order, -1.8V for 2.5 minutes followed by +1.8V for 2.5 minutes showed a very different result. It resulted in a surface elemental composition comparable to that of clean controls (Fig. 5.5.2 a and c). Moreover, this treatment increased TiO_2 levels on the surface at the expense of metallic Ti and TiO as shown by the deconvoluted Ti2p spectra (Fig. 5.5.2 b and d), indicating thickening of the TiO_2 layer covering the metals. Cleaning the contaminated discs with additional cycles with this treatment did not provide further cleaning benefits (Fig. 5.11.3 Supp.). Consequently, this last treatment; the combination of cathodic/anodic potentials for 5 minutes was selected for all subsequent experiments.

5.4.2.2 Effect of potential magnitude (voltage)

Exposing the contaminated Ti surfaces to a potential of 1.8 V (cathodic/ anodic combination) resulted in a significant change in all surface elements concentration in favor of cleaning. Alternatively, exposing them to a potential of 1.0 V did not induce any change in the levels of

surface elements while increasing the potential up to 2.8 V only induced reduction of N1s concentration (Fig. 5.5.3 a and c). On the other hand, the deconvoluted data of Ti2p spectra showed the oxidation of Ti surface with all potentials as expressed by a significant increase of TiO_2 concentration. However, the highest level of TiO_2 was obtained with 1.8V potential. The C1s deconvoluted spectra showed a significant decrease of C-O and C=O with 1.0 V and 2.8V potentials respectively, but no significant change in C1s functional groups was observed with 1.8V potential, all in comparison to contaminated surfaces (Fig. 5.5.3 b and d). Therefore, the 1.8V potential was selected for the subsequent experiment.

5.4.2.3 Effect of electrolyte solution

The use of NaCl electrolyte (cathodic/ anodic combination of charge at 1.8 V) induced a lower change in the atomic concentrations of C and O in comparison to KOH and Na_2SO_4. However, no significant difference was recorded between the three electrolytes in the atomic concentration of N. In addition, both electrolytes KOH and Na_2SO_4 showed similar result in favor of cleaning despite the difference in their normality (Fig. 5.11.4 Supp.).

5.4.2.4 Optimized cleaning method

The combination of cathodic (2.5 minutes) and anodic (2.5 minutes) potentials of 1.8V in Na_2SO_4 (0.1 Molar) provided the best cleaning outcomes at a low generated current (cathodic 2.3mA, anodic 22.5 μA). Consequently, these optimized parameters were used to evaluate the changes in the elemental composition of biofilm-contaminated Ti surfaces, the surface chemistry and morphology of Ti, and for the analysis of bacterial viability and attachment after the surfaces decontamination.

5.4.3 Effect of the electrochemical treatment (optimized method) on the surface chemistry of biofilm-contaminated Ti before and after cleaning with Ti brush

The optimized electrochemical treatment did not induce any change in the levels of surface elements of biofilm-contaminated Ti. Cleaning these surfaces with Ti brushes alone was also not sufficient to completely remove the biofilm contamination. However, cleaning them with the electrochemical treatment followed by Ti brushes significantly changed the concentrations of all surface elements to comparable levels to those of clean Ti (Fig. 5.5.5). This observation indicated that the complete elimination of a thick biofilm from Ti surfaces require the combination of the electrochemical and brushing techniques.

5.4.4 Effect of the electrochemical treatment (optimized method) on surface chemistry of Ti

The exposure of clean (control) Ti to the optimized electrochemical treatment significantly increased the surface concentration of O1s and decreased that of N1s (Fig. 5.11.5 Supp.). It also oxidized Ti and TiO oxides to TiO_2, indicating the thinking of TiO_2 layer (Table 5.11.1 Supp.).

5.4.5 Ti surface morphology, bacterial attachment and viability on contaminated and decontaminated Ti surfaces

SEM images showed that the optimized electrochemical treatment was able to remove bacteria and other surface contaminants from the saliva-contaminated surfaces without morphological changes to their surface topography (Fig. 5.5.4 a and b). The decontamination of the biofilm-contaminated surfaces with the electrochemical treatment followed by brushing was also efficient in removing the surface contaminants, but it induced a substantial change in the surface morphology (Fig. 5.5.6 a and b). Fluorescence microscope images showed the presence of bacteria on both saliva- (Fig. 5.5.4 c and e) and biofilm-contaminated surfaces (Fig. 5.5.6 c and e). However, the decontamination of the saliva-contaminated surfaces with the electrochemical

treatment (Fig. 5.5.4 d and e) and the biofilm-contaminated surfaces with the combined electrochemical/Ti brush technique (Fig. 5.5.6 d and e), resulted in a significant reduction in the number of attached bacteria (fluorescence green) and their viability ($p < 0.05$; Fig. 5.5.4 and 5.5.6 f).

5.5 Figures

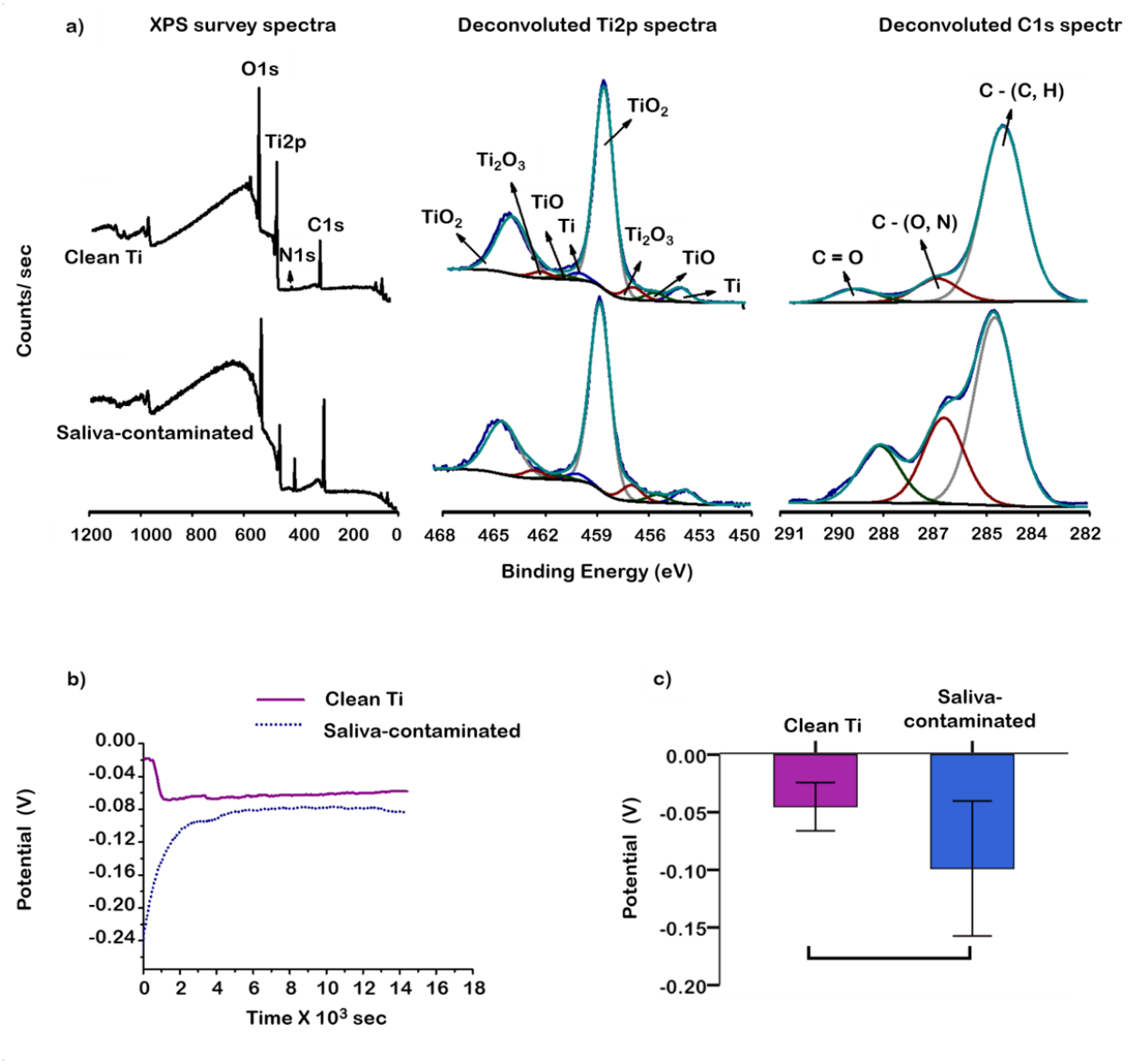

Fig. 5.5.1. (a) X-ray photoelectron spectroscopy survey and deconvoluted high-resolution spectra of C1s, Ti2p for clean (top) and saliva-contaminated Ti surfaces (bottom). (b, c) Open Circuit Potential (OCP) measurements for clean (control) and saliva-contaminated Ti samples: (b) line chart illustrating OCP variation with time; (c) Bar chart illustrating the comparison of OCP before and after contamination. Line indicates significance difference between contaminated vs. clean discs ($p < 0.05$).

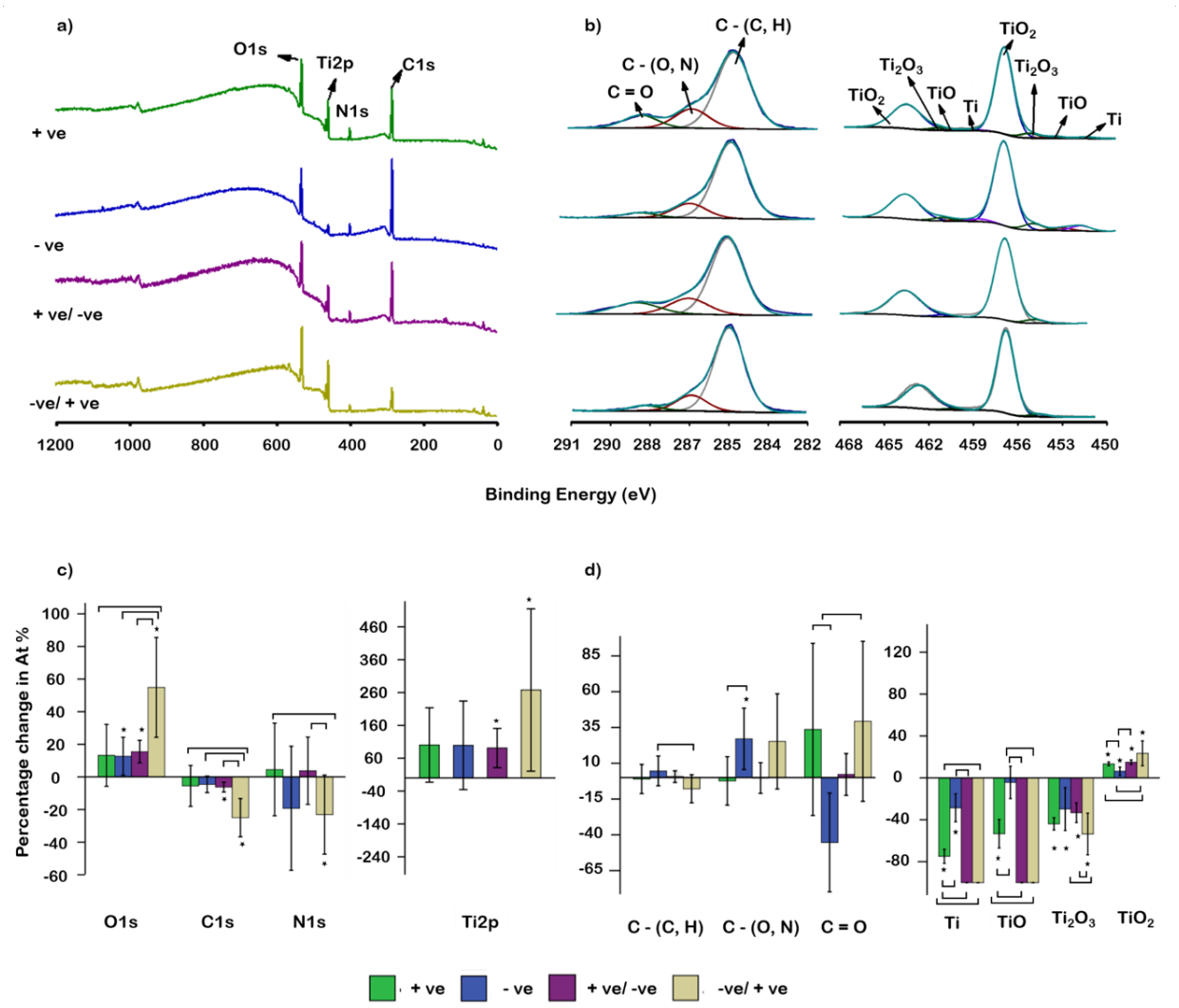

Fig. 5.5.2. X-ray photoelectron spectroscopy spectra (a, b) and bar charts (c, d) illustrating the effect of potential charge on the elemental composition of contaminated Ti surfaces: comparison of the percentage change in the elemental composition of Ti surfaces after electrochemical decontamination (1.8V, 5mins, Na_2SO_4 electrolyte solution). (a) XPS survey spectra; (b) deconvoluted high-resolution spectra of C1s and Ti2p; (c) main elements detected with XPS-low resolution scan; (d) functional groups detected with deconvoluted XPS-high resolution spectra of C1s and Ti2p. Lines indicate significance difference between different groups ($p < 0.05$); * indicates significance difference between electrochemically treated vs. saliva-contaminated discs ($p < 0.05$).

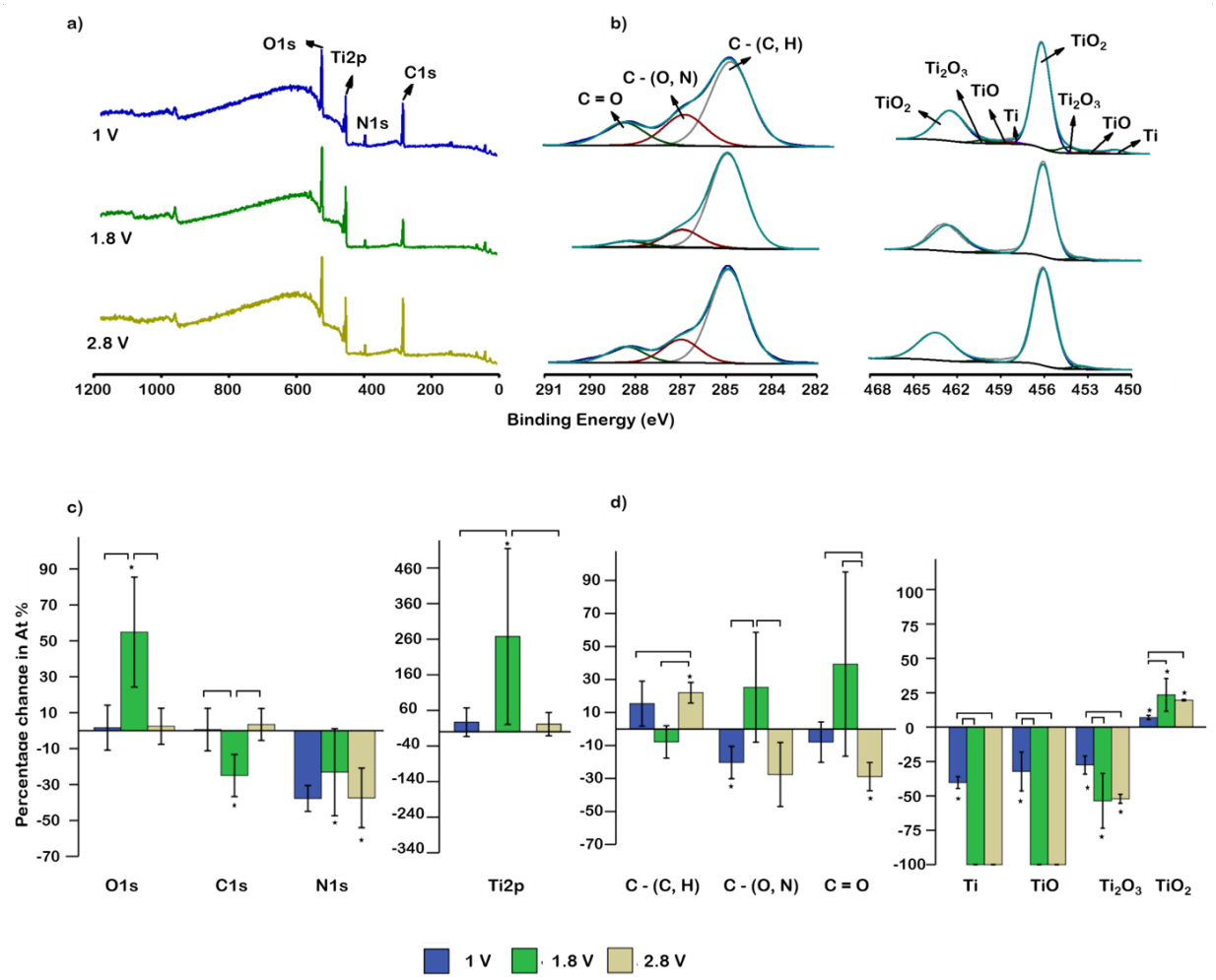

Fig. 5.5.3. X-ray photoelectron spectroscopy spectra of (a, b) and bar charts (c, d) illustrating the effect of voltage magnitude on the elemental composition of contaminated Ti surfaces: comparison of the percentage change in the elemental composition of Ti surfaces after electrochemical decontamination (cathodic/anodic potential combination, 5mins, Na_2SO_4 electrolyte solution). (a) XPS survey spectra; (b) deconvoluted high-resolution spectra of C1s and Ti2p; (c) main elements detected with XPS-low resolution scan; (d) functional groups detected with deconvoluted XPS-high resolution spectra of C1s and Ti2p. Lines indicate significance difference between different groups ($p < 0.05$); * indicates significance difference between electrochemically treated vs. saliva-contaminated discs ($p < 0.05$)

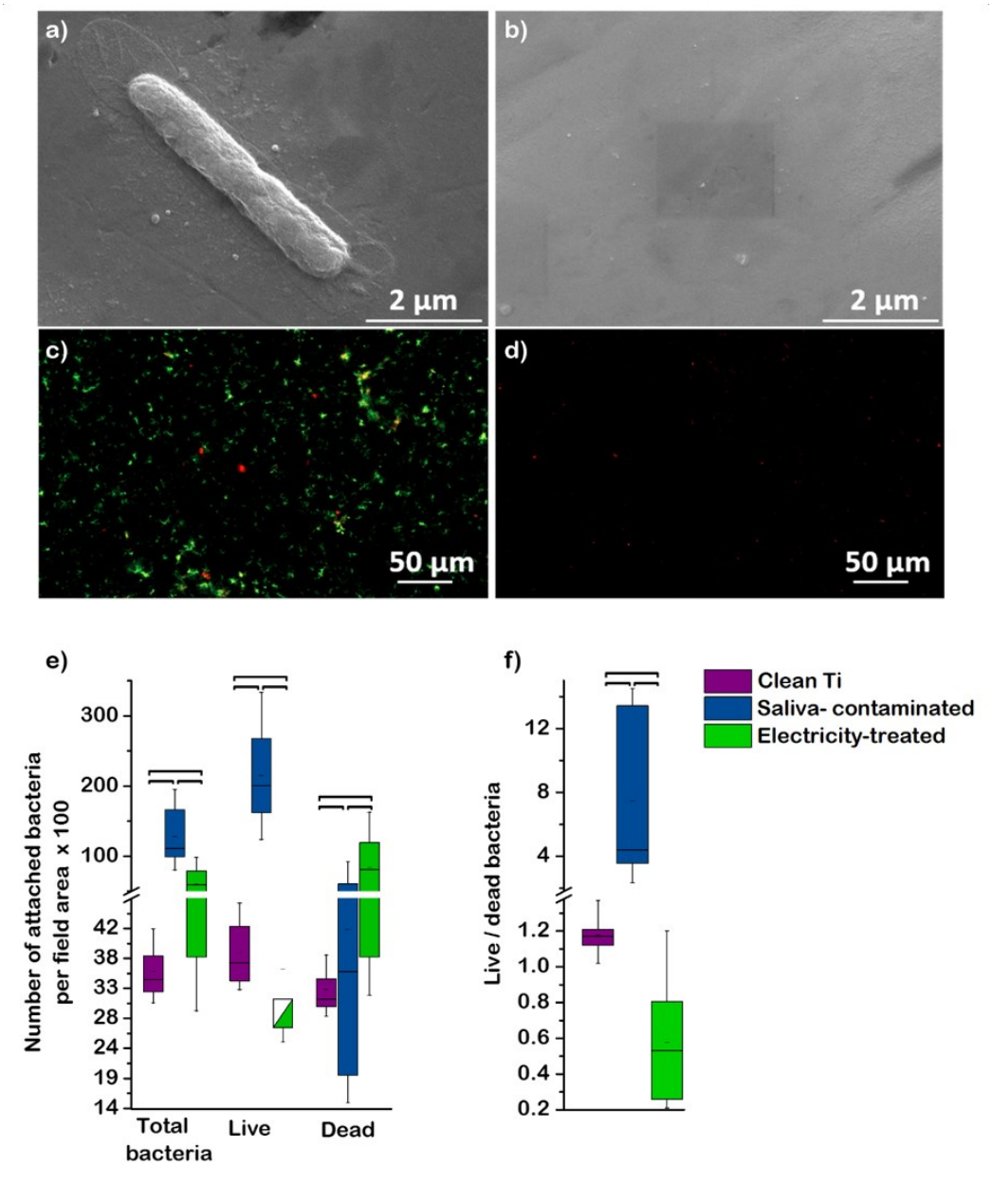

Fig. 5.5.4. (a, b) Scanning Electron Microscope images (x20,000 magnification) illustrating the morphology of Ti surfaces: (a) Saliva-contaminated Ti surface showing a bacterium and surrounding matrix; (b) the surface after the electrochemical decontamination. (c) Live/ dead staining (fluorescence) images of bacteria on Ti surface before and (d) after the electrochemical decontamination; green color represents live bacteria while red color represents dead or inactivated bacteria. (e) Comparison of the number of the attached bacteria (per field area of 0.15 mm^2) and (f) viability (live/dead ratio) on Ti surfaces before and after contamination and decontamination. The applied decontamination protocol (cathodic/anodic potentials combination, 1.8V, 5mins Na_2SO_4 electrolyte solution). Lines indicate significance difference between different groups ($p < 0.05$).

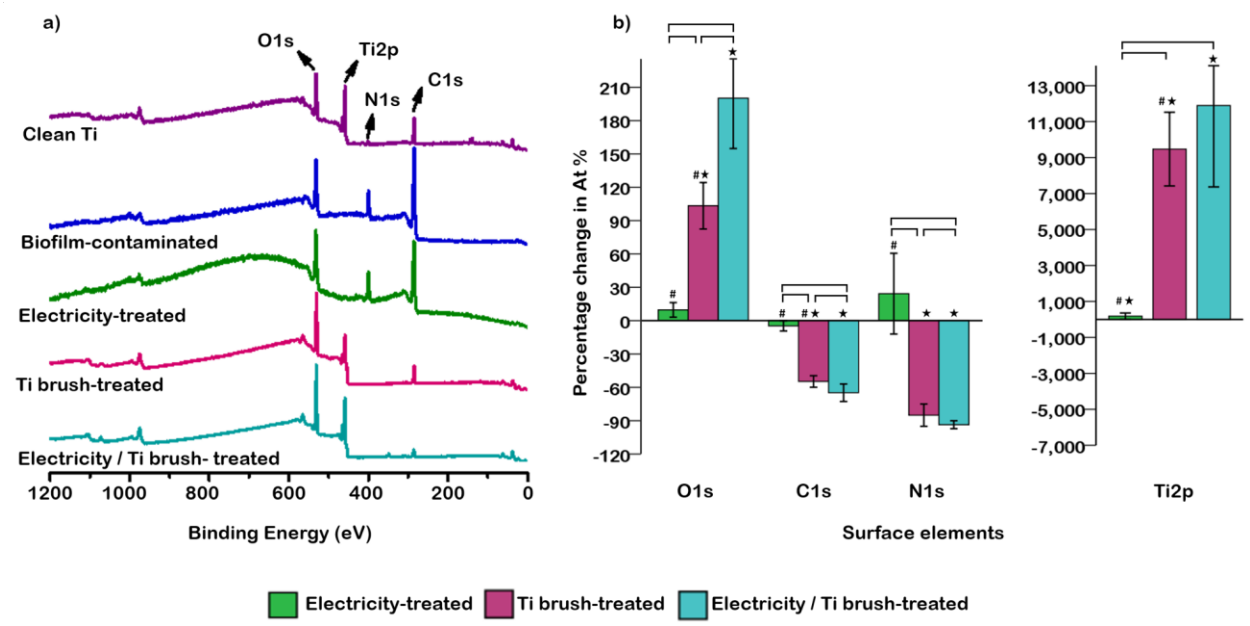

Fig. 5.5.5. X-ray photoelectron spectroscopy survey spectra (a) and bar chart (b) illustrating the cleaning effect of Ti brush alone and the combined protocol of the electrochemical (optimized method: cathodic/anodic potentials, 1.8V, 5mins, Na_2SO_4 electrolyte solution) followed by Ti brush, on the elemental surface composition of biofilm-contaminated surfaces: Comparison of the percentage change in the composition of the main elements on Ti surfaces. Lines indicate significance difference between different groups ($p < 0.05$); * indicates significance difference between treated vs. biofilm-contaminated discs ($p < 0.05$); # indicates significance difference between treated vs. clean discs ($p < 0.05$).

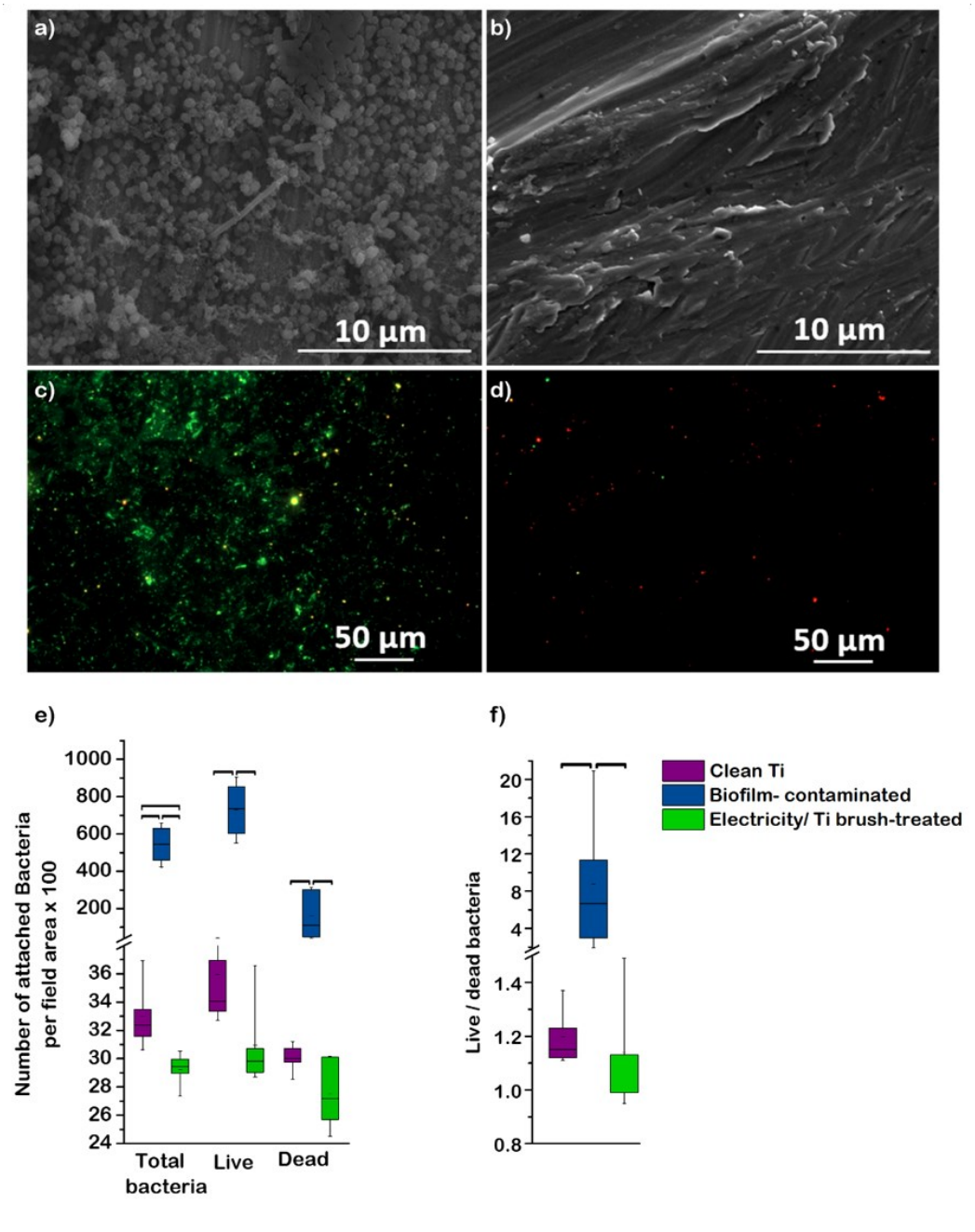

Fig. 5.5.6. (a, b) Scanning Electron Microscope images (x10,000 magnification) illustrating the morphology of Ti surfaces: (a) Biofilm-contaminated Ti surface; (b) the surface after decontamination. (c) Live/ dead staining (fluorescence) images of bacteria on Ti surface before and (d) after the decontamination; green color represents live bacteria while red color represents dead or inactivated bacteria. (e) Comparison of the number of attached bacteria (per field area of 0.15 mm^2) and (f) viability (live / dead ratio) on Ti surfaces before and after biofilm contamination and decontamination. The applied decontamination protocol was the optimized electrochemical method: cathodic/anodic potentials combination, 1.8V, 5mins Na_2SO_4 electrolyte solution; followed by Ti brush. Lines indicate significance difference between different groups ($p < 0.05$).

5.6 Discussion

The current study presents a new decontamination approach (the electrochemical treatment) that was able to disinfect contaminated Ti surfaces with low electrical alternating current (2.3mA, 22.5 μA). Exposing the contaminated surfaces to the combination of a cathodic and an anodic potentials of 1.8 V, each for 2.5 minutes was able to eliminate salivary contaminants and established biofilms with the aid of mechanical brushing. The electrochemical treatment was non-destructive, it did not alter the Ti surface morphology, and it increased the concentration of TiO_2 on the metal surface, which is known to enhance the mechanical properties of Ti [207], promote faster bone healing [208] and greater bone-to-implant contact [209].

5.6.1 Electrochemical properties of contaminated surfaces

The surface contamination of Ti with saliva reduced the corrosion resistance of the metal (Fig. 5.5.1, b and c). This could result from breakdown of the protective Ti oxide layer by saliva electrolytes and biofilm bacteria and it can have negative impact on Ti biocompatibility [210]. Indeed, previous reports showed that Ti corrosion is accelerated in the presence of inflammation [211], bacteria [212] and their products mainly lipopolysaccharides [213], especially in the gaps between implants and prosthetic structures where saliva penetrates [214]. The implanted bone plate and screws also corrode and release Ti ions, due to the adsorption of amino acids and proteins on the metal surfaces, despite the absence of wear and fretting [215].

5.6.2 Electrochemical decontamination of titanium

The electro-conductivity of Ti implants facilitates the use of electrochemical disinfection as an alternative and probably more effective approach to decontaminate Ti implants. The favorable cleaning outcomes of this method could be due to the collective effect of low direct currents, the electrical forces that control bacterial adhesion [195], and the induced electrolytic reactions.

Underneath we discuss the effect of the different electrochemical parameters and their optimization for Ti decontamination.

5.6.2.1 The current

Our results seem to indicate that the low current generated by the cathodic and anodic potentials (2.3mA and 22.5 μA respectively) have a disruptive effect on bacteria cells. Previous studies have also shown that, an electric cathodic current as low as 10 μA was found to repel and kill bacteria attached to catheter surfaces by interrupting the electrostatic charges and potentials of the cells membranes [216]. Moreover, detachment of oral bacteria has been induced by applying direct anodic current of 800 μA [195]. Nevertheless, these studies have tested the effect of electric currents on some bacterial strains, and such currents might not have the same outcomes on other bacterial strains or an established biofilm. In addition, in both studies, the inhibition of bacteria due to current was evaluated with bacterial smear tests, however, this technique does not assess if the tested currents can effectively remove the organic contaminants produced by bacteria (endotoxins).

5.6.2.2 The potential charge

Anodic potentials were found to inactivate bacteria and eliminate their biomolecules by generating bactericidal oxidative species through the following electrolytic reactions [196].

(a) $2H_2O \rightarrow O_2 + 4H^+ + 4e^-$, (b) $H_2O \rightarrow HO^{\cdot} + H^+ + e^-$

In this study, we found that using anodic potentials increased TiO_2 surface concentration (Fig. 5.5.2 b and c) that probably suggest oxidation of Ti metal and subsequent thickening of TiO_2 layer, however, high levels of contamination (Fig. 5.5.2 c and d) were sustained on these surfaces. In agreement with previous studies, this potential could have bactericidal effect but the adsorption and oxidation of the negatively charged bacteria on the anodic surface most probably

result in accumulation of dead bacteria that blocks further reactions and limits the removal of contaminants [153].

Cathodic potentials can generate electro-repulsive forces between the negatively charged surface and bacteria, resulting in their detachment [195]. It can also induce water hydrolysis that produces hydrogen gas and increased pH (alkaline) as described above. The alkaline pH has bactericidal effects mainly through hydrolysis of the bacterial polysaccharide matrix [217] while the generated H_2 gas bubbles adjuncts the detachment of surface contaminants and bacteria [218]. However, our results were in contrast to the previous findings, cathodic potentials increased the levels of reduced organic molecules and decreased the N levels, although this decrease is not significant (Fig. 5.5.2 a and c). This insignificant cleaning effect of cathodic potentials could be attributed to the re-deposition of detached bacteria and biomolecules on the cleaned surfaces once the applied potential is terminated [219].

Alternating currents (charges) can induce both bacterial detachment and degradation [199]. However, the fast repetitive change of the electric current could alter the charge of the electrode surface (metal) before reaching the effective potential that could detach or degrade bacteria [219], thus limiting its cleaning efficiency. Therefore, in the present study, two modes of alternating current (cathodic, anodic) were applied. The anodic/cathodic combination had no effect on the surface contamination (Fig. 5.5.2). This could be explained by the adsorption and neutralization of the negatively charged bacteria and biomolecules in the anodic phase of the cycle [196]. This in turn blocks the reaction of the electrode with these contaminants during the cathodic phase [153], inhibiting their detachment and resulting in a limited removal of the surface contaminants especially the nitrogen based compounds.

On the other hand, the cathodic/anodic combination resulted in a significant decontamination (Fig. 5.5.4). It was probably caused by extensive detachment of the surface contaminants and partial bacteria inactivation during the initial cathodic phase [195], before eradicating the remaining bacteria and their organic products in the following anodic phase. This observation was confirmed by the significant reduction in number and viability of the attached bacteria when this treatment was applied. Our results are in agreement with previous studies that reported the effectiveness of cathodic potentials in bacterial detachment from metallic surfaces [188, 198], and anodic potentials in bacterial inactivation [201, 202, 219]. However, this is the first study, to our knowledge, that assess the combination of cathodic and anodic potentials in the same treatment to achieve both biofilm removal and inactivation.

Extending the treatment time to 10 and 15 minutes by exposing the contaminated surfaces to additional cycles didn't change the decontamination outcomes. This is attributed to the complete elimination of surfaces contaminants in the first cycle, which presented as comparable elemental composition of the treated and clean uncontaminated surfaces (Fig. 5.11.3 Supp.).

5.6.2.3 The voltage

The most effective potential for cleaning contaminated Ti surfaces was 1.8 V. This could be explained by the fact that this potential is just above the standard electrode potential of Ti metal. Ti metal can be negatively or positively charged by reaching the standard electrode potential (cathodic or anodic) [111]. The charging of the surface could result in electrostatic forces between the charged Ti and surface contaminants as well as electrolytic reactions. Indeed, lower potentials (1.0V) did not clean the Ti, probably because it was not sufficient to induce electro-repulsion or electrolysis reactions.

On the other hand, the application of higher potential (2.8V) decreased the surface nitrogen but did not change the concentrations of the other surface elements (Fig. 5.5.3). This result could probably suggest burning of the organic contaminants on Ti surfaces by the high generated current (Cathodic 32.5mA, Anodic 0.13mA) rather than removing them.

5.6.2.4 The electrolyte solution

We used three different electrolyte solutions to test the decontamination effect of some electrolytes that present in human body. Na_2SO_4 showed comparable decontamination outcomes to that of KOH, and both were more efficient that NaCl in terms of removing carbon compounds. We used Na_2SO_4 for the optimized protocol because it is less toxic. Generally, all tested electrolytes increased the concentration of Ti, indicating their cleaning efficiency. However, the human body has complex composition in terms of electrolytes and its conductivity varies from one tissue to another. Therefore, additional research is needed to anticipate their clinical performance.

5.6.3 Complete biofilm decontamination

The optimized electrochemical parameters achieved complete elimination of thin biofilm layers such as salivary pellicle. These parameters included cathodic/ anodic potentials combination (1.8 V), 5 minutes exposure time and Na_2SO_4 electrolyte solution. However, complete removal of thick biofilms required adjunctive mechanical cleaning using Ti brushes (Fig. 5.5.5 and 5.5.6). It seems that our electrochemical treatment causes polarization of Ti surfaces that destabilizes the initially adhering contaminants and microorganisms [191], and this facilitates their removal with Ti brush. The surface roughness was not evaluated in the current study but a previous study, using both profilometry and confocal microscopy, found that Ti brushes induce surfaces morphological changes without changing surface roughness [152].

5.7 Clinical Implications

From a clinical point of view, this novel decontamination technique using a short treatment time (5 minutes) of low potentials (1.8V) and currents (2.3mA, 22.5 μA) could be safe and appropriate for clinical application. The currents involved are way below the hazardous current limit (>10.5 mA of AC or >88 mA of DC) and it should be tolerable for human beings [203, 220]. However, the human body resistance (1-2 KΩ) could present technical difficulty regarding its clinical implementation. To circumvent this limitation, an electric device could be modified to produce the optimized electrochemical parameters in which the electrodes are placed close to each other.

5.8 Study Limitations and Future Studies

The present study has several limitations. We used polished Ti discs while implants have screw-shaped designs and modified rough surfaces that could complicate the surface cleaning. However, the mirror polished surfaces allowed for accurate assessment of the effect of the electrochemical treatments by eliminating the potential confounder of surface irregularity.

We evaluated the decontamination efficiency of electricity on Ti surfaces contaminated with saliva and in vivo oral biofilm to mimic the clinical situation of initial and prolonged exposure of implant surface to oral environment. We used in vivo biofilm model because it offers the opportunity to evaluate implant surfaces in realistic clinical conditions; formation of composite plaque, co-adherence of microorganisms and salivary pellicle under the removal forces of salivary flow and chewing activities [135]. Several in vitro biofilm models have been tested and validated to study the implant surface bacterial interactions [97, 136, 137]. This includes for instance the commonly used microtiter plate-based systems [138]. However, they fail to simulate the complex structure of biofilm, the dynamics of its pathogenicity and ecological determinants

[139, 140]. Nevertheless, we recommend further studies to assess the decontamination effectiveness of the electrochemical approach on surfaces contaminated with pathogenic biofilms and virulent bacteria involved in orthopedic infections such as Staphylococcus aureus. We also recommend future animal studies to test the performance of the electrochemical treatment and its impact on mammalian cells before implementing this method clinically.

Furthermore, in this study live/dead bacterial assays were used to investigate the antibacterial efficacy of the electrochemical treatment on the biofilm. However, we recommend future studies to further investigate specific bacterial species using quantitative methods such as fluorescence in situ hybridization (FISH) or quantitative real-time polymerase chain reaction (qPCR) [157-159].

5.9 Conclusions

Here we present a new electrochemical decontamination method that effectively cleans contaminated titanium surfaces without affecting their morphology. This optimized method is based on the use of a low electrical alternating current and potential that is sufficient to achieve decontamination. The combination of cathodic/anodic potentials reduces bacterial number and viability from saliva-contaminated surfaces (thin biofilms). And by combining this method with subsequent brushing, it is also possible to completely remove bacteria and surface contaminants from biofilm-contaminated surfaces.

5.10 Acknowledgements

The authors would like to thank BIOMET *3i* (LLC, USA) for their generous donation of implants discs. We also gratefully acknowledge Evelin Barbosa de Melo for her assistance with the electrochemical experiments. This research was supported by grants received from FODQ (Fondation de l'Ordre des dentistes du Québec), RSBO (Le Réseau de recherche en santé

buccodentaire et osseuse), Alpha Omega Foundation of Canada (A.A.), Canadian Foundation for Innovation (F.T.), NSRC-Discovery (F.T.), Canada Research Chair (Tire 2, F.T.) and Islamic Development Bank scholarship (A.A.)

5.11 Supplementary Information

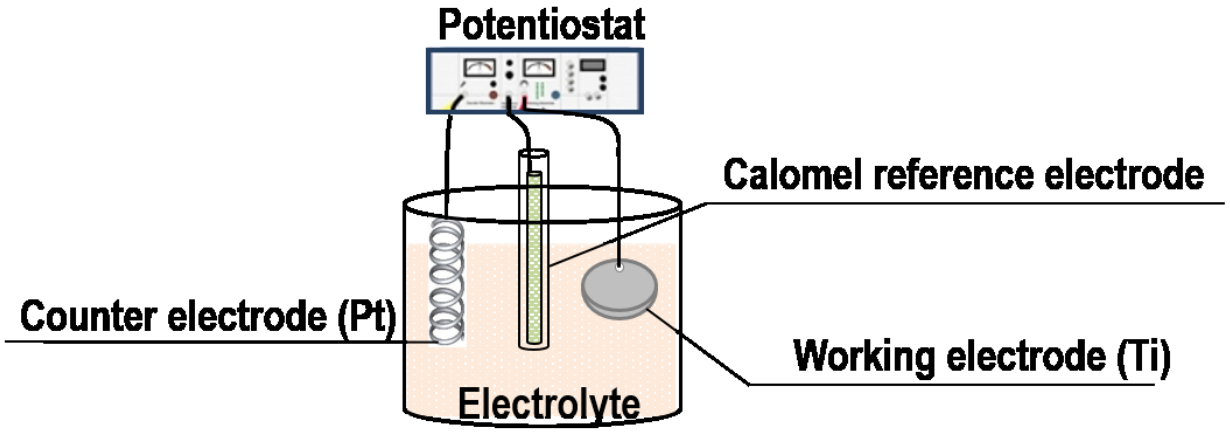

Fig. 5.11.1. Schematic diagram representing the three-electrode electrochemical cell setup employed in the study.

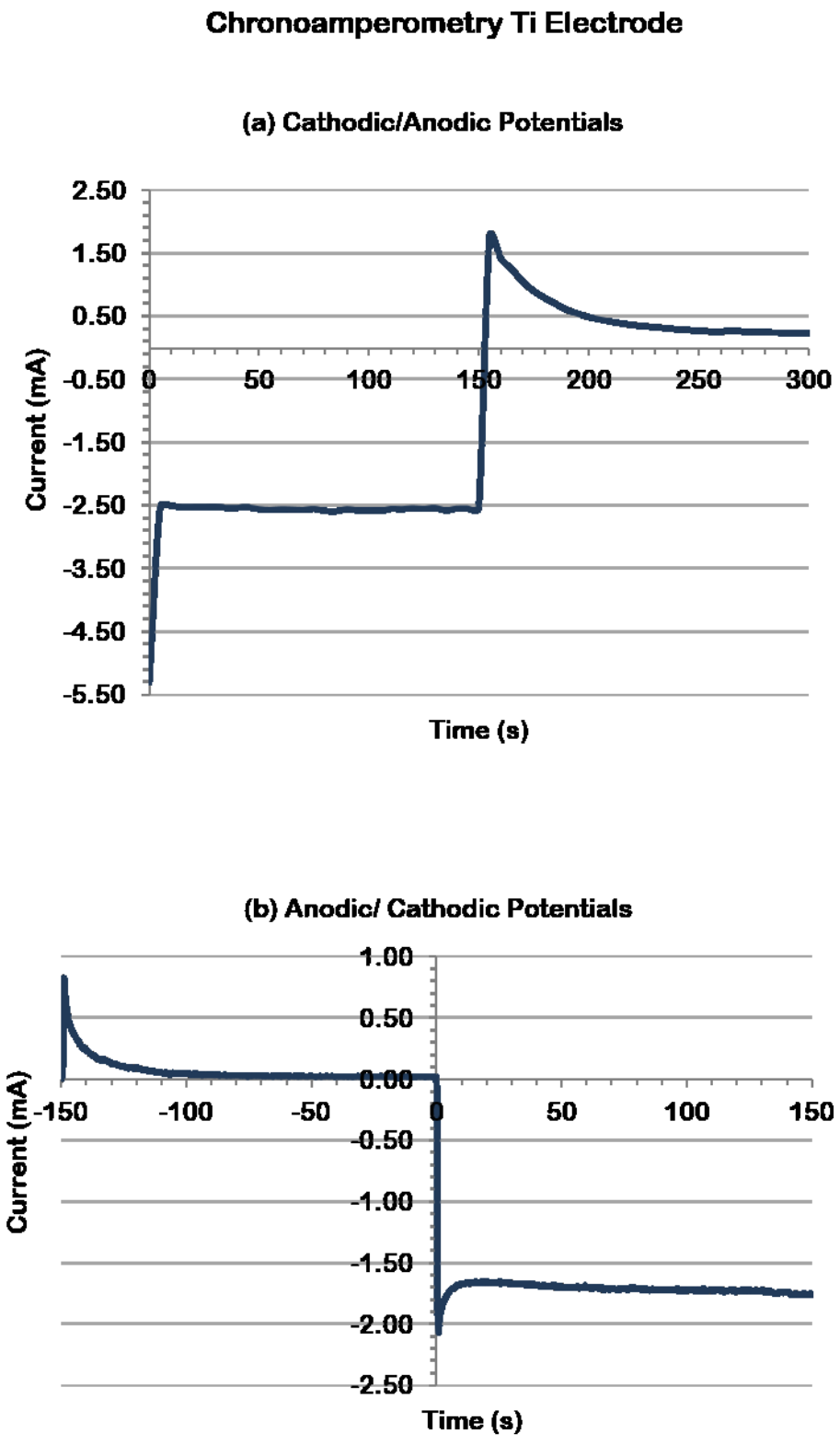

Fig. 5.11.2. Current-time response for a double-potential step chronoamperometry. (a) Optimized method: cathodic/anodic potential combination, 1.8V, 5mins, Na_2SO_4 electrolyte solution, (b) anodic/cathodic potential combination, 1.8V, 5mins, Na_2SO_4 electrolyte solution.

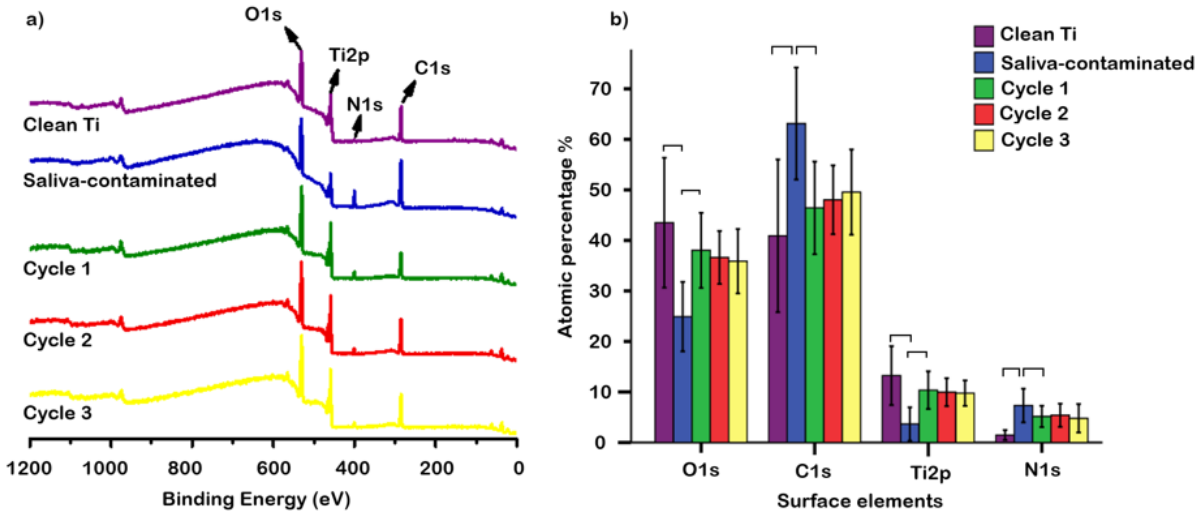

Fig. 5.11.3. X-ray photoelectron spectroscopy survey spectra (a) and bar chart (b) illustrating the effect of number of the electrochemical treatment cycles on the elemental composition of saliva-contaminated Ti surfaces using cathodic/anodic potentials combination, 1.8V, 5mins and Na_2SO_4 electrolyte solution: comparison of the surface elemental composition before and after the electrochemical treatment as detected with XPS-low resolution scan. Lines indicate significance difference between groups ($p < 0.05$).

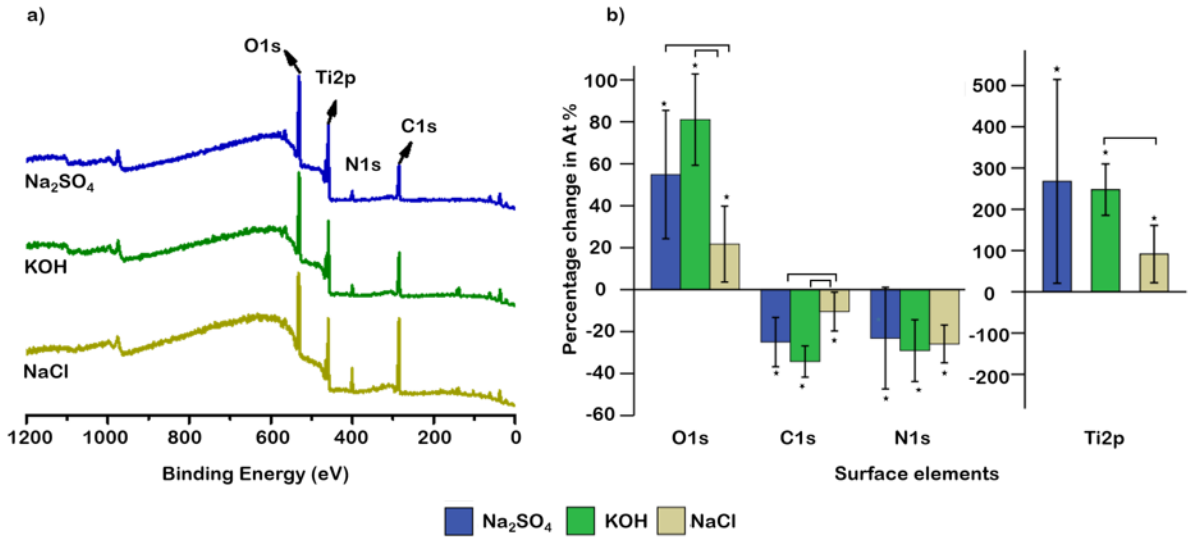

Fig. 5.11.4. X-ray photoelectron spectroscopy survey spectra (a) and bar chart (b) illustrating the effect of different electrolyte solutions on the elemental composition of saliva-contaminated Ti surfaces: comparison of the percentage change in the composition of the main elements of Ti surfaces after electrochemical decontamination (cathodic/anodic potential combination, 1.8V, 5mins), as detected with XPS-low resolution scan. Lines indicate significance difference between different groups ($p < 0.05$); * indicates significance difference between electrochemically treated vs. contaminated discs ($p < 0.05$).

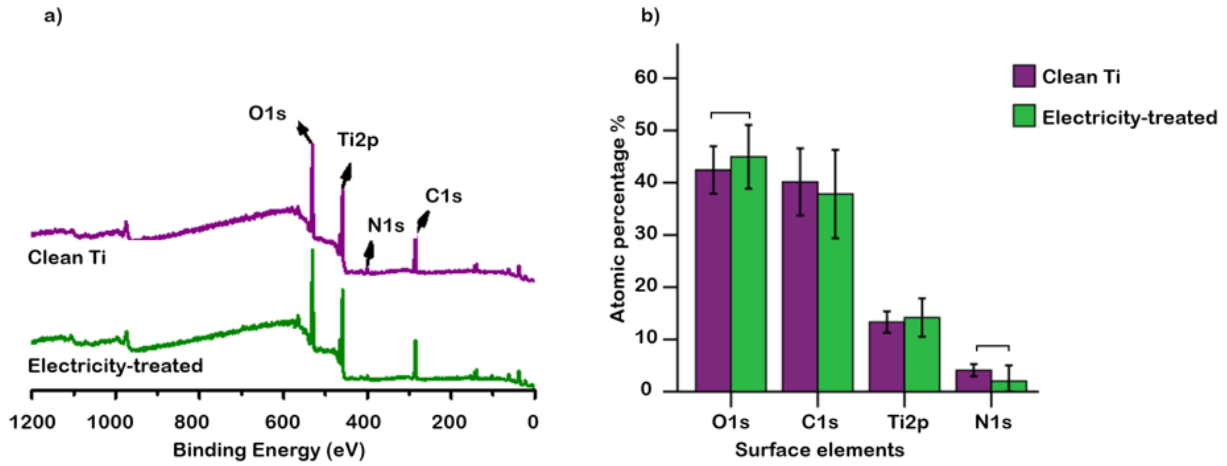

Fig. 5.11.5. X-ray photoelectron spectroscopy survey spectra (a) and bar chart (b) illustrating the effect of the electrochemical treatment (optimized method: cathodic/anodic potential combination, 1.8V, 5mins, Na_2SO_4 electrolyte solution) on the elemental surface composition of Ti discs. Lines indicate significance difference between electrochemically treated vs. clean (control) samples ($p < 0.05$).

Table 5.11.1. The effect of the optimized electrochemical treatment on the surface elemental composition of Ti.

Elements	Functional groups	Clean (control)	Electrochemically treated
Ti2p	Metallic Ti	7.7 ± 0.9	0.0 ± 0.0
	TiO	4.2 ± 0.5	0.0 ± 0.0
	Ti_2O_3	7.4 ± 0.1	3.1 ± 0.4*
	TiO_2	80.6 ± 1.6	96.6 ± 0.5*

Values were obtained from the deconvoluted XPS-High resolution spectra of Ti2p and presented as mean ±SD of the atom concentration (At %) of each functional group before and after the electrochemical treatment.
* indicates the significant difference between groups at $p < 0.05$.

CHAPTER 6: From Toothpaste to "Implant-paste": A New Product for Cleaning Dental Implants

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6.1 Abstract

Introduction: Peri-implant infections caused by oral biofilm compromise dental implant survival, a problem faced by ~ 5 million implant patients worldwide. Available prophylaxis and toothpastes are made of organic thickeners and surfactants that contaminate titanium surfaces; these products, optimized for cleaning teeth, present limitations in cleaning implants. We hypothesized that pastes free of organic thickeners and surfactants could be more effective in cleaning dental implants than organic-based toothpastes.

Objectives: This study aimed at developing an organic-free paste for cleaning dental implants (implant-paste) using a new inorganic thickening agent.

Materials and Methods: The implant-paste was made of an inorganic thickening agent (nanocrystalline magnesium phosphate) and polishing nanoparticles (hydrated silica). The implant-paste formula was optimized to decontaminate titanium surfaces coated with oral biofilm, and compared to a commercial toothpaste (Colgate Total; Colgate-Palmoliven, USA). Surface morphology, bacterial load and chemical properties of titanium surfaces were analyzed, and comparisons between different products were performed using one-way ANOVA and independent samples t-tests.

Results: The optimized inorganic implant-paste made of nanocrystalline magnesium phosphate gel (10% w/w) and (30% w/w) hydrated silica was superior than brushing alone and Colgate toothpaste in removing titanium surfaces contaminants and it did not cause surface alteration. The thixotropic and inorganic nature of the implant-paste is ideal for cleaning implant surfaces because unlike the Colgate toothpaste it does not contain organic-based thickeners that adhere tightly to titanium surfaces and change their surface chemistry.

Conclusion: An implant-paste based on inorganic thickening agent is more efficient in decontaminating implant surfaces than a commercial toothpaste with organic thickening agents.

Keywords: Dental implants, Toothpaste, Implant-paste, Surface chemistry, Thickening agent.

6.2 Introduction

The accumulation of bacterial biofilm on Ti implants changes the surface biocompatibility and initiates peri-implant diseases (peri-implant mucositis and peri-implantitis) [70]. Therefore, personal and professional oral hygiene measures are highly indicated to prevent or manage these infections in order to increase the implant survival. These techniques should be capable of removing bacterial biofilm without negatively affecting implant biocompatibility [64, 221]. However, the complete removal of biofilm from implant surfaces is still unachievable goal by any of the available methods [132, 222].

Toothpastes have been developed to promote dental health and assist the mechanical removal of biofilm with brushes. Most of the toothpastes are composed of 20-42% water and other ingredients including abrasives (Hydrated silica, calcium carbonate), surfactants (sorbitol), organic thickeners (xanthan, cellulose gums), and antimicrobials (fluoride, triclosan) [223]. Colgate Total toothpaste is a representative toothpaste that is used for personal daily care to reduce plaque and prevent gum infections. It is composed of antimicrobials (sodium fluoride, triclosan), organic thickeners (cellulose gum and copolymers), abrasives (hydrated silica and titanium dioxide), and humectants (glycerin and sorbitol). However, the toothpastes ingredients have a negative impact on the stability and chemical properties of implant surfaces [224-228]. The organic macromolecules are known to spontaneously adsorb to metals and tightly attach to their surfaces causing alteration in their physical chemistry and surface charge [185]. Fluoride ions initiate surface corrosion of Ti metal and alloys [229, 230], altering their surface chemistry [229, 231], topography and roughness [224]. The effect of fluoride is not limited to the time of oral hygiene procedure because the fluoride could be retained and concentrated in dental plaque [228], and it can be found in saliva 24 hours after the use of fluoridated oral hygiene products

[232]. In addition, the abrasives incorporated into the regular tooth or polishing pastes can cause damage to the implant surfaces and increase their roughness [225]. Consequently, the toothpastes have to be carefully selected when implant restorations are present. Unfortunately, no specific prophylaxis paste that would satisfy this requirements exists.

Natural and synthetic inorganic clays such as Laponite (crystalline layered magnesium-silicate colloid) are used in some prophylaxis and toothpastes as binders or thickeners. However, they are commonly combined with other organic thickeners (i.e. xanthan gum), to obtain the optimal consistency of a dentifrice [233, 234]. Nanocrystalline magnesium phosphate (NMP) gel is a novel inorganic colloidal suspension developed and patented in our lab. It is a hydrogel with high stability, biocompatibility and rheological properties that are ideal for prophylaxis pastes applications (Fig. 6.5.1). Unlike clays, NMP gel is silicate-free thus it is less abrasive to implants surfaces. The gel formulation was optimized to obtain an alkaline pH of 9.6 and a high content of nanocrystals. The NMP gel is also rich in Na^{+} cations, which have toxic effect on bacteria [235] and can disturb the biofilm structure by displacing the divalent cations (Ca^{++}) [236].

Available toothpastes contain fluoride ions that corrode Ti, organic compounds that alter its surface chemistry and abrasives that damage its surface microtexture. Accordingly, we hypothesized that prophylaxis pastes free of fluoride and organic compounds would be more efficient for cleaning dental implants. Thus, this study aimed at developing and optimizing a new "Implant-paste" specifically designed for decontamination of dental implants.

6.3 Materials and Methods

The study design was reviewed and approved by the Research Ethics Board Committee of McGill University (application 14-464 GEN). All subjects participating in this study have signed informed written consents before their participation.

6.3.1 Materials synthesis

The implant-paste was developed by combining a thickening agent made of inorganic nanocrystalline magnesium phosphate (NMP) gel and an abrasive agent of hydrated silica nanoparticles. In a typical procedure to synthesize the NMP gel, 270 mg of $Mg(OH)_2$ (4.62 mmol) were dissolved in 7.5 mL of H_3PO_4 1.5 M (11.25 mmol), followed by the addition of 13.5 mL NaOH solution 1.5 M (20.25 mmol). The addition of the NaOH solution provokes the instantaneous formation of a white liquid suspension made of nanocrystals with a uniform size of 50 nm. The pH of the suspension remains constant for 4 minutes (10.1) then slowly decreases and stabilizes at 9.6 after 30 minutes. The liquid suspension changes its color from white to grey possessing a solid and thixotropic behaviour with the final suspension composed of 2D nanocrystals. The solid content of the paste was modified by adding 20, 30, 50, and 60% of hydrated silica nanoparticles with average aggregate particles size of 200-300 nm. The addition of hydrated silica nanoparticles increased the viscosity of the gel depending on the concentration used, however, the thixotropic behaviour and pH of the initial gel was not affected by their incorporation (Fig. 6.5.1).

6.3.2 Samples preparation

Machined titanium discs (grade 2, Ø 5.0 and 1.0 mm thick; McMaster-Carr, Cleveland, OH, USA) were used in this study. The discs were sequentially ultrasonicated in deionised water,

acetone and ethanol for 15 minutes each, before drying over-night in a vacuum oven (Isotemp, Fisher Scientific, US).

6.3.3 Biofilm contamination

The biofilm was developed following a previously described standard protocol [117, 126, 132, 133]. Alginate impressions were taken to produce study models for each participant's upper jaw. A thermoplastic copolyester splints (1-mm thick) covering all maxillary teeth were produced. The splints were used to fix the Ti discs at the buccal aspect of premolar and molar areas, each splint had room for 12 Ti discs. The participants were asked to wear the splints for 24 hours in order to allow for soft biofilm to accumulate on Ti surfaces. The participants were instructed to remove and store the splints during drinking or eating in phosphate buffered solution. After 24 hours, the splints were collected and the discs were washed with sterile saline solution (0.9%), then stored for further analysis.

6.3.4 Samples cleaning

A rotary brush was used to clean biofilm-contaminated samples with water-intensive cooling at a speed of ~2500 rpm. The brush was held perpendicularly in gentle contact with the contaminated surfaces while moving in circular motion. The samples were initially brushed without paste for 1, 2 and 5 minutes in order to optimize the brushing time to the one that caused the least damage to the surfaces (n=6 for each group). The implant-paste formulations; 10% (w/w) NMP gel containing 20, 30, 50, and 60% (w/w) of hydrated silica were then assessed with the optimized brushing parameters (n=3 for each group). After that, the samples were brushed with the optimized implant-paste and compared to surfaces cleaned with rotary brushes alone and to others brushed with a commercial toothpaste (Colgate Total; Colgate-Palmolive, New York, US; n=6 for each group).

6.3.5 Analysis methods

Ti surfaces were analyzed before and after the biofilm contamination and subsequent brushing using the following methods:

6.3.5.1 X-ray Photoelectron Spectroscopy (XPS)

XPS is the most widely used surface analysis technique that measures the elemental composition, chemical state and electronic state of the elements within a material [237]. The chemical composition of Ti surfaces was analyzed using X-ray Photoelectron Spectrometer (Thermo Fischer Scientific Inc., East Grinstead, UK). The instrument is equipped with a monochromatic Al *Kα* X-Ray radiation source (1486.6 eV, λ= 0.834 nm) and an ultrahigh vacuum chamber (10^{-9} torr). For all discs, survey scans were acquired over the range of 0−1350 eV with a pass energy of 200 eV and a resolution of 1.0 eV. A flood gun was used to neutralize the surface charging in all samples. Binding energies, peak areas and atom concentration ratios were obtained using the curve fitting function of Avantage (5.932v) analysis software (Thermo Fisher Scientific, Waltham, MA US).

6.3.5.2 Live/ Dead bacterial assays and Fluorescence Microscopy (FM)

Live/dead staining kit (BacLight Bacterial Viability Kit L7012, Molecular Probes, Carlsbad, USA) and fluorescence microscopy were used to evaluate the viability and attachment of bacteria on the contaminated and cleaned Ti discs (n=6 for each group). The live/dead stain was prepared by diluting 1 μL of SYTO 9 (excitation (λ) =485 nm, emission=498 nm) and 1 μL of propidium iodide (excitation=535 nm, emission=617 nm) in 1 mL of distilled water. Discs were placed in 48-well plate, and 500 μL of the staining mixture was added to each well followed by incubation in the dark at room temperature for 15 min. Each disc was then carefully placed on a glass slide, covered with mounting oil and stored in a dark space at 4˚C until further processing.

Discs were evaluated 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) and operated with an image processing software (ZEN; Carl Zeiss Microscopy GmbH, Gottingen, Germany). For each disc, five randomly-selected sites were captured; one from the centre and the other four from the quarters of the Ti surface using a 20x objective. Means of red fluorescent areas (dead cells), green fluorescent areas (viable cells), and total fluorescence (total bacteria) per standard microscopic field area ($448\times335= 0.15$ mm^2) were calculated using Cell Profiler image analysis software (Broad Institute of MIT and Harvard, Massachusetts, US).

6.3.5.3 Scanning Electron Microscope (SEM)

Ti surfaces were scanned before and after biofilm contamination, and after each cleaning procedure to visualize the surface contaminants or topographical changes. Clean Ti discs were scanned with SEM (FE-SEM S-4700, Hitachi, Japan) without further preparation while the contaminated discs were prepared as follows; the discs were fixed in glutaraldehyde (2.5% in phosphate buffered solution (PBS); PAA Laboratories GmbH, Pasching, Austria) for 2 hours and washed 5 times for 10 minutes in PBS, before dehydrating them in ascending concentrations of ethanol (30 –100 v/v %, 15 min each). The discs were then dried using critical point CO_2 (Ladd Research Critical Point Dryer). All discs were mounted on SEM-sample stubs and sputtered with gold. The SE mode with an acceleration voltage of 20 kV was selected, and the vacuum pressure was maintained below 1×10^{-5} torr. For direct comparison of surface topography, the same magnification of x10,000 was selected for all samples.

6.3.6 Statistical analysis

The primary outcome variables were surface chemical composition, bacterial attachment and viability. For each cleaning technique, data of the primary variables was statistically analyzed based on paired design for comparison of the measurements from before and after contamination and decontamination. The outcomes of different decontamination methods were also analyzed and compared. Data were expressed as mean, standard deviation, and tested for the normality of distribution using Shapiro-Wilk test. All data were normally distributed, therefore repeated measures ANOVA and paired-sample t-test were used to compare the outcomes of the same groups at different time points while one-way ANOVA and independent samples t-test were performed to compare the outcomes of different groups and techniques. The data analyses were carried out using SPSS software version 22 (SPSS Inc., IBM Corporation, Somers, NY, US) and Origin 9.0 (Origin lab, Northampton, MA, US). A p-value of < 0.05 was set to represent a statistically significant difference between groups.

6.4 Results

6.4.1 Surface chemistry of clean and biofilm-contaminated surfaces

The XPS survey spectra of clean surfaces showed the presence of the following major peaks: O1s (O), C1s (C), Ti2p (Ti) and N1s (N) (Fig. 6.5.2 A and B). C1s and N1s signals indicate the surface contamination while Ti2p signals demonstrate the presence of the TiO_2 oxide layer [238, 239]. Biofilm contamination of Ti surfaces significantly increased C and N levels at the expense of O and Ti, indicating that the contaminants were mainly organic in nature (Fig. 6.5.2 A and B). Ti2p signal almost disappeared from the spectra surveys of biofilm-contaminated surfaces indicating that the biofilm covers entirely the Ti surfaces with the organic contaminants (Fig. 6.5.2 A).

6.4.2 Optimization of brushing time

Brushing Ti surfaces for 1 minute significantly decreased the levels of C and N and increased the concentrations of O and Ti (Fig. 6.5.2 B). Increasing the brushing time to 2 and 5 minutes did not achieve further cleaning benefits but it induced surface scratches as seen on SEM images (Fig. 6.5.2 C). Thus, the brushing time was fixed to 1 minute.

6.4.3 Optimization of implant-paste formulation

The composition of the NMP gel was optimized to 10% w/w, respect to the water content, to obtain an alkaline pH of 9.6. The biofilm-contaminated surfaces were then brushed using this optimized NMP gel (10%) with and without hydrated silica. SEM images showed that surfaces brushed with the NMP gel alone and the gel with 30% of hydrated silica were clean without noticeable changes in their topography (Fig. 6.5.3). Samples cleaned with the gel containing 30% of hydrated silica were also significantly different from the contaminated samples in terms of elemental composition as shown by XPS; they showed higher levels of Ti, O and lower levels of C, N, silica (Si) and magnesium (Mg).

The MNP gel with less silica also decreased the levels of surface contaminants (C and N), however, this formula was less efficient than the gel containing 30% of hydrated silica. On the other hand, higher concentrations of silica (> 30%) did not improve the cleaning performance and caused surface contamination with implant-paste residues including Si (Fig. 6.5.3 and 6.5.4). Based on these results, the optimized implant-paste formulation was the one containing 10% NMP gel and 30% hydrated silica.

6.4.4 Cleaning uncontaminated (control) samples with the optimized implant-paste

Brushing uncontaminated Ti with the optimized implant-paste increased the surface levels of Ti, decreasing those of C (Fig. 6.5.5). This indicates that the optimized implant-paste is also able to

remove the carbon-containing compounds that are commonly adsorbed and detected on clean Ti surfaces **[240]**.

6.4.5 Optimized implant-paste vs Colgate toothpaste

The optimized implant-paste significantly reduced the atomic concentration of surfaces' contaminants C and N, and increased the O and Ti levels in comparison to the surfaces cleaned with the brush alone or the brush with Colgate toothpaste (Fig. 6.5.6 A and B). Both the optimized implant-paste and Colgate toothpaste were able to remove bacteria from biofilm-contaminated Ti surfaces reaching comparable levels of bacteria to those found prior to biofilm contamination (Fig. 6.5.7 A and B). SEM images showed surfaces scratches and toothpaste residues on surfaces cleaned with Colgate toothpaste while no scratches or residues could be seen with the optimized implant-paste (Fig. 6.5.3).

6.5 Figures

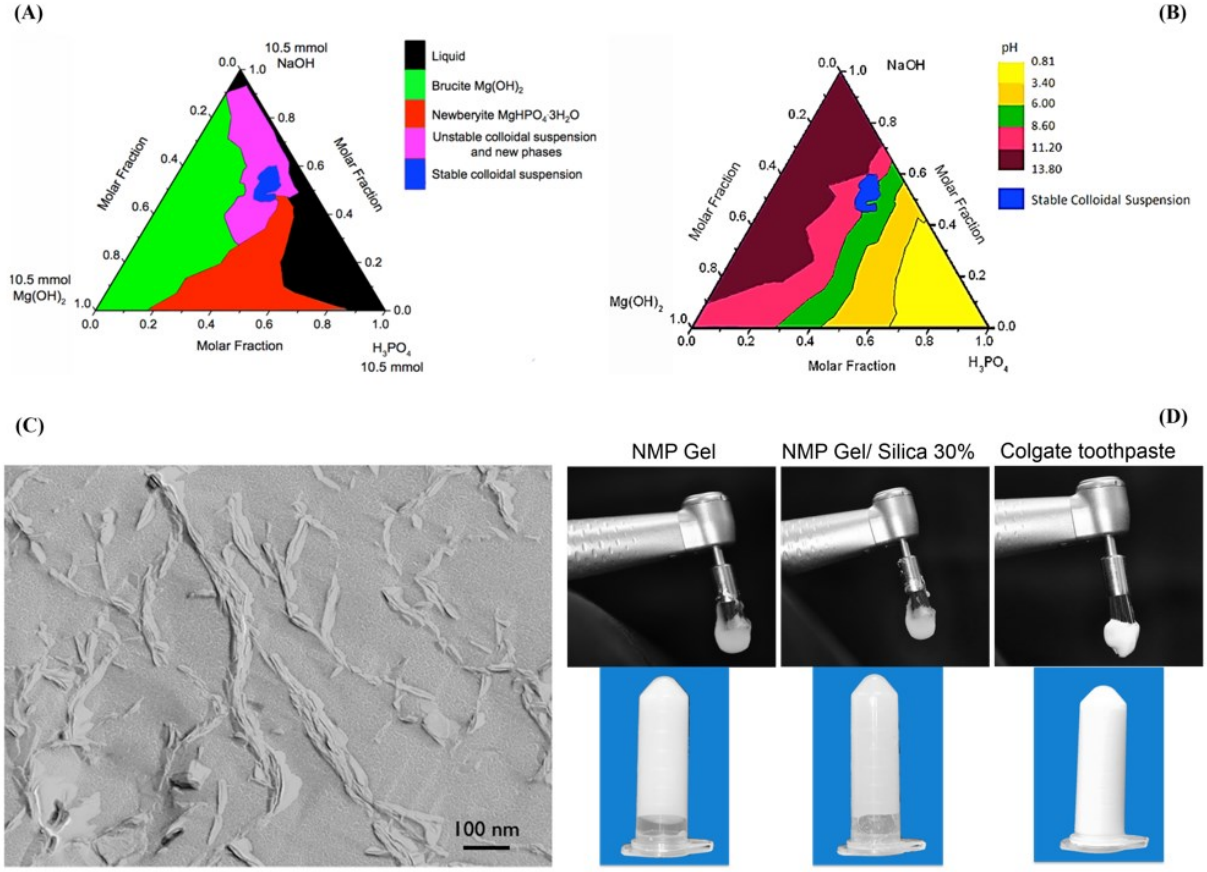

Fig. 6.5.1. Identification, characterization, nanocrystals morphology, and structure of the "clay-like" NMP implant-paste. (A) Ternary diagram of the pH as a function of the molar fraction of $Mg(OH)_2$, NaOH, and H_3PO_4. The stable NMP suspension can be obtained in a range of pH comprised between 7.80 and 11.20. (B) Representative TEM micrograph of a freeze-fractured carbon-platinum replica of a 10% w/w NMP suspension showing the 3D structure and interactions of the nanocrystals composing the NMP gel. (C) From the left to right; photographs of the rotary brush loaded with the NMP gel, developed implant-paste, and Colgate toothpaste. Eppendorf tubes showing the physical aspect of the NMP gel, implant-paste, and Colgate toothpaste; Colgate toothpaste flows without applying mechanical shear while the other pastes do not flow

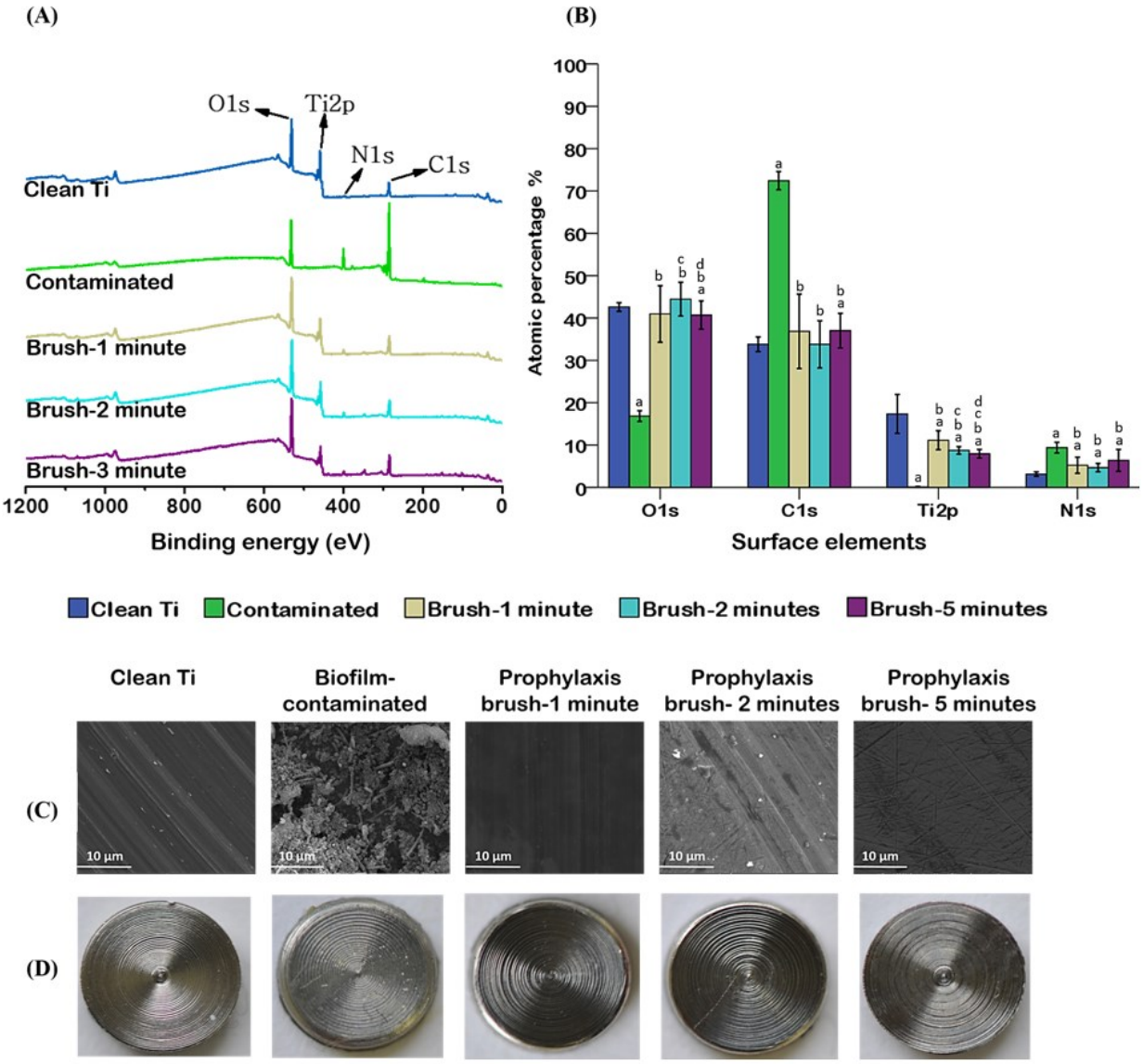

Fig. 6.5.2. X-ray Photoelectron Spectroscopy (XPS) surveys (A), bar chart (B), Scanning Electron Microscope images at a magnification of x10, 000 (C) and photographs (D), illustrating the cleaning effect of rotary prophylaxis brush at different brushing time on the elemental composition and topography of biofilm-contaminated Ti surfaces. a: significantly different from clean Ti, b: significantly different from biofilm- contaminated group, c: significantly different from Ti surfaces brushed for 1 minute, d: significantly different from Ti surfaces brushed for 2 minutes ($p < 0.05$).

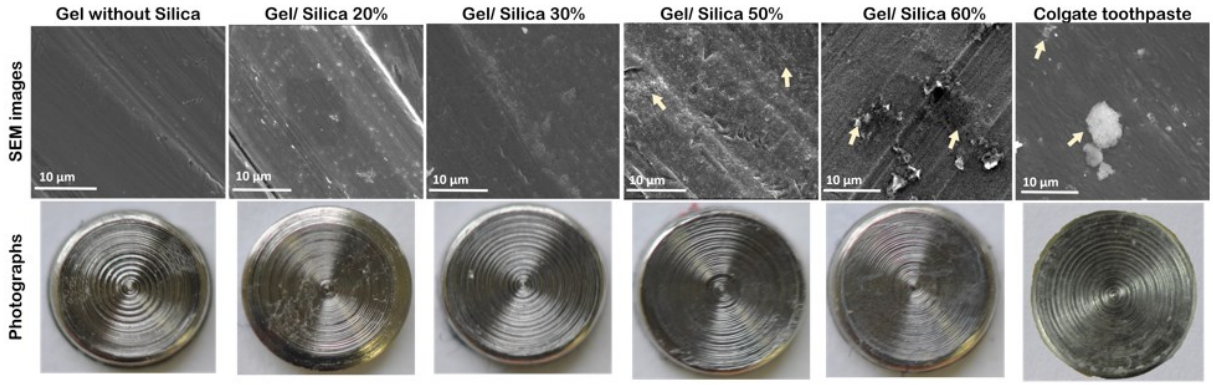

Fig. 6.5.3. Scanning Electron Microscope images (magnification x10,000) and photographs showing the topography of the biofilm-contaminated Ti surfaces after brushing with the NMP gel, the gel containing different concentrations of hydrated silica and Colgate toothpaste (Brushing time is 1 minute). Small arrows indicate the areas where the remnant silica and toothpaste residues accumulate on Ti surfaces.

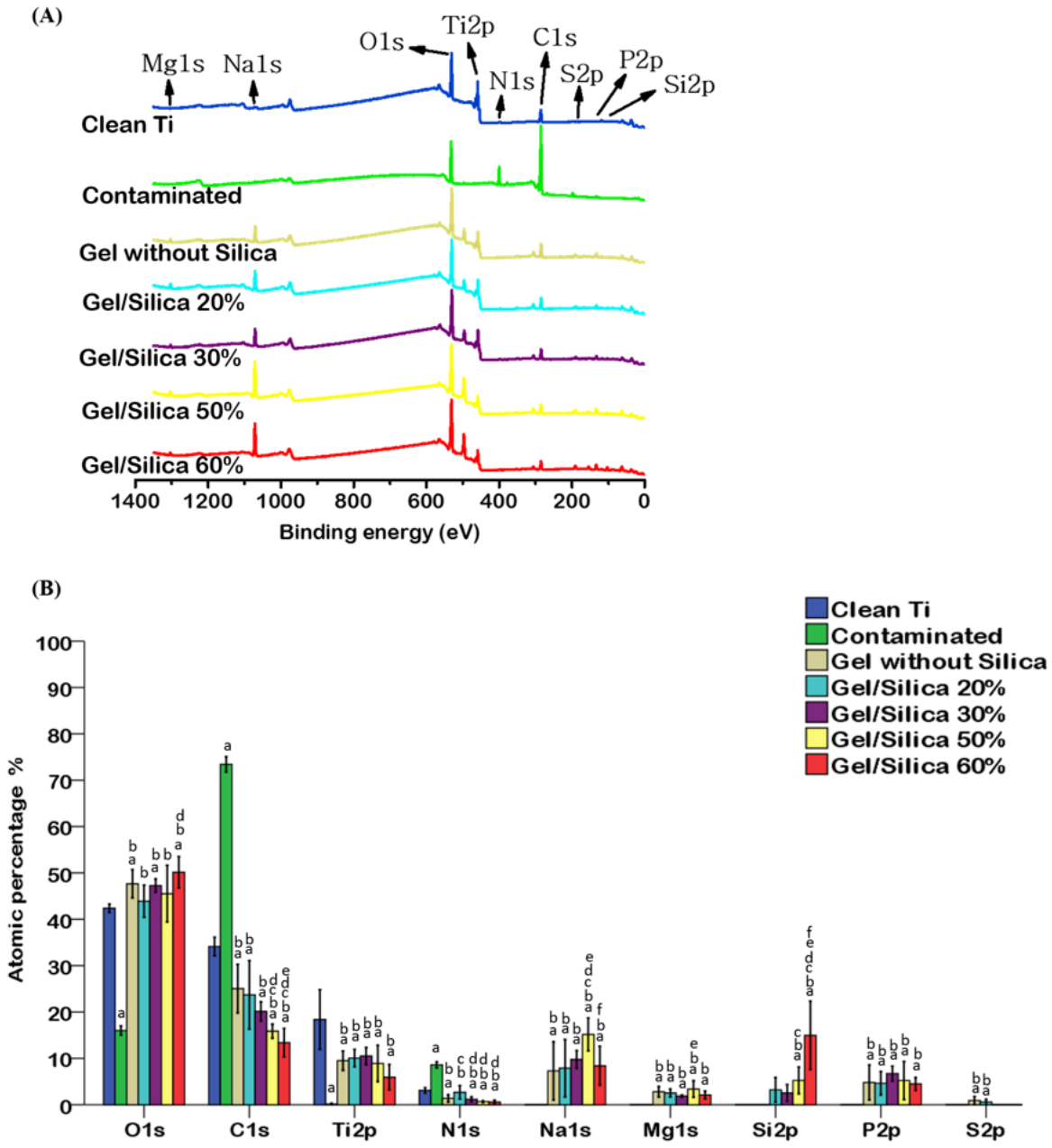

Fig. 6.5.4. XPS surveys (A) and bar chart (B), comparing the cleaning efficiency of the NMP gel and the gel containing different concentrations of hydrated silica (Brushing time is 1 minute). a: significantly different from clean Ti, b: significantly different from biofilm contaminated group, c: significantly different from Ti surfaces brushed with NMP gel alone, d: significantly different from Ti surfaces brushed with the gel containing 20% hydrated silica, e: significantly different from Ti surfaces brushed with the gel containing 30% hydrated silica, f: significantly different compared to Ti surfaces brushed with the gel containing 50% hydrated silica ($p < 0.05$).

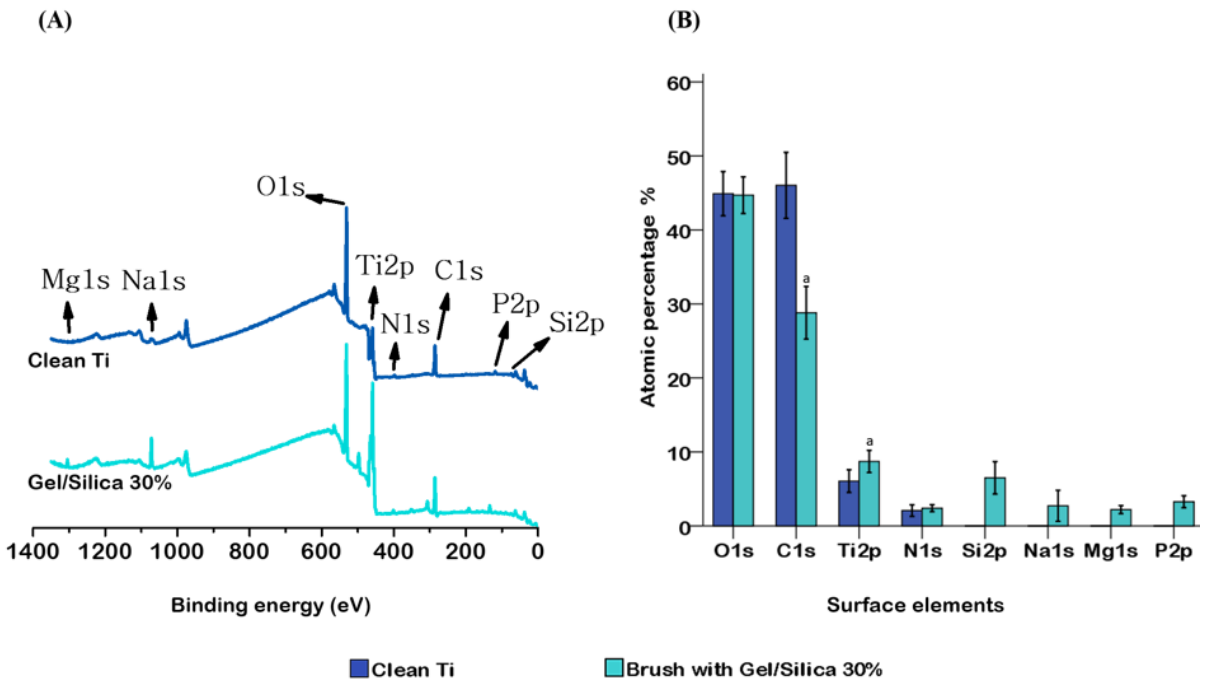

Fig. 6.5.5. XPS surveys (A) and bar chart (B), showing the change in the elemental composition of uncontaminated Ti surfaces after cleaning with the rotary brush and optimized implant-paste (NMP gel containing 30% hydrated silica, brushing time is 1 minute). a: significantly different from control group.

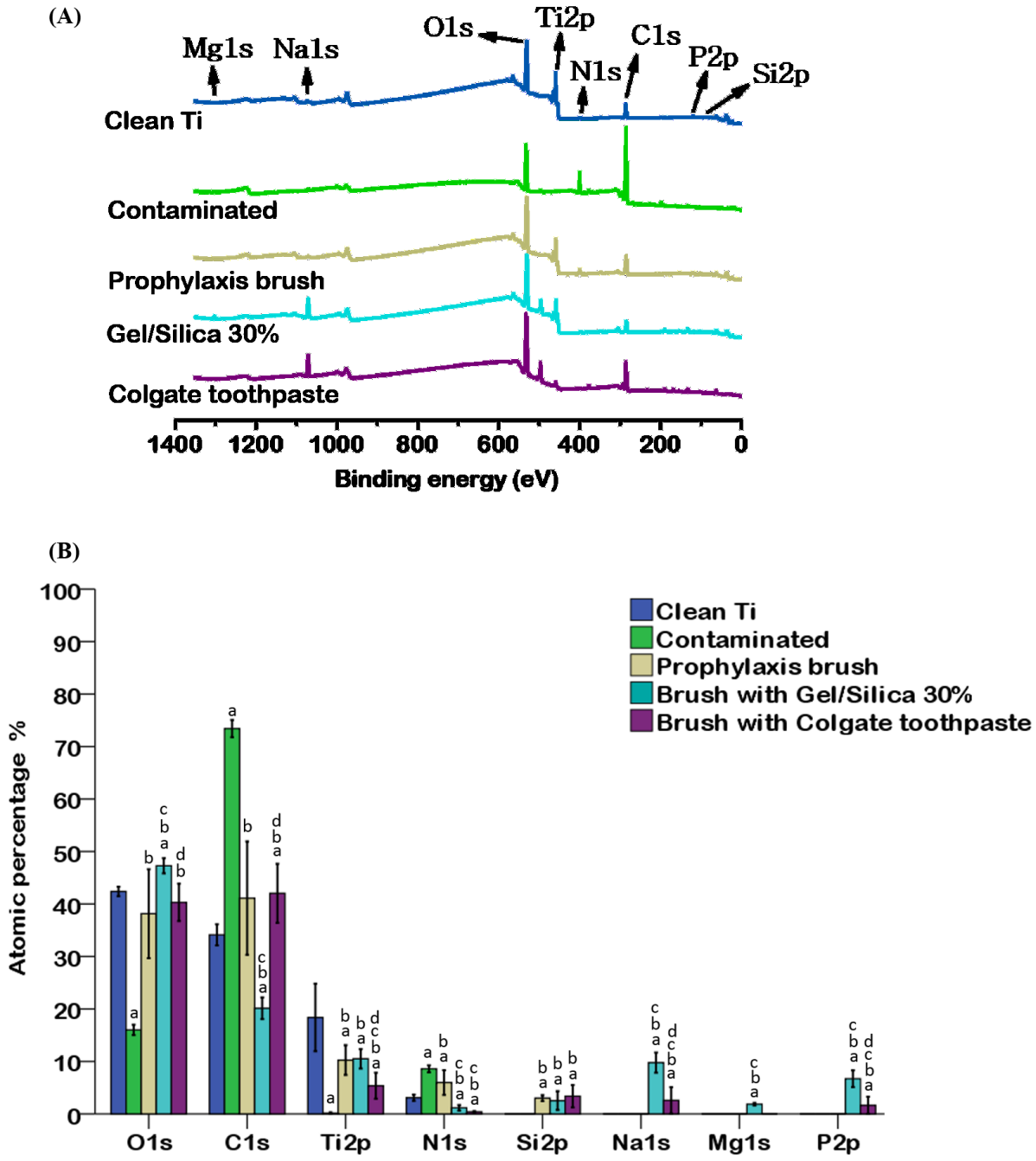

Fig. 6.5.6. XPS surveys (A) and bar chart (B), comparing the cleaning efficacy of the prophylaxis brush, the optimized implant-paste and Colgate toothpaste (Brushing time is 1 minute). a: significantly different from clean Ti, b: significantly different from biofilm-contaminated group, c: significantly different from Ti surfaces cleaned with the prophylaxis brush, d: significantly different from Ti surfaces brushed with the optimized implant-paste ($p < 0.05$).

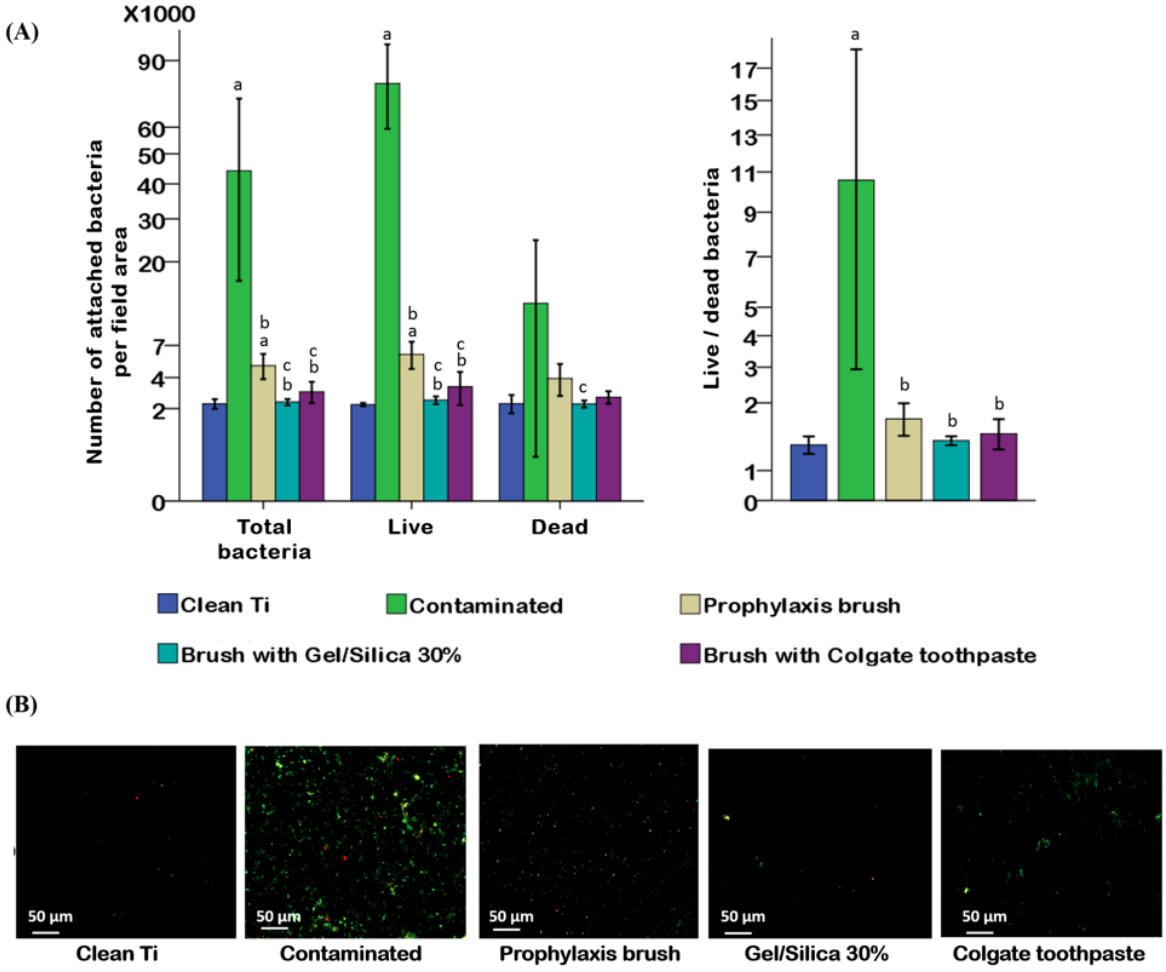

Fig. 6.5.7. Bar charts (A) and Live/Dead staining (fluorescence) images (B), comparing the bacterial removal efficiency of the prophylaxis brush, the optimized implant-paste and Colgate toothpaste (Brushing time is 1 minute). a: significantly different from clean Ti, b: significantly different from biofilm- contaminated group, c: significantly different from Ti surfaces cleaned with the prophylaxis brush, d: significantly different from Ti surfaces brushed with optimized implant-paste ($p < 0.05$). Field area is 0.15 mm^2.

6.6 Discussion

Here we present a novel implant-paste specially designed and optimized for implant surface decontamination. The implant-paste was able to effectively disinfect contaminated Ti without having a negative impact on its surface. It also showed superior decontamination efficiency than the rotary brush and the brush with a commercial toothpaste.

In this study, we used in vivo biofilm model because it offers the opportunity to evaluate implant surfaces in realistic clinical conditions; formation of composite plaque, co-adherence of microorganisms and salivary pellicle under the removal forces of salivary flow and chewing activities [135]. Several in vitro biofilm models have been tested and validated to study the implant surface bacterial interactions [97, 136, 137]. This includes for instance the commonly used microtiter plate-based systems [138]. However, they fail to precisely simulate the complex structure of biofilm, the dynamics of its pathogenicity and ecological determinants [139, 140].

Prophylaxis instruments such as brushes and rubber cup are used to remove biofilms attached to implant surfaces with or without using prophylaxis pastes. In this study, we used rotary brushes for cleaning Ti surfaces because they are inexpensive and accessible compared to titanium brushes and their plastic bristles should be gentile on Ti. Rotating cups were found to leave remnants of rubber particles on the implant surfaces after cleaning [222, 241]. In addition, some cup materials are too abrasive and can cause Ti surface damage [242].

The prophylaxis bushes were initially used to decontaminate the discs without a paste. The purpose of this procedure was to optimize the brushing time and exclude the possible damaging effect of brushing technique. To the best of our knowledge, this is the first study that optimized the time required for Ti decontamination using the prophylaxis brush. Brushing the contaminated samples for one minute was able to remove contaminants (Fig. 6.5.2 A and B) without inducing

surface damage (Fig. 6.5.2 C and D). However, brushing for more than one minute induced visible scratches on the Ti surfaces without improving the cleaning outcomes (Fig. 6.5.3). This finding could be attributed to the softness of Ti metal and its poor resistance to physical wear [243, 244]. Therefore, the brushes induced surface scratches on Ti surface when brushing strokes have been increased more than 2500 rpm. This result is in agreement with previous reports that toothbrush bristles causes changes of the Ti surface texture [245] and can produce superficial grooves on the Ti abutments [246].

Accordingly, brushing contaminated Ti surfaces for one minute (2500 rpm) was able to decontaminate them without causing mechanical abrasion, though the complete removal of contaminants and re-establishment of the Ti original chemistry were not achieved. This indicates the limited effectiveness of brushes in decontaminating Ti surfaces, which calls for the use of a dentifrice or a prophylaxis paste.

A dentifrice is usually combined with brushes to adjunct the physical removal of plaque and stains through their chemical and physical additives, or to apply therapeutic and preventive agents to tooth surfaces [243, 247]. The dentifrice needs two main ingredients to achieve the mechanical cleaning; an abrasive agent and thickener to hold the abrasives in suspension during brushing [248]. In this study, we developed and optimized prophylaxis paste to decontaminate implant surfaces "implant-paste" and enhance the cleaning efficiency of the brush. For the development of this implant-paste, the thickener is composed of an inorganic, silicate free Nanocrystalline Magnesium Phosphate (NMP) gel. The gel composition was optimized to obtain an alkaline pH of 9.6 because the corrosion resistance of Ti is high at this pH [249]. In addition, the implant-paste can be in contact with intraoral structures and teeth for several hours when

used for daily cleaning of Ti implants. Consequently, it is important for this optimized implant-paste to have a relatively alkaline pH to minimize potential tooth or implant damage.

The optimized NMP gel has similar biocompatibility and thixotropic properties of Laponite (silicate clays); the most used inorganic thickener in toothpastes. However, the optimized NMP gel has a stable consistency without the need for additional organic thickeners. This is an advantage of our novel gel over the clays-based toothpastes that require organic thickeners (i.e. xanthan gum) to provide optimal consistency [233, 234]. The incorporated organic compounds could adhere to the implant surfaces [185] complicating their decontamination.

The other key component of the implant-paste that contributes to the physical removal of biofilm is the abrasive agent. The abrasives are usually added to physically scrub the external surface of tooth or implant, and remove bacteria as well as other extrinsic stains. Carbonates, silica, phosphates and metal oxides are the common abrasive elements used in the current dentifrices [233, 247]. For our implant-paste, hydrated silica nanoparticles were chosen as abrasives. We used silica because it is a relatively safe, nontoxic ingredient and mostly compatible with other ingredients, such as glycerine and fluoride [250]. Moreover, low concentration of silicates show osteoconductive properties that help to induce and accelerate bone regeneration [251].

We used silica nanoparticles (~200-300 nm) and we also optimized their content in the implant-paste in order to obtain mild abrasiveness that removes plaque without damaging the Ti surfaces. It was demonstrated that polishing pastes and dentifrices containing hard abrasive materials with larger particle sizes induced scratches or rounded edges on implant surfaces, increasing plaque accumulation [242, 252]. Furthermore, it is always recommended to use toothpastes with low abrasiveness for the daily oral care of subjects' with Ti implants [253]. The abrasives optimization results showed that the best decontamination outcomes were obtained with the

combination of 10% w/w NMP gel and 30% w/w hydrated silica. This formula showed an efficient removal of the organic contaminants from Ti surfaces and the least topographical changes (Fig. 6.5.3 and 6.5.4). The cleaning effectiveness of this formula further confirmed by its ability to remove carbon-containing compound from clean (controls) Ti surfaces (Fig. 6.5.5). This finding confirms our hypothesis that the inorganic paste would be more efficient in cleaning Ti. Accordingly, this formula is considered the optimized implant-paste.

In the present study, we also demonstrated the superior decontamination efficiency of the optimized implant-paste on biofilm-contaminated surfaces in comparison to the rotary prophylaxis brush alone, and the brush with a commercial toothpaste (Fig. 6.5.6). The significant increase in the carbon levels after cleaning with Colgate toothpaste (the representative toothpaste) indicates that regular toothpastes further contaminate the Ti surfaces. This result could owed to the organic content of the toothpastes that are usually incorporated for thickening, binding or flavouring benefits [223]. It supports the previous findings on high affinity of the organic macromolecules to adsorb and bind to the metal surfaces, changing their chemistry [185]. It also confirms the superiority of the implant-paste developed in this study over the currently available pastes due to its inorganic nature.

Moreover, the bacteria attached to the surfaces brushed with the optimized implant-paste and Colgate toothpaste were found to be comparable to that found before contamination (Fig. 6.5.7). This result is in agreement with a previous study that indicated the superiority of a toothbrush and dentifrices over different ultrasonic scalers in reducing bacterial load from contaminated implant surfaces [254]. However, surface chemistry analysis of the Ti surfaces was not evaluated in this study to show the removal of other organic surface contaminants (bacterial endotoxins).

6.7 Clinical Implications and future studies

The new inorganic implant-paste developed in this study is able to remove biofilm from contaminated Ti implants without affecting their surface integrity. Cleaning dental implants with current organic-based toothpastes contaminates the implants surfaces, changing their surface charge and chemistry, which could have negative impact on re-osseointegration.

This is the first paste ever specially designed and optimized for implant surface decontamination. It could allow dentists and patients to remove biofilm from Ti implants and abutments, control the peri-implant infections and favor re-osseointegration in case of bone loss, although further clinical studies are required to confirm this. Accordingly, this novel implant-paste could be recommended for surgical decontamination of implant surfaces and professional cleaning of implants during maintenance visits mainly for patients with implant overdentures. The incorporation of flavouring agents and fluoride has not been tested in this study therefore further investigations are needed before using the implant-paste for daily personal care.

Furthermore, in this study live/dead bacterial assays were used to investigate the antibacterial efficacy of the implant-paste on the biofilm. However, we recommend future studies to further investigate specific bacterial species using quantitative methods such as fluorescence in situ hybridization (FISH) or quantitative real-time polymerase chain reaction (qPCR) [157-159].

6.8 Conclusions

The optimized inorganic implant-paste shows superior efficiency in decontaminating Ti implants than organic-based toothpaste without damaging their surfaces.

6.9 Acknowledgements

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CHAPTER 7: General Conclusions

From the results of this thesis, we can draw the following conclusions:

- Even though many decontamination techniques can eliminate bacteria, our data demonstrated that this does not necessarily indicate cleanliness of the Ti surfaces. All the investigated techniques (metal and plastic curettes, Ti brush and laser) fail to remove the other organic contaminants (bacterial endotoxins) or restore the surfaces original properties.

  Also, different decontamination techniques interact differently with the biofilm accumulated on Ti surfaces; Ti brush was superior for mechanical decontamination and laser treatment for bacterial eradication.

- Investigating the electrochemical properties of the biofilm growing on titanium surfaces can help to develop new electrochemical decontamination treatments. Our results indicated that an optimized decontamination method based on low electrical alternating current and potential is able to completely remove surface contaminants, reduce the bacterial load, and restore the original surface chemistry of saliva-contaminated surfaces. Also, combining this optimized electrochemical treatment with subsequent brushing can achieve complete decontamination of oral biofilm from Ti surfaces.

- Cleaning Ti implants with organic-based toothpastes contaminates their surfaces and changes their chemistry. Our results demonstrated that the developed implant-paste based on an inorganic thickening agent decontaminate Ti surfaces without affecting their integrity. Furthermore, this implant-paste is more efficient in decontaminating implant surfaces than brushing alone or brushing with organic-based toothpaste.

APPENDIX I: List of References

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APPENDIX II: Consent Forms

Research Participant Informed Consent and Privacy Authorization Form

Protocol Title: "Removal of Oral Biofilm from Dental Surfaces"

Sponsor: Alpha Omega Foundation of Canada.

Principal Investigators (PIs):

Faleh Tamimi
Faculty of Dentistry, McGill University
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Faculty of Dentistry, McGill University
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BIOFILM PREPARATION

INTRODUCTION

You are being invited to take part in this research study because you are a healthy adult and we would like to collect oral biofilm samples from healthy volunteers.

Before deciding to participate in the study you should clearly understand its requirements, risks and benefits. This document provides information about the study. It may contain words you do not fully understand. Please read it carefully and ask the study staff any questions you may have. They will discuss the study with you in detail. If you decide to participate, you will be asked to sign this form and a copy will be given to you.

BACKGROUND

The accumulation of oral biofilm on dental implants causes inflammation of the surrounding soft tissue that could progress to involve the underlying bone and result in implants loss. Regular removal of the biofilm is critical to maintain the oral and systemic health, however, complete biofilm removal was not achieved by any of the available cleaning methods. Therefore, in this study we are testing the ability of currently available cleaning methods such as metal and plastic curettes in addition to new methods such as the electrochemical treatment and a new toothpaste, to remove the oral biofilm accumulated on the dental implant surfaces.

PURPOSE OF THE STUDY

The purpose of this study is to investigate the properties of the soft oral biofilm that form on dental implants, in order to develop an optimal cleaning methods for complete biofilm removal.

STUDY DURATION

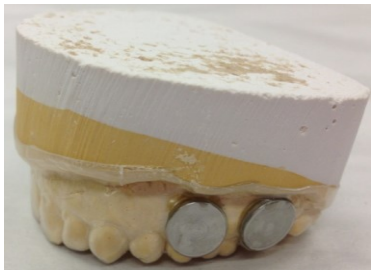
An initial visit will be required to explain this study to you. If you agree to participate, an alginate impression will be taken in a second visit to fabricate the upper jaw removable splint. You will be given the splint in the same visit and asked to wear it for 24 hours. After that, you will be called for the third visit to collect the splints.

Your participation will remain anonymous and your samples will be labeled by a specific number code for data collection purposes. The expected duration of the study is 1 year, however, we will keep the samples for 2 years and then dispose them in a safety container used for biological hazards.

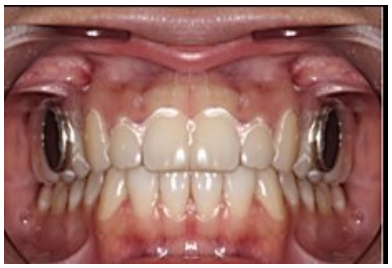
STUDY PROCEDURES

If you agree to participate in this study, the following procedures will be performed:

First, an alginate impression will be taken to produce a study model for your upper jaw on which a custom-made removable upper jaw splint will be fabricated.
Twelve titanium discs (5mm in diameter and 1mm in thickness) will be fixed to the splint at the outer surfaces of the upper jaw posterior teeth. You will be asked to wear the splint for 24 hours and only remove it during drinking or eating, meanwhile they should be stored in a phosphate buffered saline. You will be supplied with the phosphate buffered saline in a closed container. After 24 hours, you will be called to collect the splint.



Titanium discs attached to the splint at the outer surfaces of upper posterior teeth



The position of the titanium discs and splint in the volunteer' mouth

POTENTIAL RISKS AND/OR DISCOMFORTS

You might suffer a slight discomfort in the first hour of wearing the splint, however, this discomfort will subside gradually with time. This method is a standard protocol for oral biofilm collection and has been extensively used in previous researches without harms. There are no known risks associated with using alginate material, taking the impression or wearing the splint for 24 hours. The participants will not have any teeth removed.

POTENTIAL BENEFITS

You may or may not directly benefit from taking part in this study. However, the information collected from this study may benefit dentists and their patients in the choice of treatment in the future.

INDEMNIFICATION/COMPENSATION IN CASE OF INJURY

By accepting to participate in this project, you are not waiving any of your legal rights nor discharging the researchers (the granting agency, if applicable, depending on the type of research) or the institution of their civil and professional responsibility.

CONFIDENTIALITY

All information will be kept strictly confidential by identifying your sample by a code to which only authorized personnel will have access. Your name will be coded and the code list will be locked in a filing cabinet in the investigator's office with limited access. The results from this research study may be published and other physicians participating in this research study may have access to your records related to this research study; however, your identity will not be revealed in the combined results.

In order to verify the research study data, monitors from the McGill University Health Centre Research Ethics Boards may review these records, or the Quality Assurance Officer at the MUHC-Research Ethics Boards may review these records.

By signing this consent form, you give us permission to release information regarding your participation in this study to these entities. Your confidentiality will be protected to the extent permitted by applicable laws and regulations.

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VOLUNTARY PARTICIPATION AND/OR WITHDRAWAL

Your participation in this study is strictly voluntary. You may refuse to participate or may discontinue your participation at any time without explanation, and without penalty or loss of benefits to which you are otherwise entitled. If you decide not to participate, or if you discontinue your participation, you will suffer no prejudice regarding your dental care or your participation in any other research studies.

FUNDING OF THIS RESEARCH PROGRAM

The study is funded by Alpha Omega Foundation of Canada and is being run by Dr. Faleh Tamimi at the McGill University Health Centre.

CONTROL OF THE ETHICAL ASPECTS OF THE RESEARCH PROJECT

The Ethics Research Board of the MUHC has reviewed this research project and ensures its follow-up. In addition, it will first approve any review and amendment made to the information/consent form and to the study protocol.

QUALITY ASSURANCE PROGRAM

The MUHC implemented a Quality Assurance Program that includes active continuing review of projects (on site visits) conducted within our establishment. Therefore, it must be noted that all human subject research conducted at the MUHC or elsewhere by its staff, is subject to MUHC Routine and Directed Quality Improvement Visits.

CONTACT INFORMATION

If you have any questions about this research study you should contact:

Prof. Faleh Tamimi
Faculty of Dentistry
Room M-64, Strathcona Anatomy & Dent
3640 University Street
Montreal, Quebec H3A 0C7
Tel: 514-398-7203 ext 09654 (collect calls will be accepted)
Fax: 514-398-8900

If you have questions about your rights as a study participant, you should contact the hospital Ombudsperson at (514)-934-8306, who will provide you with independent advice.

DECLARATION OF CONSENT

I have read the contents of this consent form, and I agree to participate in this research study. I have had the opportunity to ask questions and all of my questions have been answered to my satisfaction. I have been given sufficient time to consider the above information and to seek advice if I choose to do so. I understand that I will be given a signed copy of this consent form. By signing this consent form, I am not giving up any of my legal rights.

For future research projects:

- I agree that my data may be used for future testing in similar research projects, after these projects have been reviewed by the Ethics Research Board of the MUHC.

Yes ☐ No ☐

I hereby consent to participate in this study:

_____	_____	_____/_____/_____
Signature of Participant	Name (Printed)	day month year*
_____	_____	_____/_____/_____
Signature of person designated by Investigator and who conducted consent discussion	Name (Printed)	day month year*

* PLEASE PERSONALLY DATE YOUR SIGNATURE

SALIVA COLLECTION

INTRODUCTION

You are being invited to take part in this research study because you are a healthy adult and we would like to collect saliva samples from healthy volunteers.

Before deciding to participate in the study you should clearly understand its requirements, risks and benefits. This document provides information about the study. It may contain words you do not fully understand. Please read it carefully and ask the study staff any questions you may have. They will discuss the study with you in detail. If you decide to participate, you will be asked to sign this form and a copy will be given to you.

BACKGROUND

The accumulation of oral biofilm on dental implant causes inflammation of the surrounding soft tissue that could progress to involve the underlying bone and result implants loss. Regular removal of the biofilm is critical to maintain the oral and systemic health, however, complete biofilm removal was not achieved by any of the available cleaning methods. Therefore, in this study we are testing the ability of currently available cleaning methods such as metal and plastic curettes in addition to new methods such as the electrochemical treatment and a new toothpaste, to remove the oral biofilm accumulated on the dental implants.

PURPOSE OF THE STUDY

The purpose of this study is to investigate the properties of salivary biofilm that form on the dental implants, in order to develop an optimal cleaning methods for its complete removal.

STUDY DURATION

An initial visit will be required to explain this study to you. If you agree to participate, we will collect the saliva sample in a second visit.
Your participation will remain anonymous and your sample will be labeled by a specific number code for data collection purposes. The expected duration of the study is 1 year, however, your saliva sample will be directly discarded after testing them. It will be disposed in a safety container used for biological hazards.

STUDY PROCEDURES

If you agree to participate in this study, the following procedures will be performed:

You will be asked to rinse your mouth with distilled water and wait for at least 10 minutes before the saliva collection to avoid sample dilution. You will be asked to sit in a slightly-inclined forward position to allow the saliva to accumulate on the floor of your mouth. The first few milliliters of your saliva will be discarded and the remainder (5 mL) will be collected in a sterile tube labeled with a number and sent to the lab for further analysis.

POTENTIAL RISKS AND/OR DISCOMFORTS

There are no known risks associated with your participation in this study or saliva collection procedure and the participants will not have any teeth removed.

POTENTIAL BENEFITS

You may or may not directly benefit from taking part in this study. However, the information collected from this study may benefit dentists and their patients in the choice of treatment in the future.

INDEMNIFICATION/COMPENSATION IN CASE OF INJURY

By accepting to participate in this project, you are not waiving any of your legal rights nor discharging the researchers (the granting agency, if applicable, depending on the type of research) or the institution of their civil and professional responsibility.

CONFIDENTIALITY

All information will be kept strictly confidential by identifying your sample by a code to which only authorized personnel will have access. Your name will be coded and the code list will be locked in a filing cabinet in the investigator's office with limited access. The results from this research study may be published and other physicians participating in this research study may have access to your records related to this research study; however, your identity will not be revealed in the combined results.

In order to verify the research study data, monitors from the McGill University Health Centre Research Ethics Boards may review these records, or the Quality Assurance Officer at the MUHC-Research Ethics Boards may review these records.

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VOLUNTARY PARTICIPATION AND/OR WITHDRAWAL

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FUNDING OF THIS RESEARCH PROGRAM

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CONTROL OF THE ETHICAL ASPECTS OF THE RESEARCH PROJECT

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DECLARATION OF CONSENT

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For future research projects:

- I agree that my data may be used for future testing in similar research projects, after these projects have been reviewed by the Ethics Research Board of the MUHC.

Yes ☐ No ☐

I hereby consent to participate in this study:

_____	_____	_____/_____/_____
Signature of Participant	Name (Printed)	day month year*
_____	_____	_____/_____/_____
Signature of person designated by Investigator and who conducted consent discussion	Name (Printed)	day month year*

* PLEASE PERSONALLY DATE YOUR SIGNATURE